UNRAVELLING NOVEL MODES OF ANTIMICROBIAL ACTION

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This thesis is dedicated to my parents

Thesis Publications

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

1. Goncalves, V. L., Nobre, L. S., Vicente, J. B., Teixeira, M., and Saraiva, L. M., 2006. Flavohaemoglobin requires microaerophilic conditions for nitrosative protection of *Staphylococcus aureus*. *FEBS Lett.* **580**:1817-1821.

2. Nobre, L. S., Seixas, J. D., Romao, C. C., and Saraiva, L. M., 2007. Antimicrobial action of carbon monoxide-releasing compounds. *Antimicrob. Agents Chemother.* **51**(12):4303-4307.

3. Nobre, L. S., Goncalves, V. L, and Saraiva, L. M., 2008. Flavohaemoglobin of *Staphylococcus aureus*. *Methods Enzymol.* **436**:203-216.

4. Nobre, L. S., Al-Shahrour, F., Dopazo, J., Saraiva, L. M, 2009. Exploring the antimicrobial action of a carbon monoxide-releasing compound through whole-genome transcription profiling of *Escherichia coli. Microbiology* **155**: 813-824.

5. Nobre, L. S., Tavares, A. F. N., Todorovic, S., Oldfield, E., Hildebrandt, P., Teixeira, M., and Saraiva, L. M., 2010. Binding of azole antibiotics to *Staphylococcus aureus* flavohaemoglobin increases intracellular oxidative stress. *J. Bacteriol.* **192**(6):1527-1533

"I´m not telling you it is going to be easy, I´m telling you it's going to be worth it"

Art Williams

Abstract

The work presented in this thesis aimed at unravelling novel modes of antimicrobial action through: i) the study of *Staphylococcus aureus* defences against nitric oxide, an antimicrobial weapon of the innate immune system, namely by performing the biochemical characterisation of *S. aureus* NO-detoxifying flavohaemoglobin and analysing the conditions under which the enzyme is operative; ii) the identification of the mechanisms beyond the antibacterial activity of azole antibiotics towards *S. aureus* that were shown to include the increase of endogenous reactive oxygen species triggered by the binding of imidazoles to flavohaemoglobin; and iii) the discovery of a novel type of bactericides, the carbon monoxide-releasing molecules, and identification of its potential cellular targets via analysis of the transcriptional response of *Escherichia coli* to CORM-2, a carbon monoxide-releasing molecule known to mimic the physiological function of carbon monoxide.

Microbes have the ability to defend themselves against an array of antimicrobial products that are generated within phagocytic cells, including nitric oxide. Flavohaemoglobins are NO-metabolising enzymes and although it is well established that they are involved in nitric oxide detoxification the dioxygen-related conditions whither their enzymatic activity is operative remain a matter of debate.

S. aureus is a pathogen that displays a high level of drug-resistance and has the ability to survive inside neutrophils. To address the *S. aureus* defence mechanisms toward nitrosative stress, the flavohaemoglobin gene was disrupted and its resistance to nitric oxide evaluated. To unmask the physiological conditions that enable *S. aureus* flavohaemoglobin to metabolise NO, the growth of *S. aureus* flavohaemoglobin null mutant was analysed under different NO/dioxygen conditions. The flavohaemoglobin protein was also cloned, expressed and purified which allowed the biochemical and spectroscopic characterisation. It was observed that: i) the over-expression of *S. aureus* flavohaemoglobin in the flavorubredoxin null mutant of *E. coli*, a strain susceptible to the deleterious effects of nitric oxide,

provides protection to *E. coli* cells against nitrosative stress under anaerobic conditions; ii) the highest induction of the flavohaemoglobin transcription is observed when *S. aureus* is grown under microaerobic conditions; and iii) a *S. aureus* strain deleted in flavohaemoglobin is susceptible to nitrosative stress exclusively under microaerobic conditions. These novel data have important implications since it shows that under physiological conditions *S. aureus* flavohaemoglobin utilises a NO denitrosylase mechanism.

S. aureus flavohaemoglobin was also demonstrated to be involved in the antimicrobial action of imidazoles antibiotics as the deletion of the flavohaemoglobin gene increases the resistance of *S. aureus* to imidazoles. To address the mechanism by which flavohaemoglobin increases *S. aureus* sensitivity to imidazoles, the binding of imidazoles to the purified flavohaemoglobin protein was analysed. Spectroscopic studies showed that imidazoles are able to bind the ferric and ferrous haem of flavohaemoglobin. Furthermore, the ligation of flavohaemoglobin to miconazole, the most active imidazole against *S. aureus*, leads to the amplification of superoxide production by the protein with consequent increase of the endogenous content of reactive oxygen species.

To examine the effect of flavohaemoglobin on the survival of miconazole-treated *S. aureus* cells in the presence of nitric oxide, the viability of *S. aureus* wild type and flavohaemoglobin null mutant was analysed after phagocytosis by LPS- and INF-γ-activated macrophages. The results showed that miconazole reduces the survival of *S. aureus* wild type, whereas the viability of miconazole-treated *S. aureus* flavohaemoglobin null mutant is higher. When *S. aureus* wild type and flavohaemoglobin null mutant infect macrophages in which the production of nitric oxide is inhibited by L-NMMA, similar results were obtained. In fact, the *S. aureus* cells expressing flavohaemoglobin display lower resistance to miconazole when compared to cells lacking the flavohaemoglobin gene.

Based on the higher content of intracellular reactive oxygen species and decreased viability of *S. aureus* cells expressing flavohaemoglobin and treated with miconazole, it is proposed that the generation of reactive oxygen species that occurs upon binding of imidazoles to flavohaemoglobin is one of the mechanisms

by which imidazoles decrease *S. aureus* survival. The fact that imidazoles inhibit the NO denitrosylase activity of flavohaemoglobin shows that the azole ligation to flavohaemoglobin also contributes to a lower ability of *S. aureus* for defence against nitrosative stress.

The carbon monoxide-releasing molecules named CO-RMs were identified as a novel group of bactericides. CO-RMs contain a transition metal coordinated to carbonyl groups and have the ability of liberating carbon monoxide to the biological systems. Several studies indicate that CO-RMs mitigate the beneficial effects of carbon monoxide on eukaryotic cells, but their role in bacteria had never been evaluated. The work presented herein shows for the first time that CO-RMs possess antimicrobial activity against Gram-negative and Gram-positive bacteria, as demonstrated by the significant decrease of cell viability of *E. coli* and *S. aureus* cells treated with CO-RMs. Furthermore, their effectiveness is reinforced towards bacteria grown under low levels of dioxygen.

Inductively coupled plasma mass spectrometry analysis revealed that after exposure to CO-RMs, *E. coli* cells display higher metal content than untreated ones, which indicates that CO-RMs are able to accumulate inside the bacterial cells where they release carbon monoxide to the cellular targets.

The antimicrobial effect of CO-RMs was shown to be related to the release of carbon monoxide since: i) the antimicrobial effect is abolished when carbon monoxide of CO-RMs is sequestered upon addition of haemoglobin to the cultures; ii) the inactive form of CO-RMs, a CO depleted form of the compound, does not have bactericidal effect; and iii) bacterial viability is lower after treatment with CO gas.

To address the mechanism that underpin the antimicrobial action of CO-RMs and to identify the bacterial targets, a transcriptomic analysis of *E. coli* exposed to tricarbonyldichlororuthenium (II) dimer, known as CORM-2, was performed. DNA microarray results obtained for *E. coli* cells grown aerobically showed that CORM-2 down-regulates the transcription of multiple enzymes of the citric acid cycle and up-regulates several methionine-related genes, indicating that the biological processes most affected by CORM-2 are the aerobic respiration and the methionine

biosynthesis. The phenotypic analysis of *E. coli* mutant strains in *metR*, the regulator of methionine biosynthesis, and in *metI* and *metN*, both described as involved in methionine transport confirmed that MetR, MetI and MetN, contribute to the lower susceptibility of *E. coli* toward CORM-2.

The data obtained for *E. coli* cells grown anaerobically revealed that upon exposure to CORM-2 the transcription of glycolytic enzymes is inhibited while several heat-shock proteins, chaperones, and proteases are induced, indicating that the pathways more affected by CORM-2 include the glycolysis and the protein folding. To address the function of protein homeostasis-related genes in the *E. coli* CORM-2 susceptibility, the phenotype of *ibpAB* and *cpxP* null mutant strains was analysed in the presence of CORM-2. The results showed that Δ*ibpAB* and Δ*cpxP* display elevated sensitivity to CORM-2 under anaerobic conditions, which reveal that IbpAB and CpxP are involved in the resistance to CORM-2.

The analysis of the results obtained for cells of *E. coli* grown aerobically and anaerobically revealed a common set of genes regulated by CORM-2. Independently of the presence of dioxygen, CORM-2 alters the transcription of several regulators namely, *frmR*, *zntR*, *narP*, *gadX, soxS* and *OxyR,* which are involved in the response to formaldehyde, zinc, nitrate/nitrite, acid and oxidative stress, respectively. Phenotypic analysis of the *soxS* null mutant*,* a strain that exhibits an increase of susceptibility to CORM-2 under aerobic and anaerobic conditions, revealed that SoxS plays an important role in protection against CORM-2 under both oxygen growth conditions. The deletion of *oxyR* decreases the viability of *E. coli* cells grown aerobically, indicating that OxyR has also a major function in protection against CORM-2. These results suggest that CORM-2 may participate in the intracellular generation of reactive oxygen species.

Since it was detected that CORM-2 affects the transcription of several genes involved in biofilm production, the evaluation of the total biofilm formed by *E. coli* cells in the presence of CORM-2 was done. The results revealed that CORM-2 does increase the biofilm production. To gain insight into the CO-RM-related function of biofilm-associated genes, the susceptibility to CORM-2 of *bshA* and *tqsA* null mutants was analysed. It was observed that the deletion of *bshA* increases the

resistance of *E. coli* to CORM-2, while the total biofilm formed upon exposure to CORM-2 is similar to that of the wild type. The deletion of *tqsA* decreases the sensitivity of *E. coli* to CORM-2 and abolishes the increase of biofilm formation trigged by the presence of CORM-2. Hence, TsqA participates in the formation of biofilm induced by CORM-2 and both TsqA and BshA contribute to the sensitivity of *E. coli* cells to CORM-2.

In conclusion, the results present in this thesis contribute to a better understanding of the role of *S. aureus* flavohaemoglobin as a fundamental bacterial enzyme involved in the detoxification of nitric oxide and reveal its potential as an antibiotic target, due to its involvement in the antibacterial activity of azoles antibiotics. A novel type of bactericides, the carbon monoxide-releasing molecules that strongly decrease the viability of Gram-negative and Gram–positive bacteria were discovered and several potential bacterial targets that contribute to CO-RMs antimicrobial action were identified. Therefore, this thesis addressed new strategies to combat the emergence and spread of drug-resistant pathogens, which due to the ineffectiveness of currently available antibiotics represents a concern for public health.

Resumo

O trabalho apresentado nesta tese teve como objectivo desvendar novos modos de acção antibacteriana através: i) do estudo das defesas de *Staphylococcus aureus* contra o óxido nítrico, uma arma antibacteriana usada pelo sistema imunitário inato, nomeadamente pela caracterização bioquímica da flavohemoglobina de *S. aureus* e pela análise das condições nas quais a atividade da flavohemoglobina opera; ii) da identificação dos mecanismos pelos quais os azoles reduzem a viabilidade de *S. aureus*, os quais incluem o aumento da produção endógena de espécies reativas de oxigénio desencadeado pela ligação dos imidazoles à flavohemoglobina; e iii) da descoberta de um novo tipo de bactericidas, as moléculas libertadoras de monóxido de carbono, e da identificação de potenciais alvos celulares através da análise da resposta transcriptional de *Escherichia coli* ao CORM-2, uma molécula libertadora de monóxido de carbono, a qual mimetiza as funções fisiológicas do monóxido de carbono.

Os micróbios têm a capacidade de se defenderem contra uma série de produtos com acção antimicrobiana que são gerados dentro das células fagociticas, os quais incluem o óxido nítrico. As flavohemoglobinas são enzimas que metabolisam o óxido nítrico, e embora esteja bem estabelecido que elas estão envolvidas na destoxificação do óxido nítrico, as condições de oxigénio em que a sua actividade catalítica opera é uma questão em debate.

S. aureus é uma bacteria patogénea que exibe um nível alto de resistência a antibióticos e tem a capacidade de sobreviver dentro dos neutrófilos. Para estudar quais os mecanismos de defesa que *S. aureus* dispõe para se defender contra o stress nitrosativo, o gene da flavohemoglobina foi interrompido e sua resistência ao óxido nítrico foi avaliada. Para conhecer as condições fisiológicas nas quais a flavohemoglobina de *S. aureus* consegue metabolizar o óxido nítrico, o crescimento de *S. aureus* foi analisado em diferentes condições de NO/oxigénio. A proteína também foi clonada, expressa e purificada de modo a proceder-se à sua

caracterização bioquímica. Foi observado que: i) a sobre-expressão da flavohemoglobina de *S. aureus* no mutante da flavorubredoxina de *E. coli*, uma estirpe suscetível aos efeitos nocivos do óxido nítrico, confere protecção às células de *E. coli* contra o stress nitrosativo, em condições anaeróbias; ii) o maior aumento da transcrição do gene da flavohemoglobina é observado quando *S. aureus* é crescida em condições microaeróbias; e iii) a estirpe de *S. aureus* deletada no gene da flavohemoglobina é suscetível ao stress nitrosativo exclusivamente em condições microaeróbias. Estes novos dados tem importantes implicações uma vez que mostram que nas condições fisiológicas a flavohemoglobina de *S. aureus* utiliza o mecanismo de NO denitrosilase.

Foi ainda demonstrado que a flavohemoglobina de *S. aureus* está envolvida na acção antimicrobiana dos antibióticos imidazoles uma vez que a deleção do gene da flavohemoglobina aumenta a resistência de *S. aureus* aos imidazoles. Para descobrir o mecanismo através do qual a flavohemoglobina aumenta a sensibilidade de *S. aureus* aos imidazoles, a ligação dos imidazoles à flavohemoglobina foi analisada. Estudos espectroscópicos mostraram que os imidazoles ligam-se ao ferro do hemo da proteina no estado férrico e no ferroso. A ligação do miconazol, o imidazol mais activo contra *S. aureus*, leva à amplificação da produção de superoxido pela proteína e aumenta consequentemente o conteúdo endógeno de espécies reativas de oxigénio em células de *S. aureus* que expressam a flavohemoglobina.

Para examinar o efeito da flavohemoglobina na sobrevivência das células de *S. aureus* tratadas com o miconazol na presença de óxido nítrico, a viabilidade da estirpe selvagem e da estirpe mutada no gene da flavohemoglobina de *S. aureus* foi analisada depois de as bacterias terem sido fagocitadas pelos macrofagos, tendo sido estes previamente activados por lipopolissacarídeos e pelo interferão-γ. Os resultados mostraram que o miconazol reduz a sobrevivência da estirpe selvagem de *S. aureus*, enquanto que a sobrevivência da estirpe mutada no gene da flavohemoglobina é maior. Quando a estirpe selvagem e a estirpe mutada no gene da flavohemoglobina de *S. aureus* infetam os macrofagos, nos quais a produção de óxido nítrico foi inibida pelo L-NMMA, resultados semelhantes foram

obtidos. De facto, as células de *S. aureus* que expressam a flavohemoglobina apresentam uma menor resistência ao miconazol do que as células que não contêm o gene que codifica a flavohemoglobina.

Baseado no alto conteúdo intracelular de espécies reativas de oxigénio e na reduzida viabilidade que as células de *S. aureus* exibem quando expressam a flavohemoglobina e são tratadas com o miconazol, é proposto que a geração de espécies reativas de oxigénio que ocorre após a ligação dos imidazoles à flavohemoglobina seja um dos mecanismos através dos quais os imidazoles diminuem a sobrevivência de *S. aureus*. Uma vez que os imidazoles inibem a actividade de NO denitrosilase da flavohemoglobina, a ligação desses compostos à flavohemoglobina, também reduz a defesa de *S. aureus* contra o stress nitrosativo, o que contribui para a diminuição da viabilidade bacteriana.

As moléculas libertadoras de monóxido de carbono denominadas de CO-RMs foram identificadas como um novo grupo de bactericidas. Os CO-RMs são constituidos por um metal de transição coordenado a grupos carbonil e tem a capacidade de libertar monóxido de carbono nos sistemas biológicos. Vários estudos mostraram que os CO-RMs mimetizam os efeitos benéficos de monóxido de carbono em células eucarióticas, mas o seu papel em bactérias nunca tinha sido avaliado. O trabalho apresentado, mostrou, pela primeira vez, que os CO-RMs possuem atividade antimicrobiana contra bactérias Gram-negativas e Gram-positivas, como concluido pela diminuição acentuada da viabilidade das células de *E. coli* e de *S. aureus* tratadas com os CO-RMs. Além disso, a eficácia dos CO-RMs é maior quando as bactérias são crescidas em baixos níveis de oxigénio.

A determinação do conteúdo de metais nas células de *E. coli* tratadas com os CO-RMs revelou que as células expostas aos CO-RMs apresentam uma maior concentração de metais do que as células não tratadas, o que indica que os CO-RMs podem se acumular dentro das células bacterianas onde libertam o monóxido de carbono para os alvos celulares.

O efeito bactericida dos CO-RMs está relacionado com a libertação de monóxido de carbono uma vez que: i) o efeito antimicrobiano é abolido quando o monóxido de carbono dos CO-RMs é sequestrado pela adição da hemoglobina às

culturas; ii) a forma inactiva dos CO-RMs, um composto que não contém grupos CO, não tem efeito bactericida; e iii) a viabilidade bacteriana é reduzida pelo tratamento com CO gás.

Para comprender o mecanismo subjacente à ação antimicrobiana dos CO-RMs e identificar os seus potenciais alvos, foi efectuada a análise transcriptomica de *E. coli* exposta ao CORM-2. Os resultados obtidos para as células de *E. coli* crescidas aerobicamente mostraram que o CORM-2 reprime a transcrição de múltiplas enzimas do ciclo do ácido cítrico e induz vários genes relacionados com a metionina, o que indica que os processos biológicos mais afectados por CORM-2 são a respiração aeróbica e a biossíntese da metionina. A análise fenotípica do mutante de *E. coli* no gene *metR*, o regulador de biossíntese da metionina, e nos genes *metI* e *metN*, ambos descritos como sendo genes envolvidos no transporte da metionina, confirmaram que o MetR, o MetI e o MetN, contribuiem para a redução da susceptibilidade de *E. coli* ao CORM-2.

Os dados obtidos para células de *E. coli* crescidas anaerobicamente e expostas ao CORM-2 revelou que a transcrição de enzimas glicolíticas foi repressa enquanto que genes codificando para proteínas de choque térmico, chaperões e proteases foram induzidos, o que indica que os processos mais afectados pelo CORM-2 incluem a glicólise e o enrolamento de proteínas. Para avaliar a função de genes relacionados com a homeostase de proteínas na susceptibilidade de *E. coli* ao CORM-2, o fenótipo de estirpes mutadas nos genes *ibpAB* e *cpxP* foi analisado na presença de CORM-2. Os resultados mostraram que a deleção desses genes aumentou a sensibilidade ao CORM-2 em condições anaeróbias, o que revela que o IbpAB e o CpxP estão involvidos na resistência ao CORM-2.

A análise comparativa dos resultados obtidos para células de *E. coli* crescidas aerobica e anaerobicamente revelou um conjunto de genes que são comumente regulados pelo CORM-2. Independentemente da presença de oxigénio, o CORM-2 alterou a transcrição de vários reguladores nomeadamente, *frmR*, *zntR*, *narP*, *gadX*, *soxS* e *OxyR*, os quais estão envolvidos na resposta ao formaldeído, zinco, nitrato/nitrito, ácido e ao stress oxidativo, respectivamente. A análise fenotípica do mutante no gene *soxS*, uma estirpe que exibe um aumento da

suscetibilidade ao CORM-2 em condições aeróbias e anaeróbias, revelaram que o SoxS tem um papel importante na proteção contra CORM-2 em ambas as condições de oxigénio usadas no crescimento. A deleção do gene *oxyR*, diminui a viabilidade das células de *E. coli* crescidas aerobicamente, indicando que o OxyR também tem uma função importante na proteção contra o CORM-2. Estes resultados sugerem que o CORM-2 poderá participar na geração intracelular de espécies reativas de oxigénio.

Uma vez que também foi detetado que o CORM-2 afeta a transcrição de vários genes implicados na produção do biofilme, o biofilme formado em células de *E. coli* na presença de CORM-2 foi avaliado. Os resultados revelaram que CORM-2 aumenta a produção de biofilme. Para compreender a função dos genes regulados pelo CORM-2 e involvidos na formação do biofilme, a susceptibilidade ao CORM-2 das estirpes mutadas no gene *bhsA* e no *tqsA* foi analisada. Observou-se que a deleção do gene *bshA* aumenta a resistência de *E. coli* ao CORM-2, enquanto que o biofilme formado após o tratamento com o CORM-2 foi semelhante ao obtido para a estirpe selvagem. A deleção do gene *tqsA* diminui a sensibilidade de *E. coli* ao CORM-2 e abole o aumento do biofilme induzido pela presença de CORM-2. Por isso, TqsA participa na formação de biofilme induzido pelo CORM-2 e tanto TqsA como BhsA contribuem para a sensibilidade das células de *E. coli* ao CORM-2.

Em conclusão, os resultados apresentados nesta tese contribuiram para um melhor conhecimento do papel da flavohemoglobina de *S. aureus*, uma enzima fundamental envolvida na destoxificação do óxido nítrico e revela o seu potencial como alvo de antibióticos, devido ao seu envolvimento na atividade antibacteriana dos azoles. Um novo tipo de bactericidas, as moléculas libertadoras de monóxido de carbono, as quais diminuem a viabilidade de bactérias Gram-negativas e Gram-positivas foram descobertos e vários potenciais alvos bacterianos que contribuiem para a sua ação antimicrobiana foram identificados. Portanto, esta tese contribuiu para revelar novas estratégias para combater o aparecimento e desenvolvimento de bactérias resistentes a antibióticos o que, devido à ineficácia dos actuais antibióticos, representa uma preocupação para a saúde pública.

Abbreviations

Δ	Deletion
ALF 062	Pentacarbonyl bromide
ALF 021	Bromo(pentacarbonyl)manganese
ATP	Adenosine triphosphate
BMPO	5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide
bp	Base pair
CFU	Colony forming units
cGMP	Cyclic guanosine monophosphate
CO	Carbon monoxide
CoA	Coenzyme A
CODH	Carbon monoxide dehydrogenase
COHb	Carboxy-haemoglobin
CO-RM	Carbon monoxide-releasing molecule
CORM-1	Dimanganese decacarbonyl
CORM-2	Tricarbonyldichloro ruthenium(II) dimmer
CORM-3	Tricarbonylchloro(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 complex
e-	Electron
E_m	Redox potencial
EPR	Electron paramagnetic resonance
ERK	Extracellular signal-regulated kinase
FAD	Flavin adenine dinucleotide
FDP	Flavodiiron protein
FDR	False discovery rate
Fe-S	Iron-Sulfur cluster
FI	Fluorescence intensity
FMN	Flavin mononucleotide
g	EPR *g*-factor
GSNO	S-nitrosoglutathione
GTP	Guanosine 5'-triphosphate

Hb	Haemoglobin
Hmp	Flavohaemoglobin
HO	Haem oxygenase
iCO-RM	Inactive form of CO-RM (depleted of CO groups)
IL-10	Interleukin 10
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IRP	Iron regulatory protein
JNK	c-Jun amino-terminal kinase
K_{Ca}	Large conductance calcium-activated potassium
Da	Dalton
LB	Luria-Bertani
L-NMMA	NG-Monomethyl-L-arginine acetate salt
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase
MBC	Minimal bactericidal concentration
MIC	Minimal inhibitory concentration
MIP-1β	Macrophage inflammatory protein 1β
MOI	Multiplicity of infection
MPO	Myeloperoxidase
MRSA	Methicillin-resistant *S. aureus*
MS	Mininal salts
MSSA	Methicillin-sensitive *S. aureus*
NAD	β-nicotinamide adenine dinucleotide, oxidized form
NADH	β-nicotinamide adenine dinucleotide, reduced form
NADP	β-nicotinamide adenine dinucleotide phosphate, oxidised form
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced form
NO	Nitric oxide
NOS	Nitric oxide synthase
OD	Optical density
PBS	Phosphate buffer
PCR	Polymerase chain reaction
Phox	NADPH oxidase
PPi	Pyrophosphate
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RR	Resonance Raman
RT-PCR	Reverse transcriptase-polimerase chain reaction

sGC	Soluble guanylate cyclase
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
TNF-α	Tumor necrosis factor α
TrHb	Truncated haemoglobin
TSA	TSB-agar
TSB	Tryptic soy broth
UV	Ultraviolet
vis	Visible
VISA	Vancomycin intermediate-resistant *S. aureus*
VRSA	Vancomycin resistant *S. aureus*
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

Strains

A.	*Alcaligene*
B.	*Bacillus*
C.	*Candida*
C. jejuni	*Campylobacter jejuni*
E. chrysanthemi	*Erwinia chrysanthemi*
E. coli	*Escherichia coli*
M.	*Mycobacterium*
N.	*Neisseria*
P.	*Pseudomonas*
P. falciparum	*Plasmodium falciparum*
R.	*Rhodospirillum*
S. aureus	*Staphylococcus aureus*
S. cerevisiae	*Saccharomyces cerevisiae*
S. coelicolor	*Streptomyces coelicolor*
S. pyogenes	*Streptococcus pyogenes*
S. Typhimurium	*Salmonella Typhimurium*

Aminoacids

A	Alanine
C	Cysteine
D	Aspartic acid
E or Glu	Glutamic acid
F or Phe	Phenylalanine
G or Gly	Glycine
H or His	Histidine
I	Isoleucine
K	Lysine
L	Leucine
M	Methionine
N	Asparagine
P or Pro	Proline
Q or Gln	Glutamine
R	Arginine
S or Ser	Serine
T or Thr	Threonine
V	Valine
W	Tryptophan
Y or Tyr	Tyrosine

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Introduction

CHAPTER 1

Resistance of *Staphylococcus aureus* to oxidative and nitrosative stress

1.1 *Staphylococcus aureus*

The name *staphylococcus* is derived from the Greek term "staphyle" meaning "a bunch of grapes". This name refers to its spherical shape (about 1 µm in diameter) arranged in pairs, short chains, or bunched in irregular groups which resemble a grape-like cluster (Figure 1.1A) [1]. The name *aureus* had its etymological origin in the Latin and means gold. The assigned name is associated with bacterium appearance as *Staphylococcus aureus* form large, round and yellow colonies on agar plates (Figure 1.1B) [2].

Staphylococci are very common in the environment and are highly resistant to hostile conditions, such as heat, desiccation, or salinity. They are readily dispersal in dust particles through the air and are often commensals on the skin or mucosa of humans and animals [3]. *S. aureus* is the best-known member of the staphylococci genus and is commonly associated with human diseases [4]. *S. aureus* is the only staphylococci that produces the enzyme coagulase, which differentiates it from the other species and is also catalase positive which allow to distinguished staphylococci from enterococci and streptococci infections [1, 4].

A B

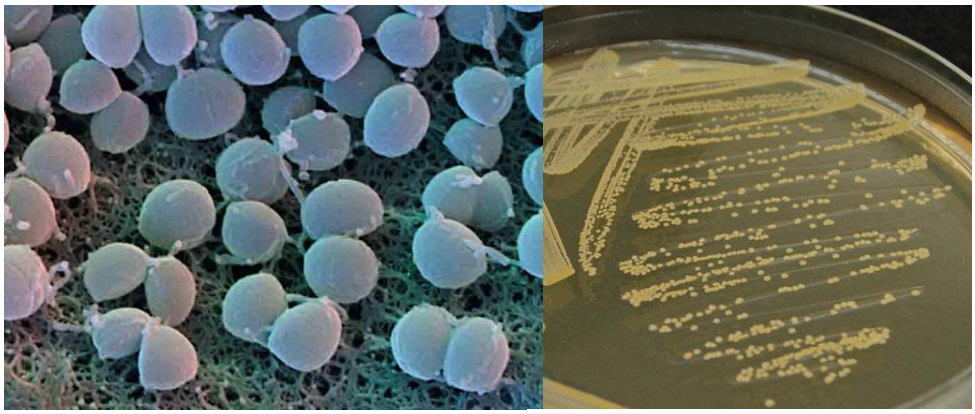

FIGURE 1.1 *S. aureus.* (A) Scanning electron microscope image of *S. aureus* (D. Scharf, http://www.scharfphoto.com). (B) *S. aureus* colonies (K. Hedetniemi and M. Liao, Furman University; ASM MicrobeLibrary.org).

S. aureus is a Gram-positive bacterium with a low GC content (~ 33 %) that can grow either in oxygenated environments or under low levels of dioxygen, being considered as a facultative anaerobe [4]. The most common habitat of *S. aureus* is the upper respiratory tract, especially the nose, throat, and the skin surface. Therefore, it is often part of the normal flora of the nasopharynx of healthy individuals (about 20 % of the population are long-term carriers of *S. aureus*) [5].

S. aureus is the most virulent staphylococci species, been frequently associated with pathological conditions ranging from mild skin infections to life-threatening diseases, including pneumonia, osteomyelitis, meningitis, arthritis, toxic shock syndrome and septicemia [4]. *S. aureus* is also a major veterinary pathogen associated with infections of an array of farm animals [6, 7].

S. aureus infections can be either acquired in the community and in association with health care, since injuries that compromise epithelial integrity, cause trauma, the surgical interventions, as well as viral infections can lead to *S. aureus* tissue invasion. Therefore, this pathogen has great importance in human medicine and the enhanced virulence that *S. aureus* has acquired through lateral gene transfer results most of the time in antibiotic resistance [8, 9]. In fact, no longer after the introduction of the first antibiotic (penicillin), the first staphylococci resistant strains appear (Table 1.1), and by late 1960s more than 80 % of both community and hospital-acquired staphylococcal isolates were resistant to penicillin [10-12]. More importantly was the emergency of methicillin-resistant *S. aureus* strains (MRSA). Methicillin is a penicillin derivative, which was introduced in 1961, and no more than one year had passed until the first case of MRSA was reported (Table 1.1) [13]. Although methicillin is no longer in use, the term MRSA continues to be

TABLE 1.1 Drugs and *S. aureus* resistance occurrence.

Drug	Year drug introduction	Years to report resistance
Penicillin	1941	1-2
Methicillin	1961	<1
Vancomycin	1956	40

Adapted from [13]

used to describe *S. aureus* strains resistant to all penicillins. MRSA strains are now worldwide spread, having a high impact on human health [14, 15].

The antibiotic vancomycin has been used as a drug of "last resort" to the treatment of serious, life-threatening infections caused by *S. aureus* and others Gram-positive bacteria [16]. However in 1997, the first report of vancomycin intermediate-resistant *S. aureus* (VISA) was identified in Japan and additional cases were subsequently reported in other countries (Table 1.1) [17, 18]. The occurrence of infections caused by vancomycin-resistant *S. aureus* (VRSA) is of great concern because, in general, these strains are also resistant to the other currently available antibiotics [12]. Nevertheless, most of *S. aureus* soft skin infections are susceptible to clindamycin or trimethoprim-sulfamethoxazole oral treatment, while intravenous therapies with vancomycin, daptomycin and linezolid are required to treat severe *S. aureus* infections [19].

TABLE 1.2 *S. aureus* completed sequenced genomes.

S. aureus	Accession Number	Length (nt)	Proteins	RNAs	Genes	Relevant Characteristics
NCTC 8325	NC_007795	2821361	2892	77	2969	MSSA
MSSA476	NC_002953	2799802	2571	104	2715	MSSA
COL	NC_002951	2809422	2612	72	2723	MRSA
MRSA252	NC_002952	2902619	2650	101	2839	MRSA
MW2	NC_003923	2820462	2624	80	2704	MRSA
N315	NC_002745	2814816	2583	79	2664	MRSA
USA300_FPR3757	NC_007793	2872769	2560	70	2648	MRSA
USA300_TCH1516	NC_010079	2872915	2654	88	2799	MRSA
Newman	NC_009641	2878897	2614	73	2687	MRSA
JH1	NC_009632	2906507	2747	81	2870	VISA
JH9	NC_009487	2906700	2697	81	2816	VISA
Mu3	NC_009782	2880168	2690	78	2768	VISA
Mu50	NC_002758	2878529	2696	77	2774	VISA
RF122	NC_007622	2742531	2509	76	2663	Bovine pathogen
ED98	NC_013450	2824404	2661	82	2752	Avian pathogen

MSSA: Methicillin-sensitive *S. aureus*
MRSA: Methicillin-resistant *S. aureus*
VISA: Vancomycin-intermediate *S. aureus* (MIC 8–16 μg/ml [12])

The dissemination of multidrug-resistant *S. aureus* represents a difficult therapeutic problem, specially because no vaccine is yet available [12, 20]. To date, the genome of 15 *S. aureus* clinical strains were sequenced and published (Table 1.2) [8].

1.2 Weapons of the innate immunity against invading pathogens

The mammalian immune system is constituted by innate and adaptive defence mechanisms and both work together protecting the host cells against microbial infections [21]. The innate immunity is mediated by pattern recognition receptors (e.g. Toll-like receptors), which bind components of the microbial cell wall such as lipopolysaccharides (LPS), peptidoglycans and lipoteichoic acids while the adaptive response is mediated by antigen receptors [21].

TABLE 1.3 Antimicrobial products of human phagocytes.

Product	Neutrophils	Macrophage*
Myeloperoxidase	+	-
Lactoferrin	+	-
Bacterial permeability increasing factor	+	-
Serprocidins	+	-
(elastase, cathepsin G, protease 3, azurocidin)		
Phospholipase A2	+	-
Cathelicidin	+	-
Lysozyme	+	-
Defensins 1-4	+	-
Reactive oxygen species	+	+
Reactive nitrogen species	+	+

* Monocytes contain some of the antimicrobial proteins of neutrophils before differentiation into macrophages

Adapted from [22]

The innate immunity is constituted by several components being phagocytes (e.g. macrophages and neutrophils) a key element whose primary function is to engulf, destroy the pathogens and digest their remains [4, 21]. On encountering bacteria, phagocytes engulf microbes into a phagosome, which fuses with

intracellular granules, e.g. lysosome, to form a phagolysosome. The first strategy of intracellular defence of phagocytes includes pathogen compartmentation and the generation of high concentrations of antimicrobials (Table 1.3) in an environment with low nutrient availability, low pH (~4.8) and iron limitation [23].

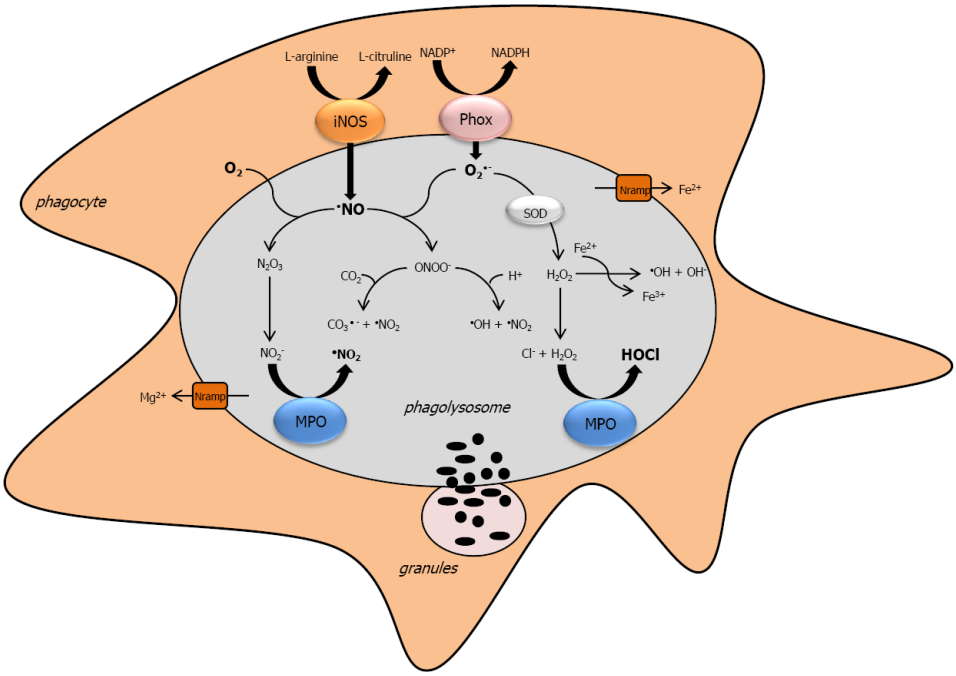

FIGURE 1.2 Schematic representation of a phagolysosome generating reactive nitrogen and oxygen species. NO synthase (NOS) and NADPH oxidase (Phox) leads to the production of nitric oxide ($^{\bullet}NO$) and superoxide ($O_2^{\bullet-}$) respectively. Superoxide is dismutated by superoxide dismutase (SOD) releasing hydrogen peroxide (H_2O_2), which in turn is converted into radical hydroxyl ($^{\bullet}OH$) and hydroxide ion (OH^-) by the Fenton reaction or is used by myeloperoxidase (MPO) to synthesise hypochlorous acid (HOCl). MPO also catalyses the reduction of nitrite (NO_2^-) to nitrogen dioxide ($^{\bullet}NO_2$). Superoxide reacts with $^{\bullet}NO$ generating peroxynitrite ($OHOO^-$). Peroxynitrite is decomposed into $^{\bullet}OH$ and nitrogen dioxide ($^{\bullet}NO_2$) or react 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). Ferroportin (Nramp) pumps Fe^{2+} and Mg^{2+} out of the phagosomal lumen and granules release their content (e.g. lysozyme, cathepsins) into the phagosome.

The sequestration of iron is an important strategy since iron is essential for cell metabolism and protection against oxidative stress [23]. Phagocytes decrease

the accessibility of iron by: i) releasing lactoferrin, an iron chelator present in granules that sequester iron in the phagosome [24]; ii) down-regulating the expression of transferrin receptor and ferritin, which is a high affinity receptor for iron and an iron storage protein, respectively [25]; and iii) removing ferrous iron from phagosomal lumen to the cytosol through ferroportin, an iron transporter that can also act as a manganese efflux pump [26, 27]. Within the phagolysosome bacteria are killed by several antimicrobial products (Table 1.3), including reactive oxygen (ROS) and reactive nitrogen species (RNS) (Figure 1.2) [28]. ROS and RNS are considered essential as antimicrobial agents of the immune system, since the simultaneously deletion of the induced nitric oxide synthase and NADPH oxidase results in massive death of mice caused by spontaneous infections with commensal microorganisms [29].

1.2.1 Oxidative stress

Production of reactive oxygen species

Oxygen toxicity has been associated with the over-production of superoxide, which leads to the production of others reactive oxygen species (Table 1.4) [30].

TABLE 1.4 Examples of reactive oxygen species.

Radicals	Non-radicals
Superoxide ($O_2^{\bullet-}$)	Hydrogen peroxide (H_2O_2)
Hydroxyl ($^{\bullet}OH$)	Hypochlorous acid (HOCl)
Peroxyl ($ROO^{\bullet}$)	
Alkoxyl ($RO^{\bullet}$)	
Hydroperoxyl ($HOO^{\bullet}$)	

Adapted from [30]

In phagocytes, the major source of reactive oxygen species is NADPH oxidase (Phox). This enzyme catalyses the reduction of dioxygen to superoxide leading to a burst of oxidative stress (Equation 1.1) [31, 32]. In resting cells, the Phox subunits (gp91, p21, p67, p47, and Rac) are present in phagocytes but are dissociated from each other. Upon phagocytosis, the cytosolic components (p67, p47 and Rac) are

translocated to the phagosome and assembled in the phagocyte membrane were the membrane components are located (gp91 and p21) which facilitated the formation of superoxide in the phagosomal lumen [33].

EQUATION 1.1 $2\ O_2 + NADPH \Rightarrow 2\ O_2^- + NADPH^+ + H^+$

EQUATION 1.2 $2\ O_2^- + 2\ H^+ \Rightarrow O_2 + H_2O_2$

EQUATION 1.3 $Cl^- + H_2O_2 \Rightarrow OCl^- + H_2O$

Superoxide dismutates spontaneously by reacting with itself (in a pH-dependent fashion) or enzymatically by superoxide dismutase (SOD), and both reactions lead to the formation of hydrogen peroxide and dioxygen (Equation 1.2) [32]. In eukaryotes two types of superoxide dismutase have been identified: a copper-zinc and a manganese-enzyme; the Cu-Zn superoxide dismutase is cytoplasmic and its expression is constitutive while the Mn-SOD is located in the mitochondria and is inducible by stress conditions [32]. In neutrophils, but not in macrophages, hydrogen peroxide together with chloride is converted into hypochlorous acid (Equation 1.3) by myeloperoxidase (MPO).

Hydroxyl radical, a highly oxidising species is formed by the reaction of superoxide with hypochlorous acid (Equation 1.4) or with hydrogen peroxide, a reaction described by Haber and Weiss (Equation 1.5) that is catalysed by transition metals (e.g. iron). In this reaction, superoxide firstly reduces ferric iron to the ferrous state forming dioxygen and subsequently hydrogen peroxide reacts with ferrous iron to form hydroxide and the hydroxyl radical while iron is reoxidised [34]. The latter reaction is denominated Fenton reaction and is the major source of hydroxyl radicals in the biological systems (Equation 1.6).

EQUATION 1.4 $HOCl + O_2^{\bullet -} \Rightarrow O_2 + Cl^- + {}^{\bullet}OH$

EQUATION 1.5 $O_2^{\bullet -} + H_2O_2 \Rightarrow O_2 + {}^{\bullet}OH + OH^-$

EQUATION 1.6 $H_2O_2 + Fe^{2+} \Rightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$

Antimicrobial action of reactive oxygen species: DNA, lipid and protein damage

Several evidences demonstrated the importance of reactive oxygen species in pathogen elimination. For example, a Phox-deficient macrophage is unable to restrict *Salmonella typhimurium* infection and is susceptible to several other pathogens, including *S. aureus* [35, 36] and mice deficient in MPO exhibit higher sensitivity to *Candida albicans* infection [32, 37]. On the other hand, the deletion of bacterial systems involved in the detoxification of reactive oxygen species (discussed forward) increases the susceptibility of pathogens to the killing by phagocytes [38, 39].

An imbalance between ROS production and antioxidant defences results in cellular oxidative stress, which causes damage to all type of biomolecules, including DNA, lipids and proteins. Much of the damage is caused by hydroxyl radicals [40].

DNA

Superoxide or hydrogen peroxide, at physiological levels do not react with DNA. However, the negatively charged of DNA molecules are prone to the binding of metal ions, namely iron and copper. The binding of metals to DNA favours the site-specific generation of hydroxyl radicals that are able to attack sugars, purines and pyrimidines on DNA, generating a multitude of products. For example, hydroxyl radical attacks the carbon-8 of guanine, generating a 8-hydroxyguanine radical, which is oxidised to 8-hydroxyguanine or reduced to give a ring-opened product (2,6-diamino-4-hydroxy-5-formamidopyrimidine) [40]; the abstraction of a hydrogen atom from the ring of thymine results in a radical that is converted into thymine peroxides or attacks the sugar moiety forming carbon-centred radicals, which in the presence of dioxygen are converted in sugar peroxyl radicals. The exposition of DNA to ROS results in single and double breaks in the backbone, adducts of base and sugar groups, and also cross-links to other molecules, interfering with the DNA repair, replication and transcription mechanisms [30].

Lipids

Polyunsaturated fatty acids in membranes are susceptible to the attack by hydroxyl radical which leads to the initiation of lipid peroxidation [40]. Lipid peroxidation is a process that is initiated by the abstraction of a hydrogen atom from an unsaturated lipid (LH) by a radical species ($R^{\bullet}$) yielding a lipidic radical ($L^{\bullet}$) (Equation 1.7). Once formed the lipidic radical, it reacts with dioxygen to form the lipidic peroxyl radical ($LOO^{\bullet}$) (Equation 1.8), which is the central specie in the propagation of lipid peroxidation (Equation 1.8-9) [41]. Therefore, a single initiation event can lead to the formation of multiple molecules of peroxyl radicals. The primary effect of lipid peroxidation is a decrease in membrane fluidity, which alters membrane proprieties and disrupts the membrane-bound proteins leading to the loss of membrane integrity [30]. As polyunsaturated fatty acids are degraded a variety of products are formed, namely aldehydes, which are very reactive molecules that damage other biomolecules such as proteins. For example, the 4-hydroxy-2-nonenal, a highly toxic product of lipid peroxidation, is a potent inhibitor of mitochondrial respiration by inhibiting α-ketoglutarate dehydrogenase, an enzyme of the citric acid cycle that catalyses the conversion of α-ketoglutarate to succinyl-CoA [40, 42].

EQUATION 1.7 $LH + R^{\bullet} \Rightarrow L^{\bullet} + RH$

EQUATION 1.8 $L^{\bullet} + O_2 \Rightarrow LOO^{\bullet}$

EQUATION 1.9 $LOO^{\bullet} + LH \Rightarrow LOOH + L^{\bullet}$

Proteins

Proteins are susceptible to be oxidised by ROS, which leads to modifications of amino acid side chains and consequently to the protein structure alteration.

Thiol groups are highly susceptible to be attack by ROS, and therefore, methionine and cysteine amino acids residues of proteins are particularly proned to be damaged by ROS. The oxidation of sulphydryl groups forms thyl radicals, especially in the presence of transition metals (Equation 1.10), which generates

disulphides (Equation 1.11). For example, methionine is oxidised by the hydroxyl radical to methionine sulphoxide (Table 1.5) [40].

EQUATION 1.10 $RSH + Cu^{2+} \Rightarrow RS^{\bullet} + 2\ H^{+} + Cu^{+}$

EQUATION 1.11 $2\ RSH \Rightarrow RS\text{-}SR + 2\ H^{+} + 2\ e^{-}$

Irreversible oxidation of amino acid residues occurs when a radical (e.g. hydroxyl) reacts with an α-hydrogen atom of an amino acid to form a carbon center radical, which in the presence of dioxygen forms an alkoxyl radical that causes cleavage of the peptide bond. In the absence of dioxygen, the carbon centered radical selft reacts generating protein-protein cross-linked derivates [40]. For example, tyrosine residues are hydroxylated to dihydroxyphenylalanine or the reaction of two tyrosine radicals results in bityrosine. Several other products of protein oxidation are shown in Table 1.5 [30].

TABLE 1.5 Amino acids oxidation products.

Amino acid	Oxidation product
Arginine	Glutamic semialdehyde
Cysteine	Disulphides, cysteic acid
Histidine	2-oxohistidine, asparagines, aspartic acid
Lysine	2-aminoadipic semialdehyde
Methionine	Methionine sulfoxide, methionine sulfone
Phenylalanine	2-3-dihydroxyphenylalanine, 2-, 3- and 4-hydroxyphenylalanine
Proline	2-pyrrolidone, 4- and 5-hydroxyproline pyroglutamic acid, glutamic semialdehyde
Threonine	2-amino-3-ketobutyric acid
Tryptophan	2-, 4-, 5-, 6-, and 7-hydroxytryptophan
Tyrosine	3,4-dihidroxyphenylalanine, tyrosine-tyrosine cross-linkages

Adapted from [40]

Iron-sulphur (Fe-S) clusters are present in several proteins that participate in many cellular processes and are very susceptible to ROS [43]. The [4Fe-4S] clusters of dehydratases are the primary intracellular target of ROS, since their clusters are substantially exposed to the solvent [44]. Superoxide and hydrogen

peroxide are small enough to invade the active site, to which they coordinate oxidising the Fe-S cluster. The loss of one or more iron ions causes the degradation of the cluster and, consequently, the inhibition of enzyme activity. Furthermore, the release of iron contributes to the increase of oxidative stress via the Fenton reaction (Equation 1.6) [30, 45-47].

In *Escherichia coli* it has been shown that superoxide is able to damage Fe-S-containing proteins that are involved in citric acid cycle (e.g. aconitase and fumarase), in amino acids biosynthesis (e.g. dihydroxyacid dehydratase) and in DNA synthesis (e.g. ribonucleotide reductase) [47, 48].

Microbial defences against oxidative stress

Microbes are able to counteract the toxic effects of ROS through the action of scavenging substrates (e.g. glutathione, ascorbic acid, α-tocopherol), DNA and proteins repairing systems, and detoxification systems [49].

Superoxide dismutase, catalase, alkyl hydroperoxidase reductase and glutathione peroxidase are important detoxification systems that afford protection against oxidative stress [50].

Superoxide dismutase efficiently dismutates superoxide into dioxygen and hydrogen peroxide (Equation 1.2). Four types of prokaryotes superoxide dismutases have been identified: the copper-zinc type (Cu/Zn-SOD), the manganese type (Mn-SOD), the iron type (Fe-SOD) and the nickel type (Ni-SOD) [17, 32]. Most bacteria possess two types of SODs: the Fe-SOD and Mn-SOD. The two enzymes are cytoplasmic and while the first is constitutive expressed the second is frequently induced by dioxygen and other stress agents (e.g. metals) [51]. The bacterial Cu/Zn-SOD are periplasmic and, as their eukaryotic counterparts, are constitutively expressed. The Ni-SOD has been identified in *Streptomyces coelicolor,* and its expression is induced by nickel [52, 53].

Catalases participate in the removal of hydrogen peroxide by converting it into dioxygen and water (Equation 1.12) [54, 55]. Deletion of this enzymatic system has been shown to increase the sensitivity of bacterial cells to hydrogen peroxide and the extension of DNA damage [56].

EQUATION 1.12 $2\ H_2O_2 \Rightarrow O_2 + 2\ H_2O$

Alkyl hydroperoxidase reductase and glutathione peroxidase reduce toxic organic hyperoxides (e.g. linoleic acid and cumene peroxide) to the corresponding alcohols. In addition, both enzymes are able to reduce hydrogen peroxide and peroxynitrite [57-60]. However, while alkyl hydroperoxidase reductase uses NADH(P)H as electron donor, glutathione peroxidase reaction is coupled to the oxidation of reduced glutathione [30]. The importance of these systems is evidenced in several microbial models. For example, the disruption of alkyl hydroperoxide reductase increases the susceptibility of *S. typhimurium* to cumene hydroperoxide, an oxidising agent that promotes lipid peroxidation [57], whereas the deletion of glutathione peroxidase increases the sensitivity of *Neisseria meningitidis* to paraquat (a potent superoxide generator) and decreases the pathogenecity of *Streptococcus pyogenes* in murine models of infection [58, 61].

1.2.2 Nitrosative stress

Production of nitric oxide in biological systems

Nitric oxide (NO) is produced non-enzymatically by mild acidification of nitrite (Equation 1.13-15). This source of NO is proposed to contribute to the killing of pathogens especially in the stomach and in urine [62].

EQUATION 1.13 $NO_2^- + H^+ \Rightarrow HNO_2$

EQUATION 1.14 $2HNO_2 \Rightarrow N_2O_3 + H_2O$

EQUATION 1.15 $N_2O_3 \Rightarrow NO + NO_2$

The major source of endogenous production of NO occurs enzymatically through the oxidation of L-arginine to citruline catalysed by NO synthase (NOS) (Figure 1.3) [63, 64]. NOSs are constituted by two domains: an oxygenase and a

reductase domain, which are linked by a regulatory calmoduline binding domain. The oxygenase domain contains a haem as the active center that binds the L-arginine substrate. The reductase domain binds co-factors and provides electrons from NADPH, via FAD and FMN, for L-arginine [64]. The synthesis of NO occurs in the presence of tetrahydrobiopterin (BH_4) via two sequential monooxygenase reactions (Figure 1.3) [63]. Mammalians contain three types of NOS: the endothelial, the neural and the inducible (eNOS, nNOS and iNOS, respectively) [65]. The two first isoforms are constitutively expressed and their activity depend on calcium concentrations, while the activity of the last one is calcium-independent and inducible by stimulus such as cytokines (e.g. IFN-γ) and microbial products (e.g. LPS) [63, 65]. Another important difference between these enzymes is the amount and the duration of NO produced by each one [49]. eNOS and nNOS release picomoles of NO during some minutes, whereas iNOS can produce NO up to five days when care is taken to replenish both the inductive stimuli and the L-arginine substrate reaching the concentration of NO the micromolar levels [63]. NO has a high diffusion coefficient (3300 $\mu m^2 s^{-1}$), and due to its lipophilic character and small size it diffuses across the biological membranes by passive transport. Consequently, the diffusion distance of NO from NO-releasing cells is 100-200 μm [66, 67]. At concentrations as low as those present in vascular or neural cells, NO has been shown to be involved in numerous biological processes such as signalling messenger, vasodilatation, inhibition of platelet aggregation, blood pressure regulation and neurotransmission [68-72]. At high

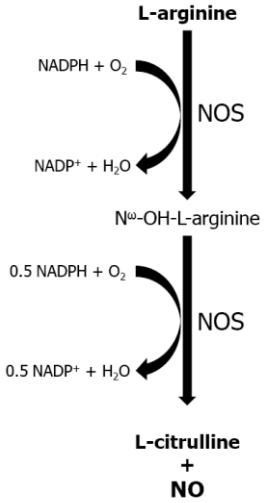

FIGURE 1.3 Synthesis of NO by NOS. L-arginine is first oxidised to N^{ω}-OH-L-arginine, which is further oxidised to L-citruline releasing NO. Both reactions require dioxygen and the NADPH is used as electron-donor. Adapted from [63].

concentrations, like the ones produced by iNOS, NO plays an important role in pathogen eradication (see below) [73-75].

In bacteria, NO can be also produced by NOS activity. Bacterial NOS proteins are smaller than their mammalian counterparts and do not contain the reductase domain which is essential to supply electrons during NO biosynthesis [76]. There are few reports on bacterial NOS and although most of the enzymes, so far identified, lack the reductase domain they successfully produce NO *in vitro* when provided with an electron donor, such as hydrogen peroxide, or with a mammalian reductase domain [77]. Nevertheless, bacterial genomes encode many reductases that might support the NOS activity. One example is the YkuNP reductase from *Bacillus subtilis* that bears NOS activity *in vitro* [78]. Recently, Gusarov and co-workers have shown that NO is indeed produced *in vivo* in *B. subtilis* [76]. Furthermore, the *S. aureus* NOS was purified in an intact functional form that contains a reductase subunit and the enzyme was shown to produce NO [79]. Although the physiological function of endogenous mammalian NO production is well established, the role of NOS enzymes in bacterial system remains to be elucidated [68].

NO is also generated in bacterial cells during the bacterial denitrification process. Denitrification is an anaerobic respiration in which N-oxide, instead of dioxygen, serves as the electron acceptor for the generation of an electrochemical gradient across the cytoplasmic membrane [80, 81]. During denitrification, nitrate is enzymatically and successively reduced via nitrite, NO and nitrous oxide to dinitrogen gas (Figure 1.4) [80].

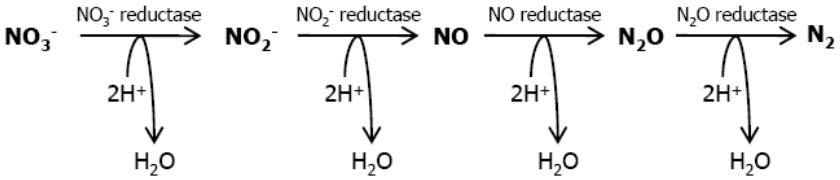

FIGURE 1.4 Enzymatic reactions involved in denitrification. Nitrate (NO_3^-) is reduced to nitrite (NO_2^-) by NO_3^- reductase. Nitrite is reduced, via the NO_2^- reductase, to nitric oxide (NO), which is reduced to nitrous oxide (N_2O) by NO reductase. Nitrous oxide yields dinitrogen gas (N_2) by the reaction of the N_2O reductase. Adapted from [80].

Although all the genes encoding the enzymes mentioned in Figure 1.4 are found in denitrifying bacteria, nitrate and nitrite reductase are also found in pathogenic bacteria and its function is associated with the persistence of pathogens in anaerobic environments [81, 82]. For example, *N. gonorrhoeae* contains a nitrite reductase, which has been associated with the ability of the bacteria to growth under anaerobic conditions, using nitrite as a terminal electron acceptor. Nitrate reductase of *Mycobacterium bovis*, an obligate aerobe that encodes both a nitrate and a nitrite reductase has been shown to be essential for the persistence of *M. bovis* in the lungs, liver, and kidneys of mice [83, 84].

Reactivity of nitric oxide

The chemical proprieties of NO (Table 1.6) were firstly described in 1772 by Joseph Priestley and until the mid-1980s it was considered only as an atmospheric pollutant [85]. The discovery of NO as the endothelium-derived relaxing factor changed the perception of NO as a toxic to a molecule of mount importance involved in a wide range of biological processes [68-70, 86]. These findings initiated a new era for NO biology and, in 1992, NO was elected the molecule of the year by Science and in 1998, R. F. Furchgott, L. J. Ignarro and F. Murad won the Nobel Prize in Physiology or Medicine for their discoveries on NO as a signalling molecule (http://nobelprize.org/nobel_prizes/medicine) [87].

NO is a diatomic paramagnetic molecule that, at room temperature and atmospheric pressure, is a colourless gas, slightly soluble in water (Table 1.6) and approximately nine times more soluble in organic solvents [67, 88].

NO is a free radical, that has one unpaired electron in an anti-bonding π orbital and, in spite of its radical character, is considered a relatively stable radical when compared with other species (Table 1.7). The product of one-electron reduction of NO is the nitroxyl ion (NO^-), which is isoelectronic to dioxygen, whereas the removal of the unpaired electron of NO yields the nitrosonium cation (NO^+) that is isoelectronic with carbon monoxide. Although NO is quite difficult to oxidise and its reduction is rather unfavourable, the interconversion of NO into NO^- and NO^+ can occur under physiological conditions, and therefore the three species must be considered in order to fully account for its biological activity. The nitroxyl ion is an unstable molecule, with a short lifetime in solution (order of milliseconds), that rapidly decomposes to give nitrous oxide. The nitrosonium cation has a even smaller lifetime (~ 3 x 10^{-10} seconds) been rapidly hydrolysed, in aqueous solutions, to give nitrous acid [90].

TABLE 1.6 Chemical and physical proprieties of NO.

Property	Value
Interatomic distance (pm)	115
Melting point (ºC)	-163.6
Boiling point (ºC)	-151.8
Density (g/L)	1.34
Solubility in water (mM)	2*

* at room temperature

Adapted from [89]

TABLE 1.7 Halflife and reactivity of oxygen and nitrogen species.

Reactive species	Half-life (s)	Reaction rate ($M^{-1}s^{-1}$)
Hydroxyl radical ($^{\bullet}OH$)	10^{-9}	10^9-10^{10}
Peroxynitrite anion ($ONOO^-$)	0.05-1.0	10^2
Peroxyl radical ($ROO^{\bullet}$)	7	10^3
Nitric oxide ($^{\bullet}NO$)	1-10	$<10^1$
Superoxide anion ($O_2^{\bullet -}$)	hours (by SOD 10^{-6})	10^1

Adapted from Mascio. P. (personal communication)

NO reacts with others radicals and gives rise to species of greater reactivity and toxicity, the so-called reactive nitrogen species, which have a relevant role in biological processes. Among them, is the oxidant peroxynitrite that results from the reaction of NO with superoxide (Equation 1.16). This reaction is extremely fast (~7 x 10^9 $M^{-1}s^{-1}$) and occurs at a rate higher than the superoxide decomposition catalysed by superoxide dismutase (Equation 1.2) [91]. Peroxynitrite can form peroxynitrous acid, an unstable molecular and reactive oxidising species that yields hydroxyl and nitrogen dioxide radicals [90].

The chemistry of NO in the presence of molecular oxygen is complex due to the large number of parallel occurring reactions. NO reacts rapidly with dioxygen to form nitrogen dioxide (Equation 1.17). Nitrogen dioxide has an unpaired electron, been unlike NO, a potent oxidant. In aqueous solution, nitrogen dioxide may react with another NO molecule yielding dinitrogen trioxide (Equation 1.18), which is the anhydride of nitrous acid and therefore the product of the reaction of NO with dioxygen in aqueous solution is indeed nitrite. With air, nitrogen dioxide dimerises to give dinitrogen tetroxide (Equation 1.19), which when dissolved in water forms nitrite and nitrate [92].

EQUATION 1.16 $NO + O_2^{\bullet -} \Rightarrow ONOO^-$

EQUATION 1.17 $2NO + O_2 \Rightarrow 2NO_2$

EQUATION 1.18 $NO + NO_2 \Rightarrow N_2O_3$

EQUATION 1.19 $NO_2 + NO_2 \Rightarrow N_2O_4$

Cellular targets of reactive nitrogen species

The ability of the host to control microbial proliferation and elimination is linked with its ability to induce the production of NO by iNOS since it has been demonstrated that: i) the *in vitro* treatment with NO gas or organochemical NO donors decreases the viability of several microorganisms [63, 68]; ii) in sites of infection of animal models the expression of iNOS is augmented [93]; iii) NOS

inhibitors contribute to worsen the course of several diseases caused by bacteria [63]; and iv) mice deficient in iNOS are more susceptible to infection caused by a wide number of intracellular pathogens such as *Salmonella enterica* [49].

The antimicrobial action of NO generated by iNOS have been attributed to the NO radical as well as to the several NO-derivatives products (Figure 1.2), which are highly toxic to biological components. RNS react with cellular components, like DNA, lipids, proteins-containing metals, thiols, and aromatic residues, leading to several cell damage (Figure 1.5).

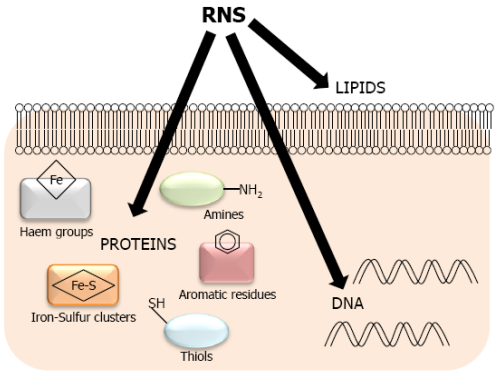

FIGURE 1.5 Microbial cellular targets of reactive nitrogen species. Adapted from [93]

DNA

NO by itself cannot damage DNA since *in vitro* experiments indicated that NO requires the presence of dioxygen to deaminate DNA [94]. However, RNS like nitrogen trioxide and peroxynitrite cause DNA injuries. Nitrogen trioxide nitrosates amines on DNA bases to form N-nitrosamines leading to deamination, mispairing and cross-links. For example, guanine can be deaminated by nitrogen trioxide to form xanthine and the mispairing of xanthine causes a G:C to A:T transition. Alternatively, xanthine can be depurinated to form an abasic site which is cleaved by endonucleases given rise to single strand breaks [95]. Peroxynitrite oxidises DNA causing single-strand breaks by attacking the sugar-phosphate backbone [96]. Unlike nitrogen trioxide, which can react with adenine, guanine and cytosine, the peroxynitrite preferentially reacts with guanine, and the two main products from the reaction of deoxyguanosine with peroxynitrite are 8-oxo-deoxyguanosine

and 8-nitro-deoxyguanosine. The first product is known to also causes G:C to A:T mutations and the second rapidly depurinates leading to the formation of abasic bases sites [95].

Lipids

NO is not a strong oxidant and cannot begin the lipid peroxidation. However, in presence of superoxide or dioxygen NO forms peroxynitrite and the nitrogen dioxide radical, respectively (Equation 1.16-17), and both are powerful oxidising species capable of initiating and propagating lipid peroxidation [41, 97, 98]. NO also reacts with organic peroxyl radicals generating alkyl peroxynitrite (LOONO) (Equation 1.20), which recombines to give unreactive alkyl nitrates ($LONO_2$) (Equation 1.21) or produces through homolytic cleavage, alkoxyl ($LO^{\bullet}$) and nitrogen dioxide radicals (Equation 1.22).

EQUATION 1.20 $^{\bullet}NO + LOO^{\bullet} \Rightarrow LOONO$

EQUATION 1.21 $LOONO \Rightarrow LONO_2$

EQUATION 1.22 $LOONO \Rightarrow LO^{\bullet} + {}^{\bullet}NO_2$

Although the radicals formed by homolytic cleavage of LOONO are expected to contribute to the propagation of lipid peroxidation, the major product, $LONO_2$, does not [99, 100]. Therefore, through radical-radical reactions, NO can also act as an inhibitor of lipid peroxidation and, in fact, it has been shown that NO is 10^4-10^5 times a more potent peroxyl radical scavenger than α-tocopherol [101]. Nevertheless, NO will react more rapidly with superoxide than with organic peroxyl radicals and hence the oxidant proprieties of NO should prevail [91, 99].

Proteins

The thiol groups of cysteine and methionine residues are among the most important protein targets of NO, and under anaerobic conditions this reaction

generates disulphide and nitrous oxide (Equation 1.23) [102]. NO also reacts with thiyl radical species to form S-nitrosothiols (Equation 1.24) [92].

EQUATION 1.23 $2\ RSH + 2\ NO \Rightarrow RSSR + N_2O + H_2O$

EQUATION 1.24 $^{\bullet}NO + RS^{\bullet} \Rightarrow RSNO$

In the presence of dioxygen, NO is oxidised to species containing NO^+ which display high electrophilicity and reactivity towards nucleophilic centers. The predominant nitrosating agent arising from NO at physiological pH is nitrogen trioxide which is a strong electrophile able to nitrosylate thiols and amines. However, under physiological conditions, nitrosylation of sulphur (S-nitrosylation) (Figure 1.6A) is preferred over nitrogen (N-nitrosylation) [90, 103]. The S-nitrosylation of sulphydryl groups leads to the formation of S-nitrosothiols (Figure 1.6A), which also contain the NO^+ group and therefore are able to nitrosylate thiol groups generating others S-nitrosothiols in a process named transnitrosylation (Figure 1.6B). The NO^- species also display reactivity toward thiol groups, particularly when in the presence of ferrous iron or other transition metals [90, 103].

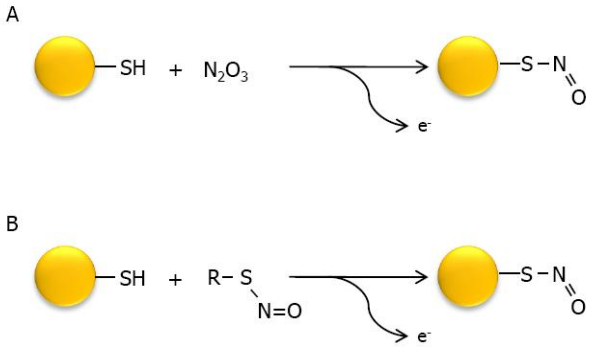

FIGURE 1.6 Schematic representation of nitrosothiols adducts formation. NO is oxidised by dioxygen to species containing NO^+ such as N_2O_3, that S-nitrosylate sulfhydryl groups forming S-nitrosothiols (upper panel). S-nitrosothiols are also able to transfere its NO^+ group to thiol-containing proteins/peptides in a process named transnitrosylation (lower panel).

S-nitrosoglutathione (GSNO) is the most abundant S-nitrosothiol in eukaryotic cells (in a concentration range of μM) and is formed by the nitrosylation of glutathione, a tripeptide with potent antioxidant proprieties that is composed by

glutamic acid, cysteine and glycine [103, 104]. In bacteria, glutathione is found predominantly in Gram-negative species and among the Gram-positive, so far, only the steptococcus and enterococcus species are able of producing glutathione [105]. Nevertheless, most bacteria encounter GSNO intracellularly at the sites of infections, since glutathione and NO are present in host cells at high concentrations. In particular, glutathione reaches concentrations as high as 10 mM in eukaryotic cells [106].

GSNO participates in protein transnitrosylation through NO^+ chemistry, and its homolytic cleavage, which occurs in the presence of cellular reductants and transition metals, generates NO. However, the transnitrosylation reaction of S-nitrosothiols generally occurs more rapidly than its decomposition [107, 108].

The aromatic residues tyrosine, tryptophan, phenylalanine and histidine are susceptible to oxidation (Table 1.5). Peroxynitrite, by reacting with carbon dioxide (Figure 1.2), mediates the nitration of tyrosine groups yielding 3-nitrotyrosine in a two-step reaction in which the carbonate abstracts a hydrogen atom from a tyrosine residue, generating a tyrosyl radical that reacts with nitrogen dioxide [100]. Nitration has an important role in biological systems since it is an irreversible process. In particular, the nitration of one tyrosine residue per subunit of the adenylated glutamine synthetase leads to the complete loss of the enzyme activity [109, 110].

NO binds to most transition metal-containing proteins [89, 111]. Of notable importance in biological processes is the reaction of NO with iron, particularly the reaction with the iron of haem groups and with iron-sulphur centers. Like other gaseous molecules, such as dioxygen and carbon monoxide, NO binds reversibly to ferrous haem (Equation 1.25) [112]. For example, the binding of NO to the ferrous haem of cytochrome *c* oxydase results in the impairment of the respiration [113]. In contrast to molecular oxygen and carbon monoxide, NO also binds to ferric haem forming ferrous-nitrosonium complex (Equation 1.26) [114]. The binding of NO to the ferric haem is less tightly than that to ferrous iron; however, once

bound, the latter reaction is less reversible [112]. An example of the deleterious effects of NO is observed upon binding to catalase that contains a ferric haem center, which leads to inhibition of the enzyme and consequently decreased of the cellular resistance to oxidative stress (see 1.2.1) [115]. NO also reacts with oxy-haem complexes generating nitrate and ferric haem (Equation 1.27), a reaction that occurs typically with haemoglobins [112].

EQUATION 1.25 $NO + Fe^{2+} \Leftrightarrow Fe^{2+}—NO$

EQUATION 1.26 $NO + Fe^{3+} \Leftrightarrow Fe^{3+}—NO\ (Fe^{2+}—NO^{+})$

EQUATION 1.27 $Fe^{3+}—O_2^{\bullet-} + NO \Rightarrow Fe^{3+}—ONOO^{-} \Rightarrow Fe^{3+} + NO_3^{-}$

The reaction of NO with iron-sulphur centers results in the formation of dinitrosyl-iron-dithiol complexes (DNIC) (Figure 1.7), which have been detected in biological systems that have a high content of NO (e.g. macrophages and tumors) [112]. As stated before, dehydratases contain a [4Fe-4S] center, in which the iron is very exposed to solvent and therefore they are a class of enzymes prone to be also damaged by NO [116]. The most studied example is aconitase, a key enzyme in the citric acid cycle that catalyses the conversion of citrate and isocitrate into aconitate [117]. The formation of DNIC leads to the disruption of the Fe-S center causing the inhibition of aconitase activity [118-120].

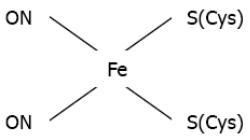

FIGURE 1.7 Dinitrosyl-iron-dithiol complex.

Bacterial defence against nitrosative stress

The three major known NO-metabolising enzymes are the NO reductases, the flavodiiron proteins and the globin-like proteins, that include the flavohaemoglobins (discussed in 1.4) [121-123].

Bacterial NO reductases (NORs) reduce NO to nitrous oxide and can be set into two distinct groups: one is the cytochrome *bc*-type complex which is typically found in denitrifiers [124]; the other group only contains a cytochrome *b*-type component and is present in several non denitrifiers pathogens [121]. The role of NORs in the protection against nitrosative stress is well established. For example, the *nor* mutant of *N. meningitidis* displays higher susceptibility to exogenously added NO and NO-related compounds and exhibits impaired survival within nasopharyngeal mucosa [125, 126]. Similar results were report for a *nor* deficient mutant of *Pseudomonas aeruginosa* which besides exhibiting high sensitivity to exogenous NO-treatment it has reduced intracellular survival within macrophages when compared to that of the parental strain [127].

Flavodiiron proteins (FDPs) are constituted by a metallo β-lactamase like-domain harboring a diiron catalytic site in the N-terminal and a flavodoxin-like domain with a FMN moiety. Additionally, several members of this family have extra domains fused at the C-terminal [122]. For example, *E. coli* FDP (also refered as flavorubredoxin) has an extra rubredoxin domain and is able to reduce NO, being the first FDP implicated in the detoxification of NO [128]. Subsequent studies have confirmed the role of other FDPs as NO detoxifiers, revealing the correspondent mutant strains increased sensitivity to nitrosative stress conditions [129-131].

1.3 *S. aureus* defence mechanisms

Although *S. aureus* represents the prototype of an extracellular pathogen, several investigations Revealed its ability to infect various types of host cells, including neutrophils [132-136].

In the first hours after bacterial infection, pathogens are cleared from the blood stream, in part by the phagocytic activity [137]. In particular, it was demonstrated that the deletion of the Toll-like receptor 2, a receptor that is implicated in the recognition of Gram-positive bacterial components such as lipoproteins, increases the mice susceptibility to *S. aureus* infections [138]. On the other hand, *S. aureus* variants lacking lipoproteins can effectively escape the immune recognition and cause lethal infection [139].

As all successful pathogens, *S. aureus* possesses several structures and biochemical features that allow to avoid phagocytosis and to resist inside the phagocytic cells. For example, *S. aureus* produces coagulase that causes deposition of fibrin in the pathogen's membrane and protects it from the attack of host cells. In addition, *S. aureus* synthesises leukocidins, proteins that are able to destroy the phagocytes. Hence, the pathogen is not killed upon ingestion; instead it lyses the phagocyte causing the release of the lysosomal content to the cytoplasm [4]. *S. aureus* is also able to neutralise the toxic products released by phagocytic cells [4]. For example, staphylokinase, a serine protease-like exoprotein, can inhibit the antibacterial effect of α-defensis on *S. aureus* cell wall [140]. Moreover, several *S. aureus* strains are proposed to produce other extracellular products such as exotoxins (e.g. haemolysin), enterotoxins (e.g. staphylococcal enterotoxin B) and proteolitic enzymes (e.g. fibrinolysin) [4].

Of particular importance are the mechanisms that *S. aureus* uses to acquire iron, an element present at low concentration inside host cells and vital to the survival of all organisms. The majority of iron in the human body (60-75 %) is present intracellularly and bound to haemoglobin (Hb), an abundant protein of the erythrocytes [141]. *S. aureus* can activate the lysis of erythrocytes by producing haemolysin, which leads to the releasing of haemoglobin/haem to the extracellular fluids [142]. The cell-wall sorted proteins of the *S. aureus* iron-regulated surface determinant system bind haemoglobin and other haemoproteins to remove the haem molecule and transport it through the cell wall and plasma membrane. Once inside the bacterium, haem is either degraded by staphylococcal haem monoxygenases (described in Chapter 2) or segregated to the bacterial membrane, where it is likely incorporated intact into the bacterial haem-binding proteins [143, 144].

Other strategy employed by *S. aureus* to acquire iron involves siderophores. Siderophores are low-molecular-weight iron-binding complexes that are secreted from the bacterial cell and are responsible for removing host iron from sources such as transferrin, lactoferrin and ferritin. *S. aureus* has been shown to contain at least four type of siderophores: staphyloferrin A, staphyloferrin B, aureochelin and

hydroxamate siderophores [145-148]. The contribution of siderophores to *S. aureus* pathogenesis is highlighted by the demonstration that a siderophore synthesis mutant exhibits a defect in virulence in a mouse model of abscess formation [149].

1.3.1 *S. aureus* defences against oxidative stress

S. aureus has efficient tools to defend against oxidative stress. It has been shown that inside neutrophils, *S. aureus* has higher mRNA expression levels of catalase, superoxide dismutase, thioredoxin, alkyl hydroperoxide reductase and glutathione peroxidase [136].

Catalase activity is associated with pathogenicity since virulent *S. aureus* strains have higher levels of catalase in opposition to non-virulent strains [150]. Exogenous addition of catalase decreases the ability of neutrophils to kill low-catalase expressing *S. aureus* strains and a catalase-deficient strain showed to be hypersensitive to hydrogen peroxide [55, 151]. However, the catalase mutant does not have attenuated pathogenicity in a murine skin abscess model of infection [151]. In *S. aureus*, catalase and alkyl hydroperoxide reductase seem to have compensatory roles since a catalase mutant contains higher levels of alkyl hydroperoxide reductase which is in agreement with the resistance studies performed in the murine skin abscess model of infection [151, 152]. In addition, alkyl hydroperoxide reductase and catalase are reported to be required for survival and persistence of nasal colonisation of *S. aureus* [152].

S. aureus has two superoxide dismutases, SodA and SodM, both with manganese as co-factor. Although only the *sodA*-mutant exhibits high susceptibility to oxidative stress, the over-expression of SodM in a double mutant was shown to decrease the susceptibility of *S. aureus* to oxidative stress [153, 154].

S. aureus produces carotenoid pigments that are proposed to be involved in protection against phagocyte generating oxidative burst as judged by the results showing that in a model mouse of subcutaneous abcess the *S. aureus crtM* mutant, that encodes an essential gene involved in carotenoid biosynthesis,

exhibits lower degree of oxidative stress resistance, neutrophil survival and pathogenicity [2].

1.3.2 *S. aureus* defences against nitrosative stress

S. aureus proteins involved in anaerobic metabolism are proposed to afford protection to *S. aureus* cells when challenged with NO. In fact, a recent study showed that when exposed to NO *S. aureus* shifts its metabolism by inducing lactate dehydrogenase (ldh1), which converts piruvate into L-lactate. The *ldh1*-mutant has high sensitivity to NO, decreased viability within macrophages and lower virulence in a murine sepsis model, demonstrating that lactate dehydrogenase activity is essential to *S. aureus* resistance to NO [155]. Another study conducted by the same authors showed that *S. aureus* deleted in *srrAB*, genes encoding a two component regulatory system that controls the transition to anaerobic metabolism, are also very sensitive to nitrosative stress [156].

S. aureus is highly resistant to nitrosative stress [156]. However, the mechanisms by which bacteria detoxify the reactive nitrogen species are almost unknown. Recently, our laboratory has identified a novel *S. aureus* nitroreductase that, apart from being involved in the activation of nitrofurans, also confers protection against GSNO. In fact, the nitroreductase-mutant strain exhibits higher growth impairment upon exposure to GSNO and enzymatic studies Revealed that the protein is able to reduced GSNO [157]. In this thesis we showed that *S. aureus* genome encodes a flavohaemoglobin that efficiently metabolise NO and afford protection to *S. aureus* cells against nitrosative stress (Chapter 3) [156, 158].

1.4 Flavohaemoglobins

Microbial haemoglobins are a diverse group of proteins which are divided into three classes (Figure 1.8) [123].

The first class is typified by the *Vitreosilla* globin (Vgb). Vgb was the first bacterial globin to be isolated and is a dimeric protein containing two haems *b* per molecule [159]. All proteins of this class are predicted to possess a typical globin fold and share amino acid sequence identities with Vgb ranging from 22 %

(Thermobifida fusca) to 66 % (Clostridium perfringens) with key amino acids around the haem pocket strictly conserved (His-F8, Phe-CD1 and Tyr-B10) [123]. The physiological function of these globins is associated with transport of dioxygen [123]. Interestingly, a chimeric protein comprising *Vitreosilla*–globin domain fused with a flavoreductase domain of *Alcaligenes eutropha* flavohaemoglobin was found to consume NO and provide *E. coli* protection from nitrosative stress [160]. This suggests that when present in the monomeric form Vgb may became associated with a reductase domain, therefore acquiring the ability to relieve the nitrosative stress [160]. The homologous globin of Vgb in *Campylobacter jejune* (Cgb) is also involved in the defence against nitrosative stress as demonstrated by the hypersensitivity of a *cgb*-mutant strain upon exposure to NO and other nitrosating agents, and by the markedly inhibition of the aerobic respiration of the *cgb*-mutant strain [161].

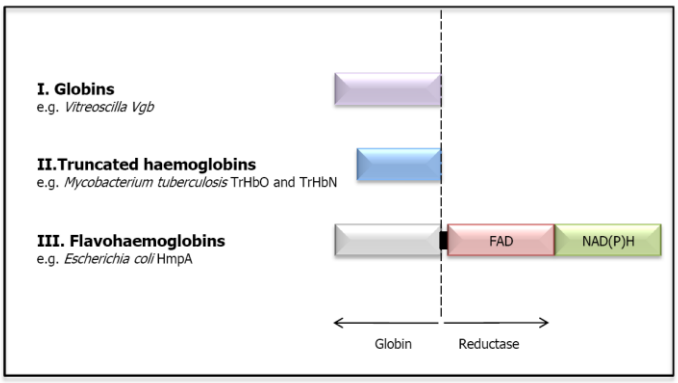

FIGURE 1.8 Classification of microbial haemoglobins by their domain architecture. The class I comprises the proteins that have a typical globin fold like the *Vitreoscilla* globin (Vgb) while class II contains the truncated haemoglobins. Class III includes the two domains proteins like flavohaemoglobins which are proteins constituted by a haem-globin domain plus a reductase domain. Adapted from [123]

The second class comprises the truncated haemoglobins (trHb), which display a smaller globin domain with a different fold but retained the key residues required for haem binding and interaction with small ligands [123, 162]. The function of these proteins is not completely understood; however, it has been demonstrated that they may be involved in dioxygen and NO metabolism. TrHb binds dioxygen

with high affinity making unlikely that it acts as an oxygen carrier. However, trHb of *M. tuberculosis* (trHbO) has been shown to enhance oxygen uptake of *E. coli* membrane fractions [163]. A second trHb was identified in *M. tuberculosis* (trHbN) and reported to detoxify NO and to protect aerobic respiration from NO inhibition [164].

Flavohaemoglobins (Hmps) are the third class of microbial haemoglobins and have a unique two-domain structure, composed by a globin fused with a reductase domain. Hmps are widely distributed in Gram-positive and Gram-negative bacteria, in yeasts and fungi [123, 162]. The first bacterial *hmp* gene discovered was that of *E. coli* [165]; although many years before a haemoglobin-like containing protein was purified from yeast, a physiological function could not be assigned at that time [166, 167].

Hmps display a globin-like domain in the N-terminal with a binding site for a haem *b* and a C-terminal domain, that resembles a ferredoxin $NADP^+$ reductase, with binding sites for flavin (FAD) and NAD(P)H (Figure 1.9) [168, 169].

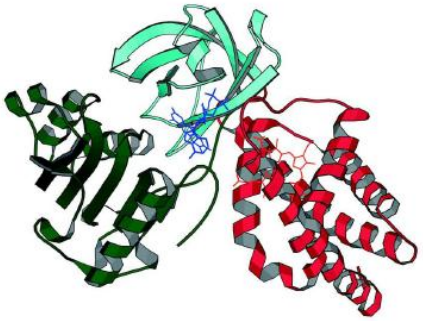

FIGURE 1.9 Structure of *E. coli* flavohaemoglobin. Heart-shaped structure in which three different domains are distinguished: the globin domain at the N-terminal (red), a flavin-binding domain (cyan), and the NAD-binding domain on the C-terminal (green). Adapted from [169].

The X-ray structures of *E. coli* (Figure 1.9) and *A. eutrophus* Hmp show that the architecture of the globin domain correspond to a classic globin fold composed by six helices (from A to H) and the amino acids sequence alignment of all known Hmps Revealed that these proteins share conserved residues in both the haem and flavin binding sites [169-171]. The conserved amino acids within the haem domain include the residues linked to the haem pocket on proximal and distal sites. Residues His85 and Phe88, located on proximal site, allow the

covalently linkage of haem to the polypeptide chain and the correct orientation of haem within the pocket, respectively [170], while the distal-side residues, Tyr29 and Gln53, control the interaction with the iron-bound ligand. Mutagenic studies Revealed that the replacement of the Tyr residue by Phe decreases the reaction rate of Hmp with NO by a factor of ~ 30 [172].

The amino acid residues responsible for the flavin binding are also strictly conserved, namely Phe290, Gly270, Glu388, Thr272, Pro273, Gly186, Gln187, Tryr188 and Ser232. These residues appear to control and shape the flavin-binding cavity, thus reducing the solvent access and providing the structural basis for the interaction with NAD(P)H as substrate [171].

1.4.1 Physiological function

The first indication that Hmp could be involved in NO metabolism arised from the observation that *hmp* expression is markedly induced by NO gas, as well as by GSNO and sodium nitroprusside (SNP) [173, 174]. Studies performed with *E. coli* mutants selected on a screening of NO-resistance genes Revealed that they exhibited an oxygen-dependent NO-consuming activity which was attributed to flavohaemoglobin [175]. Further work reported that *hmp*-mutants of *E. coli* are sensitive to nitrosative stress and presently, the role of Hmp-mediating NO protection is well established for a large array of microorganisms [175-177]. In particular, Hmps have been shown to protect *S. typhimurium*, *P. aeruginosa*, *Erwinia chrysanthemi*, *B. subtilis*, *Saccharomyces cerevisiae* and *Cryptococcus neoformans* from nitrosative stress generate by different agents [178-184]. The deletion of *hmp* gene results in strains hyper-sensitive to the killing by macrophages [178, 184-186] and Hmp is required to confer virulence in mice and to allow *E. chrysanthemi* infection of *Saintpaulia ionantha* plants [156, 178, 181]. Therefore, Hmp is considered a NO-detoxifying enzyme that provides protection against nitrosative stress under aerobic and anaerobic conditions [187].

Interestingly, the *E. coli* Hmp was also shown to interact with bacterial lipid membranes and is able to specifically bind unsaturated or cyclopropanated fatty acids [188, 189]. Hmps are also able to mediate the electron transfer to an array

of molecules/enzymes when haem is blocked (e.g. with CO). Under such conditions, FAD is able to transfer electrons from NAD(P)H to a number of external acceptors like cytochrome *c* and paraquat, among others [190-192].

1.4.2 Enzymatic activity

Flavohaemoglobins are able to detoxify NO either aerobically by oxidising NO to nitrate and anaerobically by reducing NO to nitrous oxide [175, 193, 194]. However, the reaction rate occurring in the presence of dioxygen is higher than that under anoxic conditions and the mechanism beyond Hmp catalytic activity is a matter of debate since two hypotheses are considered: the NO dioxygenase and the NO denitroxylase (Figure 1.10) [175, 193-196].

Gardner and co-workers proposed a mechanism analogous to that described for haemoglobin, in which the dioxygen that binds to the Hmp ferrous haem is reduced to superoxide while the haem is oxidised. Then, NO reacts with the bounded superoxide producing nitrate and regenerating the ferric haem. This reaction is denominated as NO dioxygenase activity (Figure 1.10 low part, right reaction) [112, 175, 194]. Since dioxygen and NO have similar association constants (17-50 $\mu M^{-1}s^{-1}$ for O_2 and 10-26 $\mu M^{-1}s^{-1}$ for NO), the binding to ferrous haem will be dependent on the relative concentrations of dioxygen and NO; if $[O_2]$ > [NO], dioxygen will bind first, whereas when $[O_2]$ < [NO] NO will preferentially bind to the haem. Furthermore, the K_M for dioxygen is higher than for NO ($K_M[O_2]$ = 30-100 μM and K_M[NO] = 0.1-0.25 μM) and, therefore, when present at similar concentrations the binding of NO is preferred over that of dioxygen [175, 197]. Considering the kinetic parameters mentioned above and the higher dissociation constant (K_d) for dioxygen when compared to that of NO, ($K_d[O_2]$ = 12 nM and K_d[NO] = 0.008 nM), the NO dioxygenase activity (Figure 1.10 low part, right reaction) will be only functional when $[O_2]$ >> [NO] [172, 175, 193, 197, 198].

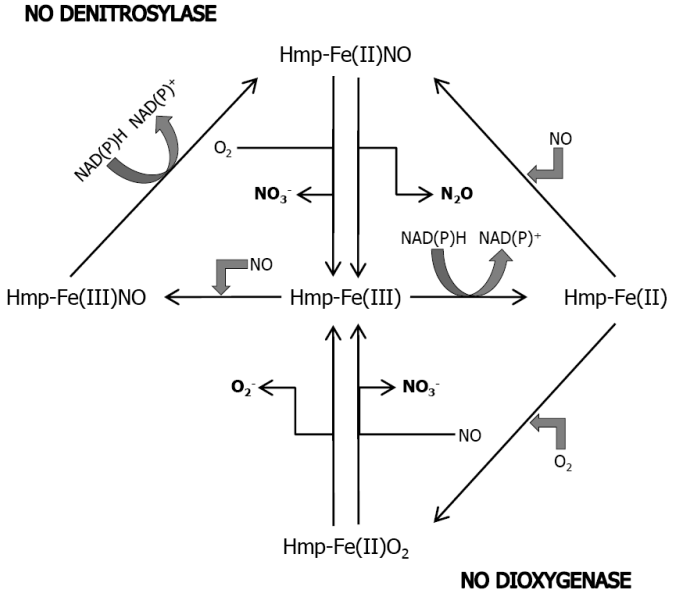

FIGURE 1.10 Catalytic activity of Hmp. Ferric Hmp (Hmp-Fe(III)) reacts with either NAD(P)H or NO, forming ferrous Hmp (Hmp-Fe(II)) or ferric-nitrosyl Hmp (Hmp-Fe(III)NO), respectively. The latter is subsequently reduced by NAD(P)H to form ferrous-nitrosyl Hmp (Hmp-Fe(II)NO). Hmp-Fe(II) reacts either with NO or O2, forming Hmp-Fe(II)NO or HmpFe(II)O2, respectively; the first reacts with O2 to give nitrate (NO_3^-) or dimerises in the absence of O_2 forming N_2O (NO denitroxylase activity, upper reactions); the second reacts with NO generating also nitrate. The oxidase activity reaction, in which dioxygen is reduced to superoxide is also shown in left site of the lower part of the figure. Adapted from [195].

However, pathogens are exposed to high concentrations of NO generated by macrophages in low dioxygen environments and since Hmp displays higher affinity for NO than for dioxygen, and NO binds both ferric and ferrous haem whereas dioxygen only binds the ferrous form, the reaction of Hmp is likely to proceed through a denitrosylase pathway, as proposed by Hausladen *et al.,* in which NO will bind to ferrous haem, being then reduced to nitroxyl anion that subsequently reacts with dioxygen generating nitrate (Figure 1.10, upper part). In the absence of dioxygen, the reaction proceeds more slowly, yielding nitrous oxide by dimerisation and dehydration of HNO [195] (Figure 1.9 upper part, right reaction). This mechanism seems to be the most probable one based on physiological conditions and, in fact, it has been shown that Hmp is able to protect microbes against high NO concentrations anaerobically, a condition under which the NO dioxygenase activity cannot be operative [172, 175, 193, 195, 197, 198].

Hmps have the potential of exhibiting different enzymatic activities by mediating the electron transfer from NAD(P)H to the haem bound-ligand. Hence, in an environment lacking NO, Hmp is also able to reduce dioxygen to superoxide (Figure 1.10 lower part, left reaction) [199-201]. Furthermore, under anaerobic conditions, Hmp reduces hydroperoxides (e.g. tert-butyl hydroperoxide, cumyl hydroperoxide and linoleic acid hydroperoxide) to the corresponding alcohols (Table 1.8), acting as an alkyl hydroperoxide reductase [202]. Since *hmp* expression is induced by paraquat and the lipids hydroperoxides can be generated during oxidative and nitrosative stress, it is also proposed that Hmp may be involved in the repair of membrane lipids [202, 203].

TABLE 1.8 Michaelis constant (K_M) for *E. coli* Hmp.

Substrate	K_M (μM at 25 °C, pH 7)
NO	0.11
O_2	27
Linoleic acid hydroperoxide	26
Cumyl hydroperoxide	55
Tert-butyl hydroperoxide	76
H_2O_2	260

Adapted from [171]

1.4.3 Regulation of flavohaemoglobin transcription

The expression of *hmp* gene is increased by several factors: by NO and NO related compounds [173, 180, 204-206]; in the stationary cell growth phase [203, 207]; by the decrease of iron generated by the iron chelator 2,2'-dipyridyl [173]; by paraquat [203]; and limitation of dioxygen [208, 209].

Microarrays studies performed in cells of a wide array of microorganisms have shown that *hmp* is one of the few genes whose expression is always induced by NO, independently of the agent used to generate nitrosative stress [130, 156, 204, 210-213]. However, the available data Reveals that the mechanism beyond the regulation of *hmp* by NO is very complex involving several regulators [173].

Most of the studies on *hmp* regulation have been performed in *E. coli* but, at least, two regulators have been shown to also regulate the *hmp* transcription in several other microorganisms, namely the transcription factor fumarate-nitrate

reduction, known as FNR, and the NO-sensitive repressor NsrR [178, 182, 214-218].

FNR is a dioxygen and a NO sensing transcription factor via its labile [4Fe-4S] cluster and it represses the *hmp* expression under anaerobic conditions [214]. Several evidences link the regulation of *hmp* to FNR: i) the *fnr* mutation enhances *hmp* expression [173, 217]; ii) the *hmp* promoter displays two putative FNR binding motifs [207, 214]; and iii) the anaerobic reaction of NO with the Fe-S cluster of FNR leads to the formation of a dinitrosyl-iron cysteine complex, decreasing the affinity of FNR to the *hmp* promoter and leading to a derepression of the *hmp* transcription [214].

NsrR, a member of the Rrf2 family of transcriptional repressors, regulates the expression of genes involved in nitrosative stress response, including *hmp* [215]. NsrR is a NO sensor that contains a [2Fe-2S] cluster whose nitrosylation results in the lost of its DNA binding activity [216]. The activity of *hmp* promoter is constitutively expressed in a *nsrR* mutant, under both aerobic and anaerobic conditions, which indicates that *hmp* promoter is a target for repression mediated by NsrR [178, 215].

In *E. coli* it has been demonstrated that, under aerobic conditions, MetR also participates in the *hmp* regulation. MetR regulates the expression of *glyA* (a gene that is divergently transcribed from *hmp* in *E. coli*, as well as in other bacterial genomes), and uses homocysteine as a cofactor [123, 210]. High levels of homocysteine decreases the expression of *hmp*, and the low levels present in *metC* mutant are associated with high levels of *hmp* expression [174]. Since GSNO can nitrosylate homocysteine, it is considered that the induction of *hmp* by nitrosating agents such as GSNO may be explained by the decrease of homocysteine levels, enhancing the binding of MetR at a proximal site of *hmp* promotor and therefore inducing the *hmp* transcription [174].

In *B. subtilis*, *hmp* transcription, which is repressed by NsrR, is also dependent of ResDE, a transduction system that is induced by oxygen limitation and by NO [182, 218]. However, while in a *nsrR* null mutant the *hmp* transcription is derepressed in the presence of NO, the NO-inducible expression of *hmp* in a

resED deleted strain is diminished but not completely abolished [219]. Interestingly, *S. aureus* genome encodes an analogue transduction system of *B. subtilis* ResDE, the SrrAB system and, like it occurs in *B. subtilis*, *S. aureus* NO-dependent *hmp* transcription is decreased in a *srrAB*-deleted strain, but not completely eliminated [156].

1.4.4 Azole antibiotics and flavohaemoglobin

Azole antibiotics are N-1 substituted compounds containing two or three nitrogens atoms in the azole ring that are thereby classified as imidazoles (e.g. miconazole and clotrimazole) or triazoles (e.g. fluconazole and itraconazole).

The azoles were initially used to treat fungal infections [220]. Clotrimazole was one of the first imidazole to be developed. It was synthesised, in 1969, by chemists at Bayer AG and it is nowadays used for the topical treatment of both cutaneous and vaginal candidiasis and other superficial fungal infections [221, 222]. However, oral administration of clotrimazole has been shown to cause side effects that include gastrointestinal disturbances and alterations in hepatic and adrenal functions [223, 224]. In the same year, Jassen Pharmaceutica synthesised miconazole [225]. In contrast to clotrimazole, miconazole exhibits low toxicity and was the first azole derivative to be applied intravenously for the therapy of systemic fungal infections. Furthermore, miconazole was the first azole reported to be active against Gram-positive bacteria [226]. The two azoles continue to be administrated today and are used as control drugs in many clinical trials of newer azole derivatives [222]. Ketoconazole was also developed by Jassen Pharmacetica in 1977 and is a broad-spectrum antifungal agent, which can be also administrated orally [227]. Ketoconazole is widely used to treated topical and systemic fungal infections, being considered the most successful azole derivative. Latter, Pfizer U.K. developed fluconazole, one of the first triazole agents to be synthesised. Triazoles have a broad range of applications in the treatment of both superficial and systemic fungal infections and display greater affinity for fungal rather than mammalian cytochrome P450 enzymes, which contributes to an improved safety profile [228].

The principal mode of action of azoles antibiotics lays on the inhibition of P450 lanosterol 14α-demethylase, which is a key enzyme in ergosterol biosynthesis, a major component of the fungal plasma membrane. The inhibition of P450 lanosterol 14α-demethylase activity occurs by the binding, as a sixth ligand, of the nitrogen atom of the imidazole ring to the haem of the enzyme causing the depletion of ergosterol and accumulation of the toxic 14α-methylated sterols, which alters the normal permeability and fluidity of the membranes [220].

The ability of azoles to coordinate the haem of several others enzymes suggests the existence of other potential cellular targets. For example, in *C. albicans* and *S. cerevisiae* catalase and peroxidase are inhibited by miconazole, with consequent increase of the endogenous ROS production [220, 229, 230]. Clotrimazole inhibits the haemoperoxidase of *Plasmodium falciparum*, which leads to the increase of oxidative stress and results in the impairment of the growth of the different strains of *P. falciparum* and contributes to the antimalary action of clotrimazole [231-233]. Recently, the action of azoles was also correlated with the inhibition of the NO scavenger activity of flavohaemoglobin in *E. coli*, *S. cerevisiae* and *C. albicans* [234].

Although azoles are also active against bacteria, in particular for Gram-positive bacteria, the cellular targets remains elusive [235, 236]. Earlier reports demonstrated that miconazole has antimicrobial activity against *S. aureus* and causes K^+ release suggesting membrane damage [237]. In the present thesis's work, the mode of action of azole antibiotics was explored in *S. aureus*, as shown in Chapter 4.

1.5 References

[1] Masoud Haghkhah, D.V.M. (2003) Study of virulence factors of *Staphylococcus aureus*. In: Division of Infection and immunity, Doctor of philosophy, Faculty of Biomedical and Life Sciences, University of Glasgow, Glasgow.

[2] Liu, G.Y., Essex, A., Buchanan, J.T., Datta, V., Hoffman, H.M., Bastian, J.F., Fierer, J. and Nizet, V. (2005) *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. Journal of Experimental Medicine 202, 209-215.

[3] Cossart, P., Boquet, P., Normark, S. and Rappuoli, R. (2005) Celular Microbiology. ASM Press Washington.

[4] Madigan, M.T., Martinko, J.M. and Parker, J. (2000) Biology of Microorganisms. Prentice-Hall, Inc, New Jersey.

[5] Wertheim, H.F., Melles, D.C., Vos, M.C., van Leeuwen, W., van Belkum, A., Verbrugh, H.A. and Nouwen, J.L. (2005) The role of nasal carriage in *Staphylococcus aureus* infections. Lancet Infect. Dis. 5, 751-762.

[6] Bergonier, D., de Cremoux, R., Rupp, R., Lagriffoul, G. and Berthelot, X. (2003) Mastitis of dairy small ruminants. Vet. Res. 34, 689-716.

[7] Hermans, K., Devriese, L.A. and Haesebrouck, F. (2003) Rabbit staphylococcosis: difficult solutions for serious problems. Vet. Microbiol.. 91, 57-64.

[8] Ben Zakour, N.L., Guinane, C.M. and Fitzgerald, J.R. (2008) Pathogenomics of the staphylococci: insights into niche adaptation and the emergence of new virulent strains. FEMS Microbiol. Lett. 289, 1-12.

[9] Fitzgerald, J.R., Sturdevant, D.E., Mackie, S.M., Gill, S.R. and Musser, J.M. (2001) Evolutionary genomics of *Staphylococcus aureus*: insights into the origin of methicillin-resistant strains and the toxic shock syndrome epidemic. Proc. Natl. Acad. Sci. U S A 98, 8821-8826.

[10] Fleming, A. (1929) On the antibacterial action of cultures of a penicillium with special reference to their use in the isolation of B. influenzae. . British Journal of Experimental Pathology 10, 226–236.

[11] Spink, W.W. and Ferris, V. (1947) Penicillin-resistant staphylococci; mechanisms involved in the development of resistance. J. Clin. Invest. 26, 379-393.

[12] Lowy, F.D. (2003) Antimicrobial resistance: the example of *Staphylococcus aureus*. J. Clin. Invest. 111, 1265-1273.

[13] Chambers, H.F. (2001) The changing epidemiology of *Staphylococcus aureus*? Emerg. Infect. Dis. 7, 178-182.

[14] Klevens, R.M., Morrison, M.A., Nadle, J., Petit, S., Gershman, K., Ray, S., Harrison, L.H., Lynfield, R., Dumyati, G., Townes, J.M., Craig, A.S., Zell, E.R., Fosheim, G.E., McDougal, L.K., Carey, R.B. and Fridkin, S.K. (2007) Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. Jama 298, 1763-1771.

[15] Grundmann, H., Aanensen, D.M., van den Wijngaard, C.C., Spratt, B.G., Harmsen, D. and Friedrich, A.W. Geographic distribution of *Staphylococcus aureus* causing invasive infections in Europe: a molecular-epidemiological analysis. PLoS Med. 7, e1000215.

[16] Feng, Y., Chen, C.J., Su, L.H., Hu, S., Yu, J. and Chiu, C.H. (2008) Evolution and pathogenesis of *Staphylococcus aureus*: lessons learned from genotyping and comparative genomics. FEMS Microbiol. Rev.. 32, 23-37.

[17] Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T. and Tenover, F.C. (1997) Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. J. Antimicrob. Chemother. 40, 135-136.

[18] Smith, T.L., Pearson, M.L., Wilcox, K.R., Cruz, C., Lancaster, M.V., Robinson-Dunn, B., Tenover, F.C., Zervos, M.J., Band, J.D., White, E. and Jarvis, W.R. (1999) Emergence of vancomycin resistance in

Staphylococcus aureus. Glycopeptide-Intermediate *Staphylococcus aureus* Working Group. N. Engl. J. Med. 340, 493-501.

[19] LaPlante, K.L., Leonard, S.N., Andes, D.R., Craig, W.A. and Rybak, M.J. (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.

[20] Projan, S.J., Nesin, M. and Dunman, P.M. (2006) Staphylococcal vaccines and immunotherapy: to dream the impossible dream? Curr. Opin. Pharmacol. 6, 473-479.

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

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

[23] Radtke, A.L. and O'Riordan, M.X. (2006) Intracellular innate resistance to bacterial pathogens. Cell Microbiol. 8, 1720-1729.

[24] Byrd, T.F. and Horwitz, M.A. (1991) Lactoferrin inhibits or promotes *Legionella pneumophila* intracellular multiplication in nonactivated and interferon gamma-activated human monocytes depending upon its degree of iron saturation. Iron-lactoferrin and nonphysiologic iron chelates Reverse monocyte activation against *Legionella pneumophila*. J. Clin. Invest. 88, 1103-1112.

[25] Byrd, T.F. and Horwitz, M.A. (1993) Regulation of transferrin receptor expression and ferritin content in human mononuclear phagocytes. Coordinate upregulation by iron transferrin and downregulation by interferon gamma. J. Clin. Invest. 91, 969-976.

[26] Forbes, J.R. and Gros, P. (2003) Iron, manganese, and cobalt transport by Nramp1 (Slc11a1) and Nramp2 (Slc11a2) expressed at the plasma membrane. Blood 102, 1884-1892.

[27] Chlosta, S., Fishman, D.S., Harrington, L., Johnson, E.E., Knutson, M.D., Wessling-Resnick, M. and Cherayil, B.J. (2006) The iron efflux protein ferroportin regulates the intracellular growth of *Salmonella enterica*. Infect. Immun. 74, 3065-3067.

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

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

[30] Halliwell, B. and Gutteridge, J.M.C. (1999) Free radicals in biology and medicine. Oxford science publications, New York.

[31] Cathcart, M.K. (2004) Regulation of superoxide anion production by NADPH oxidase in monocytes/macrophages: contributions to atherosclerosis. Arterioscler. Thromb. Vasc. Biol. 24, 23-28.

[32] Babior, B.M. (2000) Phagocytes and oxidative stress. Am. J. Med. 109, 33-44.

[33] Diacovich, L. and Gorvel, J.P. Bacterial manipulation of innate immunity to promote infection. Nat. Rev. Microbiol. 8, 117-128.

[34] Koppenol, W.H. (2001) The Haber-Weiss cycle-70 years later. Redox Rep. 6, 229-234.

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

[36] Jackson, S.H., Gallin, J.I. and Holland, S.M. (1995) The *p47phox* mouse knock-out model of chronic granulomatous disease. J. Exp. Med. 182, 751-758.

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

[38] Basu, M., Czinn, S.J. and Blanchard, T.G. (2004) Absence of catalase reduces long-term survival of Helicobacter pylori in macrophage phagosomes. Helicobacter 9, 211-216.

[39] La Carbona, S., Sauvageot, N., Giard, J.C., Benachour, A., Posteraro, B., Auffray, Y., Sanguinetti, M. and 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.

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

[41] Hogg, N. and Kalyanaraman, B. (1999) Nitric oxide and lipid peroxidation. Biochim. Biophys. Acta 1411, 378-384.

[42] Humphries, K.M. and Szweda, L.I. (1998) Selective inactivation of alpha-ketoglutarate dehydrogenase and pyruvate dehydrogenase: reaction of lipoic acid with 4-hydroxy-2-nonenal. Biochemistry 37, 15835-15841.

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

[44] Lauble, H., Kennedy, M.C., Beinert, H. and Stout, C.D. (1992) Crystal structures of aconitase with isocitrate and nitroisocitrate bound. Biochemistry 31, 2735-2748.

[45] Jang, S. and Imlay, J.A. (2007) Micromolar intracellular hydrogen peroxide disrupts metabolism by damaging iron-sulfur enzymes. J. Biol. Chem. 282, 929-937.

[46] Kuo, C.F., Mashino, T. and Fridovich, I. (1987) alpha, beta-Dihydroxyisovalerate dehydratase. A superoxide-sensitive enzyme. J. Biol. Chem. 262, 4724-4727.

[47] Flint, D.H., Tuminello, J.F. and Emptage, M.H. (1993) The inactivation of Fe-S cluster containing hydro-lyases by superoxide. J. Biol. Chem. 268, 22369-22376.

[48] Gaudu, P., Niviere, V., Petillot, Y., Kauppi, B. and Fontecave, M. (1996) The irreversible inactivation of ribonucleotide reductase from *Escherichia coli* by superoxide radicals. FEBS Lett. 387, 137-140.

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

[50] Farr, S.B. and Kogoma, T. (1991) Oxidative stress responses in *Escherichia coli* and *Salmonella typhimurium*. Microbiol. Rev. 55, 561-585.

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

[52] Barondeau, D.P., Kassmann, C.J., Bruns, C.K., Tainer, J.A. and Getzoff, E.D. (2004) Nickel superoxide dismutase structure and mechanism. Biochemistry 43, 8038-8047.

[53] Tietze, D., Breitzke, H., Imhof, D., Kothe, E., Weston, J. and Buntkowsky, G. (2009) New insight into the mode of action of nickel superoxide dismutase by investigating metallopeptide substrate models. Chemistry 15, 517-523.

[54] Papp-Szabo, E., Sutherland, C.L. and Josephy, P.D. (1993) Superoxide dismutase and the resistance of *Escherichia coli* to phagocytic killing by human neutrophils. Infect. Immun. 61, 1442-1446.

[55] Mandell, G.L. (1975) Catalase, superoxide dismutase, and virulence of *Staphylococcus aureus*. *In vitro* and *in vivo* studies with emphasis on staphylococcal-leukocyte interaction. J. Clin. Invest. 55, 561-566.

[56] Hong, Y., Wang, G. and Maier, R.J. (2007) A Helicobacter hepaticus catalase mutant is hypersensitive to oxidative stress and suffers increased DNA damage. J. Med. Microbiol. 56, 557-562.

[57] Chen, L., Xie, Q.W. and Nathan, C. (1998) Alkyl hydroperoxide reductase subunit C (AhpC) protects bacterial and human cells against reactive nitrogen intermediates. Mol. Cell 1, 795-805.

[58] Brenot, A., King, K.Y., Janowiak, B., Griffith, O. and Caparon, M.G. (2004) Contribution of glutathione peroxidase to the virulence of *Streptococcus pyogenes*. Infect. Immun. 72, 408-413.

[59] Poole, L.B. (2005) Bacterial defenses against oxidants: mechanistic features of cysteine-based peroxidases and their flavoprotein reductases. Arch. Biochem. Biophys. 433, 240-254.

[60] Bryk, R., Griffin, P. and Nathan, C. (2000) Peroxynitrite reductase activity of bacterial peroxiredoxins. Nature 407, 211-215.

[61] Moore, T.D. and Sparling, P.F. (1996) Interruption of the *gpxA* gene increases the sensitivity of *Neisseria meningitidis* to paraquat. J. Bacteriol. 178, 4301-4305.

[62] Weitzberg, E. and Lundberg, J.O. (1998) Nonenzymatic nitric oxide production in humans. Nitric Oxide 2, 1-7.

[63] MacMicking, J., Xie, Q.W. and Nathan, C. (1997) Nitric oxide and macrophage function. Annu. Rev. Immunol. 15, 323-350.

[64] Stuehr, D.J. (1999) Mammalian nitric oxide synthases. Biochim. Biophys. Acta 1411, 217-230.

[65] Alderton, W.K., Cooper, C.E. and Knowles, R.G. (2001) Nitric oxide synthases: structure, function and inhibition. Biochem. J. 357, 593-615.

[66] Malinski, T., Taha, Z., Grunfeld, S., Patton, S., Kapturczak, M. and Tomboulian, P. (1993) Diffusion of nitric oxide in the aorta wall monitored *in situ* by porphyrinic microsensors. Biochem. Biophys. Res. Commun. 193, 1076-1082.

[67] Lancaster, J.R., Jr. (1997) A tutorial on the diffusibility and reactivity of free nitric oxide. Nitric Oxide 1, 18-30.

[68] Moncada, S., Palmer, R.M. and Higgs, E.A. (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. 43, 109-142.

[69] Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E. and Chaudhuri, G. (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc. Natl. Acad. Sci. U S A 84, 9265-9269.

[70] Bredt, D.S. and Snyder, S.H. (1989) Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. Proc. Natl. Acad. Sci. U S A 86, 9030-9033.

[71] Radomski, M.W., Palmer, R.M. and Moncada, S. (1990) An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. Proc. Natl. Acad. Sci. U S A 87, 5193-5197.

[72] Moncada, S., Radomski, M.W. and Palmer, R.M. (1988) Endothelium-derived relaxing factor. Identification as nitric oxide and role in the control of vascular tone and platelet function. Biochem. Pharmacol. 37, 2495-2501.

[73] Hibbs, J.B., Jr., Taintor, R.R., Vavrin, Z. and Rachlin, E.M. (1988) Nitric oxide: a cytotoxic activated macrophage effector molecule. Biochem. Biophys. Res. Commun. 157, 87-94.

[74] Anggard, E. (1994) Nitric oxide: mediator, murderer, and medicine. Lancet 343, 1199-1206.

[75] Moncada, S. (1992) Nitric oxide gas: mediator, modulator, and pathophysiologic entity. J. Lab. Clin. Med. 120, 187-191.

[76] Gusarov, I., Starodubtseva, M., Wang, Z.Q., McQuade, L., Lippard, S.J., Stuehr, D.J. and Nudler, E. (2008) Bacterial nitric-oxide synthases operate without a dedicated redox partner. J. Biol. Chem. 283, 13140-13147.

[77] Adak, S., Aulak, K.S. and Stuehr, D.J. (2002) Direct evidence for nitric oxide production by a nitric-oxide synthase-like protein from Bacillus subtilis. J. Biol. Chem. 277, 16167-16171.

[78] Wang, Z.Q., Lawson, R.J., Buddha, M.R., Wei, C.C., Crane, B.R., Munro, A.W. and Stuehr, D.J. (2007) Bacterial flavodoxins support nitric oxide production by *Bacillus subtilis* nitric-oxide synthase. J. Biol. Chem. 282, 2196-2202.

[79] Hong, I.S., Kim, Y.K., Choi, W.S., Seo, D.W., Yoon, J.W., Han, J.W., Lee, H.Y. and Lee, H.W. (2003) Purification and characterization of nitric oxide synthase from *Staphylococcus aureus*. FEMS Microbiol. Lett. 222, 177-182.

[80] Zumft, W.G. (1997) Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. 61, 533-616.

[81] Philippot, L. (2005) Denitrification in pathogenic bacteria: for better or worst? Trends Microbiol. 13, 191-192.

[82] Ferguson, S.J. (1994) Denitrification and its control. Antonie Van Leeuwenhoek 66, 89-110.

[83] Knapp, J.S. and Clark, V.L. (1984) Anaerobic growth of *Neisseria gonorrhoeae* coupled to nitrite reduction. Infect. Immun. 46, 176-181.

[84] Fritz, C., Maass, S., Kreft, A. and Bange, F.C. (2002) Dependence of *Mycobacterium bovis* BCG on anaerobic nitrate reductase for persistence is tissue specific. Infect. Immun. 70, 286-291.

[85] Schofield, R.E. (1966) A scientific autobiogrphy of Joseph Priestley (1733-1804): selected scientific correspondence. MIT Press, Cambridge, MA.

[86] Ignarro, L.J. (2000) Nitric Oxide - Biology and Pathobiology. Academic Press.

[87] Culotta, E. and Koshland, D.E., Jr. (1992) NO news is good news. Science 258, 1862-1865.

[88] Ford, P.C. and Lorkovic, I.M. (2002) Mechanistic aspects of the reactions of nitric oxide with transition-metal complexes. Chem. Rev. 102, 993-1018.

[89] Greenwood, N.N. and Earnshaw, A. (1997) Chemistry of the elements. Reed educational and professional publishing Ltd.

[90] Hughes, M.N. (1999) Relationships between nitric oxide, nitroxyl ion, nitrosonium cation and peroxynitrite. Biochim. Biophys. Acta 1411, 263-272.

[91] Huie, R.E. and Padmaja, S. (1993) The reaction of no with superoxide. Free Radic. Res. Commun. 18, 195-199.

[92] Hughes, M.N. (2008) Chemistry of nitric oxide and related species. Methods Enzymol. 436, 3-19.

[93] Fang, F.C. (1997) Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity. J. Clin. Invest. 99, 2818-2825.

[94] Wink, D.A., Kasprzak, K.S., Maragos, C.M., Elespuru, R.K., Misra, M., Dunams, T.M., Cebula, T.A., Koch, W.H., Andrews, A.W., Allen, J.S. and et al. (1991) DNA deaminating ability and genotoxicity of nitric oxide and its progenitors. Science 254, 1001-1003.

[95] Burney, S., Caulfield, J.L., Niles, J.C., Wishnok, J.S. and Tannenbaum, S.R. (1999) The chemistry of DNA damage from nitric oxide and peroxynitrite. Mutat. Res. 424, 37-49.

[96] Beckman, J.S. (1996) Oxidative damage and tyrosine nitration from peroxynitrite. Chem. Res. Toxicol. 9, 836-844.

[97] Radi, R., Beckman, J.S., Bush, K.M. and Freeman, B.A. (1991) Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. Arch. Biochem. Biophys. 288, 481-487.

[98] Church, D.F. and Pryor, W.A. (1985) Free-radical chemistry of cigarette smoke and its toxicological implications. Environ. Health Perspect. 64, 111-126.

[99] Padmaja, S. and Huie, R.E. (1993) The reaction of nitric oxide with organic peroxyl radicals. Biochem. Biophys. Res. Commun. 195, 539-544.

[100] Pryor, W.A., Houk, K.N., Foote, C.S., Fukuto, J.M., Ignarro, L.J., Squadrito, G.L. and Davies, K.J. (2006) Free radical biology and medicine: it's a gas, man! Am. J. Physiol. Regul. Integr. Comp. Physiol. 291, R491-511.

[101] Goss, S.P., Hogg, N. and Kalyanaraman, B. (1997) The effect of nitric oxide release rates on the oxidation of human low density lipoprotein. J. Biol. Chem. 272, 21647-21653.

[102] DeMaster, E.G., Quast, B.J., Redfern, B. and Nagasawa, H.T. (1995) Reaction of nitric oxide with the free sulfhydryl group of human serum albumin yields a sulfenic acid and nitrous oxide. Biochemistry 34, 11494-11499.

[103] Gaston, B. (1999) Nitric oxide and thiol groups. Biochim. Biophys. Acta 1411, 323-333.

[104] Gaston, B., Reilly, J., Drazen, J.M., Fackler, J., Ramdev, P., Arnelle, D., Mullins, M.E., Sugarbaker, D.J., Chee, C., Singel, D.J. and et al. (1993) Endogenous nitrogen oxides and bronchodilator S-nitrosothiols in human airways. Proc. Natl. Acad. Sci. U S A 90, 10957-10961.

[105] Smirnova, G.V. and Oktyabrsky, O.N. (2005) Glutathione in bacteria. Biochemistry (Mosc) 70, 1199-1211.

[106] Singh, S.P., Wishnok, J.S., Keshive, M., Deen, W.M. and Tannenbaum, S.R. (1996) The chemistry of the S-nitrosoglutathione/glutathione system. Proc. Natl. Acad. Sci. U S A 93, 14428-14433.

[107] Hess, D.T., Matsumoto, A., Kim, S.O., Marshall, H.E. and Stamler, J.S. (2005) Protein S-nitrosylation: purview and parameters. Nat. Rev. Mol. Cell Biol. 6, 150-166.

[108] Arnelle, D.R. and Stamler, J.S. (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.

[109] Berlett, B.S., Friguet, B., Yim, M.B., Chock, P.B. and Stadtman, E.R. (1996) Peroxynitrite-mediated nitration of tyrosine residues in *Escherichia coli* glutamine synthetase mimics adenylylation: relevance to signal transduction. Proc. Natl. Acad. Sci. U S A 93, 1776-1780.

[110] Tien, M., Berlett, B.S., Levine, R.L., Chock, P.B. and Stadtman, E.R. (1999) Peroxynitrite-mediated modification of proteins at physiological carbon dioxide concentration: pH dependence of carbonyl formation, tyrosine nitration, and methionine oxidation. Proc. Natl. Acad. Sci. U S A 96, 7809-7814.

[111] Thomas, J.L.C., Bauschlicher, C.W. and Hall, M.B. (1997) Binding of Nitric Oxide to First-Transition-Row Metal Cations: An ab Initio Study. J. Phys. Chem. 101, 8530–8539.

[112] Cooper, C.E. (1999) Nitric oxide and iron proteins. Biochim. Biophys. Acta 1411, 290-309.

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

[114] Addison, A.W. and Stephanos, J.J. (1986) Nitrosyliron(III) haemoglobin: autoreduction and spectroscopy. Biochemistry 25, 4104-4113.

[115] Brown, G.C. (1995) Reversible binding and inhibition of catalase by nitric oxide. Eur. J. Biochem. 232, 188-191.

[116] Gardner, P.R., Costantino, G., Szabo, C. and Salzman, A.L. (1997) Nitric oxide sensitivity of the aconitases. J. Biol. Chem. 272, 25071-25076.

[117] Flint, D.H. and Allen, R.M. (1996) Ironminus signSulfur Proteins with Nonredox Functions. Chem. Rev. 96, 2315-2334.

[118] Drapier, J.C. (1997) Interplay between NO and [Fe-S] clusters: relevance to biological systems. Methods 11, 319-329.

[119] Kennedy, M.C., Antholine, W.E. and Beinert, H. (1997) An EPR investigation of the products of the reaction of cytosolic and mitochondrial aconitases with nitric oxide. J. Biol. Chem. 272, 20340-20347.

[120] Tortora, V., Quijano, C., Freeman, B., Radi, R. and Castro, L. (2007) Mitochondrial aconitase reaction with nitric oxide, S-nitrosoglutathione, and peroxynitrite: mechanisms and relative contributions to aconitase inactivation. Free Radic. Biol. Med. 42, 1075-1088.

[121] Hendriks, J., Oubrie, A., Castresana, J., Urbani, A., Gemeinhardt, S. and Saraste, M. (2000) Nitric oxide reductases in bacteria. Biochim. Biophys. Acta 1459, 266-273.

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

[123] Wu, G., Wainwright, L.M., Membrillo-Hernandez, J. and Poole, R.K. (2004) Bacterial haemoglobins: old proteins with new functions? Roles in respiratory and nitric oxide metabolism. Respiration in Archaea and Bacteria Diversity of Prokaryotic Electron Transport Carriers, ed Davide Zanoni 251-284.

[124] Zumft, W.G. (2005) Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme-copper oxidase type. J. Inorg. Biochem. 99, 194-215.

[125] Anjum, M.F., Stevanin, T.M., Read, R.C. and Moir, J.W. (2002) Nitric oxide metabolism in *Neisseria meningitidis*. J. Bacteriol. 184, 2987-2993.

[126] Stevanin, T.M., Moir, J.W. and Read, R.C. (2005) Nitric oxide detoxification systems enhance survival of *Neisseria meningitidis* in human macrophages and in nasopharyngeal mucosa. Infect. Immun. 73, 3322-3329.

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

[128] Gomes, C.M., Giuffre, A., Forte, E., Vicente, J.B., Saraiva, L.M., Brunori, M. and Teixeira, M. (2002) A novel type of nitric-oxide reductase. *Escherichia coli* flavorubredoxin. J. Biol. Chem. 277, 25273-25276.

[129] Mills, P.C., Richardson, D.J., Hinton, J.C. and Spiro, S. (2005) Detoxification of nitric oxide by the flavorubredoxin of *Salmonella enterica serovar Typhimurium*. Biochem. Soc. Trans. 33, 198-199.

[130] Justino, M.C., Vicente, J.B., Teixeira, M. and Saraiva, L.M. (2005) New genes implicated in the protection of anaerobically grown *Escherichia coli* against nitric oxide. J. Biol. Chem. 280, 2636-2643.

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

[132] Menzies, B.E. and Kourteva, I. (1998) Internalization of *Staphylococcus aureus* by endothelial cells induces apoptosis. Infect. Immun. 66, 5994-5998.

[133] Krut, O., Sommer, H. and Kronke, M. (2004) Antibiotic-induced persistence of cytotoxic *Staphylococcus aureus* in non-phagocytic cells. J. Antimicrob. Chemother. 53, 167-173.

[134] Kapral, F.A. and Shayegani, M.G. (1959) Intracellular survival of staphylococci. J. Exp. Med. 110, 123-138.

[135] Gresham, H.D., Lowrance, J.H., Caver, T.E., Wilson, B.S., Cheung, A.L. and Lindberg, F.P. (2000) Survival of *Staphylococcus aureus* inside neutrophils contributes to infection. J. Immunol. 164, 3713-3722.

[136] Voyich, J.M., Braughton, K.R., Sturdevant, D.E., Whitney, A.R., Said-Salim, B., Porcella, S.F., Long, R.D., Dorward, D.W., Gardner, D.J., Kreiswirth, B.N., Musser, J.M. and DeLeo, F.R. (2005) Insights into mechanisms used by *Staphylococcus aureus* to avoid destruction by human neutrophils. J. Immunol. 175, 3907-3919.

[137] Lowy, F.D. (1998) *Staphylococcus aureus* infections. N. Engl. J. Med. 339, 520-532.

[138] Takeuchi, O., Hoshino, K. and Akira, S. (2000) Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to *Staphylococcus aureus* infection. J. Immunol. 165, 5392-5396.

[139] Bubeck Wardenburg, J., Williams, W.A. and Missiakas, D. (2006) Host defenses against *Staphylococcus aureus* infection require recognition of bacterial lipoproteins. Proc. Natl. Acad. Sci. U S A 103, 13831-13836.

[140] Jin, T., Bokarewa, M., Foster, T., Mitchell, J., Higgins, J. and Tarkowski, A. (2004) *Staphylococcus aureus* resists human defensins by production of staphylokinase, a novel bacterial evasion mechanism. J. Immunol. 172, 1169-1176.

[141] Maines, M.D., Trakshel, G.M. and Kutty, R.K. (1986) Characterization of two constitutive forms of rat liver microsomal heme oxygenase. Only one molecular species of the enzyme is inducible. J. Biol. Chem. 261, 411-419.

[142] Skaar, E.P. and Schneewind, O. (2004) Iron-regulated surface determinants (Isd) of *Staphylococcus aureus*: stealing iron from heme. Microbes Infect. 6, 390-397.

[143] Skaar, E.P., Gaspar, A.H. and Schneewind, O. (2004) IsdG and IsdI, heme-degrading enzymes in the cytoplasm of *Staphylococcus aureus*. J. Biol. Chem. 279, 436-443.

[144] Wu, R., Skaar, E.P., Zhang, R., Joachimiak, G., Gornicki, P., Schneewind, O. and Joachimiak, A. (2005) *Staphylococcus aureus* IsdG and IsdI, heme-degrading enzymes with structural similarity to monooxygenases. J. Biol. Chem. 280, 2840-2846.

[145] Konetschny-Rapp, S., Jung, G., Meiwes, J. and Zahner, H. (1990) Staphyloferrin A: a structurally new siderophore from staphylococci. Eur. J. Biochem. 191, 65-74.

[146] Drechsel, H., Freund, S., Nicholson, G., Haag, H., Jung, O., Zahner, H. and Jung, G. (1993) Purification and chemical characterization of staphyloferrin B, a hydrophilic siderophore from staphylococci. Biometals 6, 185-192.

[147] Courcol, R.J., Trivier, D., Bissinger, M.C., Martin, G.R. and Brown, M.R. (1997) Siderophore production by *Staphylococcus aureus* and identification of iron-regulated proteins. Infect. Immun. 65, 1944-1948.

[148] Sebulsky, M.T., Hohnstein, D., Hunter, M.D. and Heinrichs, D.E. (2000) Identification and characterization of a membrane permease involved in iron-hydroxamate transport in *Staphylococcus aureus*. J. Bacteriol. 182, 4394-4400.

[149] Dale, S.E., Doherty-Kirby, A., Lajoie, G. and Heinrichs, D.E. (2004) Role of siderophore biosynthesis in virulence of *Staphylococcus aureus*: identification and characterization of genes involved in production of a siderophore. Infect. Immun. 72, 29-37.

[150] Kanafani, H. and Martin, S.E. (1985) Catalase and superoxide dismutase activities in virulent and nonvirulent *Staphylococcus aureus* isolates. J. Clin. Microbiol. 21, 607-610.

[151] Horsburgh, M.J., Clements, M.O., Crossley, H., Ingham, E. and Foster, S.J. (2001) PerR controls oxidative stress resistance and iron storage proteins and is required for virulence in *Staphylococcus aureus*. Infect. Immun. 69, 3744-3754.

[152] Cosgrove, K., Coutts, G., Jonsson, I.M., Tarkowski, A., Kokai-Kun, J.F., Mond, J.J. and Foster, S.J. (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.

[153] Clements, M.O., Watson, S.P. and Foster, S.J. (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.

[154] Valderas, M.W. and Hart, M.E. (2001) Identification and characterization of a second superoxide dismutase gene (sodM) from *Staphylococcus aureus*. J. Bacteriol. 183, 3399-3407.

[155] Richardson, A.R., Libby, S.J. and Fang, F.C. (2008) A nitric oxide-inducible lactate dehydrogenase enables *Staphylococcus aureus* to resist innate immunity. Science 319, 1672-1676.

[156] Richardson, A.R., Dunman, P.M. and Fang, F.C. (2006) The nitrosative stress response of *Staphylococcus aureus* is required for resistance to innate immunity. Mol. Microbiol. 61, 927-939.

[157] Tavares, A.F., Nobre, L.S., Melo, A.M. and Saraiva, L.M. (2009) A novel nitroreductase of *Staphylococcus aureus* with S-nitrosoglutathione reductase activity. J. Bacteriol. 191, 3403-3406.

[158] Goncalves, V.L., Nobre, L.S., Vicente, J.B., Teixeira, M. and Saraiva, L.M. (2006) Flavohaemoglobin requires microaerophilic conditions for nitrosative protection of *Staphylococcus aureus*. FEBS Lett. 580, 1817-1821.

[159] Wakabayashi, S., Matsubara, H. and Webster, D.A. (1986) Primary sequence of a dimeric bacterial haemoglobin from *Vitreoscilla*. Nature 322, 481-483.

[160] Kaur, R., Pathania, R., Sharma, V., Mande, S.C. and Dikshit, K.L. (2002) Chimeric *Vitreoscilla* haemoglobin (VHb) carrying a flavoreductase domain relieves nitrosative stress in *Escherichia coli*: new insight into the functional role of VHb. Appl. Environ. Microbiol. 68, 152-160.

[161] Elvers, K.T., Wu, G., Gilberthorpe, N.J., Poole, R.K. and Park, S.F. (2004) Role of an inducible single-domain haemoglobin in mediating resistance to nitric oxide and nitrosative stress in *Campylobacter jejuni* and *Campylobacter coli*. J. Bacteriol. 186, 5332-5341.

[162] Wittenberg, J.B., Bolognesi, M., Wittenberg, B.A. and Guertin, M. (2002) Truncated haemoglobins: a new family of haemoglobins widely distributed in bacteria, unicellular eukaryotes, and plants. J. Biol. Chem. 277, 871-874.

[163] Pathania, R., Navani, N.K., Rajamohan, G. and Dikshit, K.L. (2002) *Mycobacterium tuberculosis* haemoglobin HbO associates with membranes and stimulates cellular respiration of recombinant *Escherichia coli*. J. Biol. Chem. 277, 15293-15302.

[164] Ouellet, H., Ouellet, Y., Richard, C., Labarre, M., Wittenberg, B., Wittenberg, J. and Guertin, M. (2002) Truncated haemoglobin HbN protects *Mycobacterium bovis* from nitric oxide. Proc. Natl. Acad. Sci. U S A 99, 5902-5907.

[165] Vasudevan, S.G., Armarego, W.L., Shaw, D.C., Lilley, P.E., Dixon, N.E. and Poole, R.K. (1991) Isolation and nucleotide sequence of the hmp gene that encodes a haemoglobin-like protein in *Escherichia coli* K-12. Mol. Gen. Genet. 226, 49-58.

[166] Oshino, R., Oshino, N. and Chance, B. (1973) Studies on yeast haemoglobin. The properties of yeast haemoglobin and its physiological function in the cell. Eur. J. Biochem. 35, 23-33.

[167] Oshino, R., Asakura, T., Takio, K., Oshino, N., Chance, B. and Hagihara, B. (1973) Purification and molecular properties of yeast haemoglobin. Eur. J. Biochem. 39, 581-590.

[168] Ioannidis, N., Cooper, C.E. and Poole, R.K. (1992) Spectroscopic studies on an oxygen-binding haemoglobin-like flavohaemoprotein from *Escherichia coli*. Biochem. J. 288 (Pt 2), 649-655.

[169] Ilari, A., Bonamore, A., Farina, A., Johnson, K.A. and Boffi, A. (2002) The X-ray structure of ferric *Escherichia coli* flavohaemoglobin Reveals an unexpected geometry of the distal heme pocket. J. Biol. Chem. 277, 23725-23732.

[170] Ermler, U., Siddiqui, R.A., Cramm, R. and Friedrich, B. (1995) Crystal structure of the flavohaemoglobin from Alcaligenes eutrophus at 1.75 A resolution. Embo J. 14, 6067-6077.

[171] Bonamore, A. and Boffi, A. (2008) Flavohaemoglobin: structure and reactivity. IUBMB Life 60, 19-28.

[172] Gardner, P.R., Gardner, A.M., Martin, L.A., Dou, Y., Li, T., Olson, J.S., Zhu, H. and Riggs, A.F. (2000) Nitric-oxide dioxygenase activity and function of flavohaemoglobins: sensitivity to nitric oxide and carbon monoxide inhibition. J. Biol. Chem. 275, 31581-31587.

[173] Poole, R.K., Anjum, M.F., Membrillo-Hernandez, J., Kim, S.O., Hughes, M.N. and Stewart, V. (1996) Nitric oxide, nitrite, and Fnr regulation of hmp (flavohaemoglobin) gene expression in *Escherichia coli* K-12. J. Bacteriol. 178, 5487-5492.

[174] Membrillo-Hernandez, J., Coopamah, M.D., Channa, A., Hughes, M.N. and Poole, R.K. (1998) A novel mechanism for upregulation of the *Escherichia coli* K-12 *hmp* (flavohaemoglobin) gene by the 'NO releaser', S-nitrosoglutathione: nitrosation of homocysteine and modulation of MetR binding to the *glyA-hmp* intergenic region. Mol. Microbiol. 29, 1101-1112.

[175] Gardner, P.R., Gardner, A.M., Martin, L.A. and Salzman, A.L. (1998) Nitric oxide dioxygenase: an enzymic function for flavohaemoglobin. Proc. Natl. Acad. Sci. U S A 95, 10378-10383.

[176] Membrillo-Hernandez, J., Coopamah, M.D., Anjum, M.F., Stevanin, T.M., Kelly, A., Hughes, M.N. and Poole, R.K. (1999) The flavohaemoglobin of *Escherichia coli* confers resistance to a nitrosating agent, a "Nitric oxide Releaser," and paraquat and is essential for transcriptional responses to oxidative stress. J. Biol. Chem. 274, 748-754.

[177] Svensson, L., Marklund, B.I., Poljakovic, M. and Persson, K. (2006) Uropathogenic *Escherichia coli* and tolerance to nitric oxide: the role of flavohaemoglobin. J. Urol. 175, 749-753.

[178] Bang, I.S., Liu, L., Vazquez-Torres, A., Crouch, M.L., Stamler, J.S. and Fang, F.C. (2006) Maintenance of nitric oxide and redox homeostasis by the salmonella flavohaemoglobin *hmp*. J. Biol. Chem. 281, 28039-28047.

[179] Crawford, M.J. and Goldberg, D.E. (1998) Role for the *Salmonella* flavohaemoglobin in protection from nitric oxide. J. Biol. Chem. 273, 12543-12547.

[180] Arai, H., Hayashi, M., Kuroi, A., Ishii, M. and Igarashi, Y. (2005) Transcriptional regulation of the flavohaemoglobin gene for aerobic nitric oxide detoxification by the second nitric oxide-responsive regulator of *Pseudomonas aeruginosa*. J. Bacteriol. 187, 3960-3968.

[181] Boccara, M., Mills, C.E., Zeier, J., Anzi, C., Lamb, C., Poole, R.K. and Delledonne, M. (2005) Flavohaemoglobin HmpX from *Erwinia chrysanthemi* confers nitrosative stress tolerance and affects the plant hypersensitive reaction by intercepting nitric oxide produced by the host. Plant J. 43, 226-237.

[182] Rogstam, A., Larsson, J.T., Kjelgaard, P. and von Wachenfeldt, C. (2007) Mechanisms of adaptation to nitrosative stress in *Bacillus subtilis*. J. Bacteriol. 189, 3063-3071.

[183] Liu, L., Zeng, M., Hausladen, A., Heitman, J. and Stamler, J.S. (2000) Protection from nitrosative stress by yeast flavohaemoglobin. Proc. Natl. Acad. Sci. U S A 97, 4672-4676.

[184] de Jesus-Berrios, M., Liu, L., Nussbaum, J.C., Cox, G.M., Stamler, J.S. and Heitman, J. (2003) Enzymes that counteract nitrosative stress promote fungal virulence. Curr. Biol. 13, 1963-1968.

[185] Stevanin, T.M., Poole, R.K., Demoncheaux, E.A. and Read, R.C. (2002) Flavohaemoglobin Hmp protects *Salmonella enterica serovar typhimurium* from nitric oxide-related killing by human macrophages. Infect. Immun. 70, 4399-4405.

[186] Stevanin, T.M., Read, R.C. and Poole, R.K. (2007) The *hmp* gene encoding the NO-inducible flavohaemoglobin in *Escherichia coli* confers a protective advantage in resisting killing within macrophages, but not *in vitro*: links with swarming motility. Gene 398, 62-68.

[187] Poole, R.K. and Hughes, M.N. (2000) New functions for the ancient globin family: bacterial responses to nitric oxide and nitrosative stress. Mol. Microbiol. 36, 775-783.

[188] Bonamore, A., Farina, A., Gattoni, M., Schinina, M.E., Bellelli, A. and Boffi, A. (2003) Interaction with membrane lipids and heme ligand binding properties of *Escherichia coli* flavohaemoglobin. Biochemistry 42, 5792-5801.

[189] D'Angelo, P., Lucarelli, D., della Longa, S., Benfatto, M., Hazemann, J.L., Feis, A., Smulevich, G., Ilari, A., Bonamore, A. and Boffi, A. (2004) Unusual heme iron-lipid acyl chain coordination in *Escherichia coli* flavohaemoglobin. Biophys. J. 86, 3882-3892.

[190] Poole, R.K., Rogers, N.J., D'Mello R, A., Hughes, M.N. and Orii, Y. (1997) *Escherichia coli* flavohaemoglobin (Hmp) reduces cytochrome *c* and Fe(III)-hydroxamate K by electron transfer from NADH via FAD: sensitivity of oxidoreductase activity to haem-bound dioxygen. Microbiology 143 (Pt 5), 1557-1565.

[191] Anjum, M.F., Ioannidis, N. and Poole, R.K. (1998) Response of the NAD(P)H-oxidising flavohaemoglobin (Hmp) to prolonged oxidative stress and implications for its physiological role in *Escherichia coli*. FEMS Microbiol. Lett. 166, 219-223.

[192] Wu, G., Wainwright, L.M. and Poole, R.K. (2003) Microbial globins. Adv. Microb. Physiol. 47, 255-310.

[193] Hausladen, A., Gow, A.J. and Stamler, J.S. (1998) Nitrosative stress: metabolic pathway involving the flavohaemoglobin. Proc. Natl. Acad. Sci. U S A 95, 14100-14105.

[194] Kim, S.O., Orii, Y., Lloyd, D., Hughes, M.N. and Poole, R.K. (1999) Anoxic function for the *Escherichia coli* flavohaemoglobin (Hmp): Reversible binding of nitric oxide and reduction to nitrous oxide. FEBS Lett. 445, 389-394.

[195] Hausladen, A., Gow, A. and Stamler, J.S. (2001) Flavohaemoglobin denitrosylase catalyzes the reaction of a nitroxyl equivalent with molecular oxygen. Proc. Natl. Acad. Sci. U S A 98, 10108-10112.

[196] Frey, A.D. and Kallio, P.T. (2003) Bacterial haemoglobins and flavohaemoglobins: versatile proteins and their impact on Microbiology and biotechnology. FEMS Microbiol. Rev. 27, 525-545.

[197] Gardner, A.M., Martin, L.A., Gardner, P.R., Dou, Y. and Olson, J.S. (2000) Steady-state and transient kinetics of *Escherichia coli* nitric-oxide dioxygenase (flavohaemoglobin). The B10 tyrosine hydroxyl is essential for dioxygen binding and catalysis. J. Biol. Chem. 275, 12581-12589.

[198] Mills, C.E., Sedelnikova, S., Soballe, B., Hughes, M.N. and Poole, R.K. (2001) *Escherichia coli* flavohaemoglobin (Hmp) with equistoichiometric FAD and haem contents has a low affinity for dioxygen in the absence or presence of nitric oxide. Biochem. J. 353, 207-213.

[199] Wu, G., Corker, H., Orii, Y. and Poole, R.K. (2004) *Escherichia coli* Hmp, an "oxygen-binding flavohaemoprotein", produces superoxide anion and self-destructs. Arch. Microbiol. 182, 193-203.

[200] Membrillo-Hernandez, J., Ioannidis, N. and Poole, R.K. (1996) The flavohaemoglobin (HMP) of *Escherichia coli* generates superoxide in vitro and causes oxidative stress *in vivo*. FEBS Lett. 382, 141-144.

[201] Gaudu, P., Touati, D., Niviere, V. and Fontecave, M. (1994) The NAD(P)H:flavin oxidoreductase from *Escherichia coli* as a source of superoxide radicals. J. Biol. Chem. 269, 8182-8188.

[202] Bonamore, A., Gentili, P., Ilari, A., Schinina, M.E. and Boffi, A. (2003) *Escherichia coli* flavohaemoglobin is an efficient alkylhydroperoxide reductase. J. Biol. Chem. 278, 22272-22277.

[203] Membrillo-Hernandez, J., Kim, S.O., Cook, G.M. and Poole, R.K. (1997) Paraquat regulation of hmp (flavohaemoglobin) gene expression in *Escherichia coli* K-12 is SoxRS independent but modulated by sigma S. J. Bacteriol. 179, 3164-3170.

[204] Moore, C.M., Nakano, M.M., Wang, T., Ye, R.W. and Helmann, J.D. (2004) Response of *Bacillus subtilis* to nitric oxide and the nitrosating agent sodium nitroprusside. J. Bacteriol. 186, 4655-4664.

[205] Hromatka, B.S., Noble, S.M. and Johnson, A.D. (2005) Transcriptional Response of *C. albicans* to Nitric Oxide and the Role of the YHB1 Gene in Nitrosative Stress and Virulence. Mol. Biol. Cell.

[206] Hu, Y., Butcher, P.D., Mangan, J.A., Rajandream, M.A. and Coates, A.R. (1999) Regulation of hmp gene transcription in Mycobacterium tuberculosis: effects of oxygen limitation and nitrosative and oxidative stress. J. Bacteriol. 181, 3486-3493.

[207] Membrillo-Hernandez, J., Cook, G.M. and Poole, R.K. (1997) Roles of RpoS (sigmaS), IHF and ppGpp in the expression of the hmp gene encoding the flavohaemoglobin (Hmp) of *Escherichia coli* K-12. Mol. Gen. Genet. 254, 599-603.

[208] Bollinger, C.J., Bailey, J.E. and Kallio, P.T. (2001) Novel haemoglobins to enhance microaerobic growth and substrate utilization in *Escherichia coli*. Biotechnol. Prog. 17, 798-808.

[209] Nakano, M.M. (2006) Essential role of flavohaemoglobin in long-term anaerobic survival of *Bacillus subtilis*. J. Bacteriol. 188, 6415-6418.

[210] Flatley, J., Barrett, J., Pullan, S.T., Hughes, M.N., Green, J. and Poole, R.K. (2005) Transcriptional responses of *Escherichia coli* to S-nitrosoglutathione under defined chemostat conditions Reveal major changes in methionine biosynthesis. J. Biol. Chem. 280, 10065-10072.

[211] Pullan, S.T., Gidley, M.D., Jones, R.A., Barrett, J., Stevanin, T.M., Read, R.C., Green, J. and Poole, R.K. (2007) Nitric oxide in chemostat-cultured *Escherichia coli* is sensed by Fnr and other global

regulators: unaltered methionine biosynthesis indicates lack of S nitrosation. J. Bacteriol. 189, 1845-1855.

[212] Firoved, A.M., Wood, S.R., Ornatowski, W., Deretic, V. and Timmins, G.S. (2004) Microarray analysis and functional characterization of the nitrosative stress response in nonmucoid and mucoid *Pseudomonas aeruginosa*. J. Bacteriol. 186, 4046-4050.

[213] Hromatka, B.S., Noble, S.M. and Johnson, A.D. (2005) Transcriptional response of *Candida albicans* to nitric oxide and the role of the YHB1 gene in nitrosative stress and virulence. Mol. Biol. Cell 16, 4814-4826.

[214] Cruz-Ramos, H., Crack, J., Wu, G., Hughes, M.N., Scott, C., Thomson, A.J., Green, J. and Poole, R.K. (2002) NO sensing by FNR: regulation of the *Escherichia coli* NO-detoxifying flavohaemoglobin, Hmp. Embo J. 21, 3235-3244.

[215] Bodenmiller, D.M. and Spiro, S. (2006) The *yjeB* (*nsrR*) gene of *Escherichia coli* encodes a nitric oxide-sensitive transcriptional regulator. J. Bacteriol. 188, 874-881.

[216] Tucker, N.P., Hicks, M.G., Clarke, T.A., Crack, J.C., Chandra, G., Le Brun, N.E., Dixon, R. and Hutchings, M.I. (2008) The transcriptional repressor protein NsrR senses nitric oxide directly via a [2Fe-2S] cluster. PLoS One 3, e3623.

[217] Gilberthorpe, N.J., Lee, M.E., Stevanin, T.M., Read, R.C. and Poole, R.K. (2007) NsrR: a key regulator circumventing *Salmonella enterica serovar Typhimurium* oxidative and nitrosative stress *in vitro* and in IFN-gamma-stimulated J774.2 macrophages. Microbiology 153, 1756-1771.

[218] Nakano, M.M., Geng, H., Nakano, S. and Kobayashi, K. (2006) The nitric oxide-responsive regulator NsrR controls ResDE-dependent gene expression. J. Bacteriol. 188, 5878-5887.

[219] Nakano, M.M. (2002) Induction of ResDE-dependent gene expression in *Bacillus subtilis* in response to nitric oxide and nitrosative stress. J. Bacteriol. 184, 1783-1787.

[220] François, I.E.J.A., Cammue, B.P., Borgers, M., Ausma, J., Dispersyn, G.D. and Thevissen, K. (2006) Azoles: Mode of Antifungal Action and Resistance Development. Effect of Miconazole on Endogenous Reactive Oxygen Species Production in *Candida albicans*. Anti-Infective Agents in Medicinal Chemistry 5, 3-13.

[221] Brugmans, J.P., Thienpont, D.C., van Wijngaarden, I., Vanparijs, O.F., Schuermans, V.L. and Lauwers, H.L. (1971) Mebendazole in enterobiasis. Radiochemical and pilot clinical study in 1,278 subjects. Jama 217, 313-316.

[222] Fromtling, R.A. (1988) Overview of medically important antifungal azole derivatives. Clin. Microbiol. Rev. 1, 187-217.

[223] Burgess, M.A. and Bodey, G.P. (1972) Clotrimazole (Bay b 5097): *in vitro* and clinical pharmacological studies. Antimicrob. Agents Chemother. 2, 423-426.

[224] Tettenborn, D. (1974) Toxicity of clotrimazole. Postgrad. Med. J. 50 Suppl. 1, 17-20.

[225] Godefroi, E.F., Heeres, J., Van Cutsem, J. and Janssen, P.A. (1969) The preparation and antimycotic properties of derivatives of 1-phenethylimidazole. J. Med. Chem. 12, 784-791.

[226] Yamaguchi, H., Hiratani, T. and Plempel, M. (1983) *In vitro* studies of a new imidazole antimycotic, bifonazole, in comparison with clotrimazole and miconazole. Arzneimittelforschung 33, 546-551.

[227] Heeres, J., Backx, L.J., Mostmans, J.H. and Van Cutsem, J. (1979) Antimycotic imidazoles. part 4. Synthesis and antifungal activity of ketoconazole, a new potent orally active broad-spectrum antifungal agent. J. Med. Chem. 22, 1003-1005.

[228] Sheehan, D.J., Hitchcock, C.A. and Sibley, C.M. (1999) Current and emerging azole antifungal agents. Clin. Microbiol. Rev. 12, 40-79.

[229] Kobayashi, D., Kondo, K., Uehara, N., Otokozawa, S., Tsuji, N., Yagihashi, A. and Watanabe, N. (2002) Endogenous reactive oxygen species is an important mediator of miconazole antifungal effect. Antimicrob. Agents Chemother. 46, 3113-3117.

[230] Thevissen, K., Ayscough, K.R., Aerts, A.M., Du, W., De Brucker, K., Meert, E.M., Ausma, J., Borgers, M., Cammue, B.P. and Francois, I.E. (2007) Miconazole induces changes in actin cytoskeleton prior to reactive oxygen species induction in yeast. J. Biol. Chem. 282, 21592-21597.

[231] Saliba, K.J. and Kirk, K. (1998) Clotrimazole inhibits the growth of *Plasmodium falciparum in vitro*. Trans. R. Soc. Trop. Med. Hyg. 92, 666-667.

[232] Tiffert, T., Ginsburg, H., Krugliak, M., Elford, B.C. and Lew, V.L. (2000) Potent antimalarial activity of clotrimazole in in vitro cultures of *Plasmodium falciparum*. Proc. Natl. Acad. Sci. U S A 97, 331-336.

[233] Trivedi, V., Chand, P., Srivastava, K., Puri, S.K., Maulik, P.R. and Bandyopadhyay, U. (2005) Clotrimazole inhibits haemoperoxidase of *Plasmodium falciparum* and induces oxidative stress. Proposed antimalarial mechanism of clotrimazole. J. Biol. Chem. 280, 41129-41136.

[234] Helmick, R.A., Fletcher, A.E., Gardner, A.M., Gessner, C.R., Hvitved, A.N., Gustin, M.C. and Gardner, P.R. (2005) Imidazole antibiotics inhibit the nitric oxide dioxygenase function of microbial flavohaemoglobin. Antimicrob. Agents Chemother. 49, 1837-1843.

[235] Rautelin, H., Vaara, M., Renkonen, O.V., Kosunen, T.U. and Seppala, K. (1992) *In vitro* activity of antifungal azoles against *Helicobacter pylori*. Eur. J. Clin. Microbiol. Infect. Dis. 11, 273-274.

[236] Van Cutsem, J.M. and Thienpont, D. (1972) Miconazole, a broad-spectrum antimycotic agent with antibacterial activity. Chemotherapy 17, 392-404.

[237] Sud, I.J. and Feingold, D.S. (1982) Action of antifungal imidazoles on *Staphylococcus aureus*. Antimicrob. Agents Chemother. 22, 470-474.

CHAPTER 2

The physiological role of carbon monoxide

2.1 Historical overview

The harmful action of carbon monoxide (CO) gas in humans was recognized for a long time. However, only in 1857 Claude Bernard showed that the toxic effects of CO were related to the reversible binding of CO to haemoglobin, forming carboxy-haemoglobin (COHb) and leading to asphyxia [1]. This gaseous molecule was considered for many years only as an environmental poison that results from the incomplete combustion of organic matter. Although in the second half of 1800s the first indication of endogenous production of CO was reported [2, 3], only fifty years later the synthesis of CO gas in the human body was indeed demonstrated [4-6]. Shortly after, the production of CO in bacteria was also reported [7].

In the late 1960s the production of CO was attributed to haem oxygenase (HO) activity [8-10]. However, it was regarded as a metabolic waste for about twenty years and the study of CO as a signaling molecule was only explored after the discoveries made in 1980s regarding the physiological role of nitric oxide [11-16]. Since then research on CO developed rapidly and, in 1993, Snyder and co-workers reported that CO could act as a neural messenger and Rattan and Chakder demonstrated that endogenous CO was involved in relaxation of the internal anal sphincter [17, 18]. Two years later, it was reported that CO, like NO, binds to soluble guanylate cyclase and activates the enzyme albeit to a minor extension than NO [19-21].

The physiological function and the pharmacological application of CO have been extensively explored [22]. The search for new compounds that could safely carry and delivery CO into a biological environment led to the development of CO-releasing molecules (CO-RMs) [23]. CO-RMs are proposed to mimic the physiological action of CO gas and are currently under preclinical evaluation since they are a promising tool for therapeutic CO-delivery [24-26].

In the last decade, it has been a huge and growing interest in the biology of endogenous generated gases, such as NO, CO and more recently hydrogen sulfite (H_2S) [27, 28]. All three gases seem to work together, exerting a similar biological role albeit via different mechanisms and its regulation seems to be intimately linked [27-29]. The multiplicity of targets and the diversity of physiological

functions of these gaseous molecules have attracted much attention and several investigations have revealed the biological significance of its endogenous production [30]. In this chapter a summary of the most important findings in CO bioactivity will be presented.

2.2 Reactivity of carbon monoxide

The direct oxidation of carbon in a limited supply of oxygen or air yields CO. Hence, the incomplete combustion of carbon-containing fuels, such as coal, petrol, natural gas and wood releases CO [31].

CO is a colourless, odourless, tasteless and flammable gas that, at standard temperature and pressure (IUPAC), has a density 1.25 g/L (about the same density as that of air). Above -205 °C, CO changes directly from a solid phase to a gaseous phase through sublimation with a boiling point of -192 ºC [31, 32] (Table 2.1).

TABLE 2.1 Chemical and physical proprieties of CO.

Property	Value
Interatomic distance (pm)	112.8
Melting point (ºC)	-205
Boiling point (ºC)	-192
Density (g/L)	1.25
Solubility in water (mM)	1*

* at room temperature
Adapted from [31]

CO has ten valence electrons, four from the carbon and six from oxygen. Two electrons are used to form the σ-bond and four to form two π-bonds. Therefore, CO has a triple bond leaving four electrons to form a lone pair on each of the carbon and oxygen atoms [31]. The lone-pair on carbon is available, albeit weakly, for donation to an acceptor. Hence, most of CO chemistry occurs via the carbon atom, which is negatively charged, and not through the oxygen. The short interatomic distance of CO (Table 2.1) and the great strength of the bond confer stability to the molecule. Thus, the chemical reduction of CO requires temperatures well above 100 ºC. For example, the catalytic reduction to methanol occurs at 230-400 ºC and 50-100 atmosphere (atm) (Equation 2.1). The oxidation of CO to carbon dioxide also takes place at high temperatures via the metal-catalysed water-gas-shift reaction (Equation 2.2) [31].

EQUATION 2.1 $CO + 2H_2 \Rightarrow CH_3OH$

EQUATION 2.2 $CO + H_2O \Rightarrow CO_2 + H_2$

CO is weakly soluble in water (Table 2.1) and is soluble in organic solvents such as chloroform, benzene, ethanol and methanol [33]. In addition, CO does not readily react with reducing agents (including molecular hydrogen) and the reaction with molecular oxygen is slow with a high activation energy (~ 213 kJ mol^{-1}) [22].

CO is considered to be a relatively inert molecule and the reduction of CO can be greatly facilitated by transition metals in low oxidation states, especially those from 6-10 group (as molybdenum, manganese, iron, cobalt or nickel), with which it forms stable complexes [31]. The bond between the transition metal and the CO ligand occurs through a donation of a pair of electrons from the lone pair on the carbon atom to the hybrid orbital of the metal to form a σ-bond, and a back donation from the filled *d*-orbital on the metal to the empty anti-bonding π-orbital on the CO [31, 34]. Consequently, the bioactivity of CO proceeds almost exclusively through the binding to transition metals which are present in structural and functional proteins, especially via binding to ferrous haem (Table 2.2). On the other hand, CO is an important organometalic ligand and the knowledge of the CO related chemistry helped in the development of CO-RMs.

TABLE 2.2 Haemoproteins reported as potential targets for CO.

Haemoproteins
Myoglobin
Soluble guanylate cyclase
Inducible nitric oxide synthase
Cytochrome P450
Cytochrome *c* oxidase
NADPH oxidase
Dopamine β hydroxylase
Tryptophan oxidase
Haem oxygenase

Adapted from [35]

2.3 Carbon monoxide toxicity

Although CO has been present in our environment since the beginning of life and might had a fundamental role, along with oxygen and nitrogen, in the creation

of amino acids and proteins, it represents today the most abundant pollutant in the lower atmosphere [36, 37]. The industrial activity and the automobile exhaustion are the major sources of CO atmospheric accumulation. Other sources include fires, stoves, portable heaters and cigarette smoke [38].

The atmospheric ambient contains approximately 10 parts per million (ppm) of CO (Table 2.3) and even though it can reach 50 ppm in metropolitan areas, acute clinical poisoning does not normally arise [39]. Nevertheless, CO is responsible for a great number of deaths every year in the United States of America through inhalation of CO that usually happens accidently in closed spaces due to inappropriate air ventilation [40, 41]. Actually, the Center for Disease Control in USA estimates that CO poisoning results in more than 20000 visits to hospital emergency departments annually. Of those more than 4000 were hospitalised and nearly 400 died (http://www.cdc.gov/co).

CO is absorbed through the lungs and diffuses across the alveolar capillary membrane. Once absorbed, CO diffuses through the plasma, passes across the red blood cell membrane and finally enters the red cells where it binds to haemoglobin [22]. CO competes with dioxygen for binding to the four haem iron centers of haemoglobin and since the CO affinity to haemoglobin-haem is approximately 250 times higher than that of dioxygen, CO reduces the number of oxygen molecules transported [42]. As a consequence, CO decreases the haemoglobin storage capacity and increases the blood pressure. The partial occupation of CO at the haemoglobin binding sites also inhibits the liberation of dioxygen from the remaining haem groups to the recipient tissues. The decrease of dioxygen supply to the tissues leads to hypoxia, and ultimately to cell death and organ failure [43, 44]. The inhalation of CO gas has toxic effects within the body, specially on the organs with the highest oxygen requirement, like the brain and the heart [44].

The adverse health effects associated with CO vary with its concentration and duration of exposure since the ligation of CO to haemoglobin is reversible, and once exposure has ceased CO dissociates from it and is eliminated through the lungs [45]. In fact, the half-life of CO in the blood of adults is 30-180 min [46]. In

severe cases of CO exposure, oxygen therapy under hyperbaric conditions (100 % oxygen in 2.5 atm) may be applied as antidote [47].

The percentage of haemoglobin occupied by CO in normal tissues is less than 1 % (0.4-0.96 % COHb) [33]. However, basal levels of carboxy-haemoglobin can reach 3 % in non-smokers and may be as high as 10-15 % in smokers [46].

No standards value for CO levels have been agreed for indoor air and owing to the lack of a consensus unit of measurement for CO, whether be in ppm, ml/h, μM or % COHb, it became a challenge to advocate and agree on a safe and tolerable dose of CO [35]. Currently, the USA Occupational Health and Safety Administration stated a limit of 50 ppm over 8 h/day (Table 2.3) which generates carboxy-haemoglobin levels of ~ 8-10 % [35]. In most animal models, CO is lethal in the upper range of 10000 ppm (Table 2.3) [26, 35]. To obtain the benefits of CO, the percentage of carboxy-haemoglobin in the blood should be 12-20 % and, in fact, most of the positive effects of CO observed in a range of 50 to 500 ppm per hour lead to a tolerable level of carboxy-haemoglobin (15-18 %) with no physiological toxicity (Table 2.3) [26, 35]. Symptoms of CO poisoning begin to appear at 20 % carboxy-haemoglobin and include, headache, dizziness, weakness, nausea, vomiting, chest pain and confusion [46]. However, an ambient level of 100 ppm of CO can generate 16 % carboxy-haemoglobin at equilibrium, which may be enough to produce some clinical symptoms [38]. Prolonged exposure to CO leads to 50-80 % of blood carboxy-haemoglobin that results in seizure, coma and can ultimately conducted to death [46].

TABLE 2.3 Carbon monoxide levels and its medical relevance.

CO concentration (ppm)	Medical Relevance
10000	Lethal in min
10-500	Preclinical efficacy (1 h)
10	Ambient air
35/50	Limit for 8 h work/day (EPA/OHSA)

EPA: Environmental protection agency, USA

OHSA: Occupational Health and Safety Administration, USA

Adapted from [26]

Although 80 % of CO loaded in our body is bound to haemoglobin, other cellular targets cannot be excluded. CO also binds to other haemoproteins (Table 2.2), namely cytochrome *c* oxidase and cytochrome P450, inhibiting their activity. However, sufficient inhalation of CO to inhibit respiration and xenobiotic metabolism is most likely preceded by tissue hypoxia that originates death [46]. Hence, the contribution of such mechanisms to the overall toxicity remains controversial.

2.4 Endogenous production of carbon monoxide

Nearly every organism generates CO as a normal cellular function. Haem oxygenase activity accounts for about 86 % of the CO produced in humans [46], with the remaining 14 % of the CO generated from the oxidation of organic molecules that include auto-oxidation of phenols, flavenoids and halomethanes, photo-oxidation of organic compounds, peroxidation of membrane lipids and xenobiotic metabolism [48]. The rate of CO production in the human body is approximately 16.4 μmol/h [49]. The production of CO can be detected in exhaled air and an adult breathes out approximately 6 ml of CO per day. This level may increase substantially under disease states such as asthma, bronchiectasis, cystic fibrosis, diabetes and rhinitis [50]

Haem oxygenase catalyses the first rate-limiting step in the degradation of the haem. The oxidation of the α-methene bridge of haem by haem oxygenase yields stoichoimetric quantities of biliverdin, ferrous iron and CO (Figure 2.1) [51]. In this reaction, haem acts as prosthetic group and as substrate and three molecules of dioxygen are consumed per haem. The catalytic activity of haem oxygenase plays a crucial role in maintaining cellular haem homeostasis and haemoprotein levels [52].

All products of haem oxygenase activity are biologically active. CO is primarily a signaling molecule that has important physiological function which will be discussed below (2.5). Iron is traditionally considered as a pro-oxidant molecule since it participates in the Fenton reaction generating the highly reactive hydroxyl radical. However, iron also regulates the transcription of several genes by binding

to iron regulatory proteins (IRP) and influencing the mRNAs stability [52]. In particular, the binding of iron to IRP releases IRP from ferritin mRNA allowing its transcription and conferring cytoprotection against oxidative stress [53]. In contrast, the removal of IRP by iron binding, destabilizes the transferrin receptor mRNA, diminishing the cellular capacity for iron uptake [52]. Biliverdin is reduced to bilirubin by biliverdin reductase (Figure 2.1) and both metabolites are believed to be potent endogenous antioxidants, which efficiently scavenges peroxy radicals inhibiting lipid peroxidation [54-56].

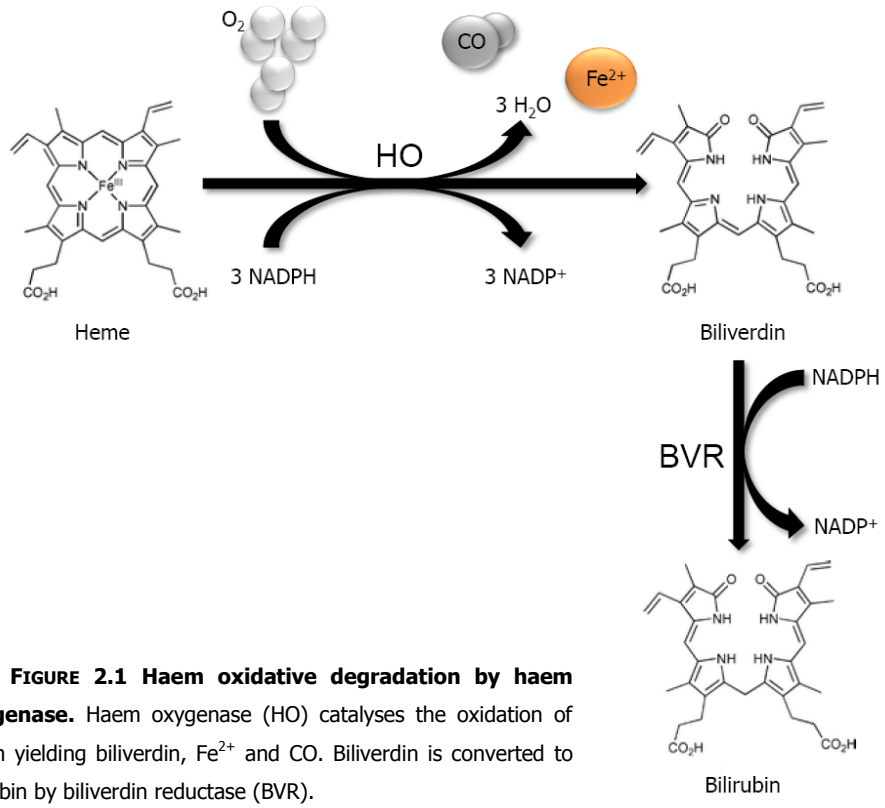

FIGURE 2.1 Haem oxidative degradation by haem oxygenase. Haem oxygenase (HO) catalyses the oxidation of haem yielding biliverdin, Fe^{2+} and CO. Biliverdin is converted to bilirubin by biliverdin reductase (BVR).

To date, three isoenzymes of haem oxygenase were identified, HO-1, HO-2 and HO-3 (Table 2.4). The HO-1 was first characterised in 1974, HO-2 was discovered about a decade after HO-1, and HO-3 was identified in 1997 [57-61].

TABLE 2.4 Characteristics of haem oxygenase isoforms.

	HO-1	HO-2	HO-3
Physiological Function	Haem degradation Anti-oxidant defence Modulation of vascular tone and liver perfusion Neural signaling Anti-inflammatory Regulation of haemoproteins activity	Haem degradation Haem binding Maintenace of vascular tone Neural signaling	Haem binding
Constitutive tissue expression	Spleen Liver	Most tissues e.g. Brain Retina Liver Spleen Testis Lungs Kidney Vasculature	Most tissues
Inducers	Endotoxin Heat – shock Heavy metals Haem Hydrogen peroxide Hyperoxia Hypoxia NO Phorbol esters Shear stress Sodium arsenite UV radiation	Adrenal glucocorticoids opiates	Not known
Enzyme activity	K_m = 0.24 μM V_{max} = 3.4 μmol/mg/h	K_m = 0.67 μM V_{max} = 0.24 μmol/mg/h	Negligible

Adapted from [52]

The HO-2 and HO-3 are constitutively expressed and display high amino acid sequence similarity among them, while HO-1 is inducible and presents low

percentage of sequence identity and similarity with the other two isoforms [61, 62]. The three proteins also differ in their physiological functions, tissue distribution and regulation (Table 2.4). HO-1 occurs at undetectable levels in most tissues and cell types until a stress condition arises. Nevertheless, HO-1 is the predominant form that contributes to the haem oxygenase activity in spleen even under normal, unstressed conditions [63]. HO-2 may exist at detectable levels in most tissues in the absence of stress and in spite of being considered as constitutive, it is responsive to adrenal glucocorticoids [62]. HO-2 is the main form in neurons and endothelial cells and its function is associated with neurotransmission and regulation of vascular tone [62, 64]. Even though HO-3 is found in most organs (brain, heart, kidney, liver, testes and spleen), its role remains unknown since besides binding haem is not able to degrade it [52, 61]. It is proposed that HO-3 may derived from the retrotransposition of HO-2 as HO-3 does not contain introns [65].

As mentioned, HO-1 is stimulated by conditions that cause a threat to cell homeostasis and survival (Table 2.4). Among the HO-1 inducers are haem and haem derivatives, UV radiation, heavy metals, NO and its derivatives, hypoxia and hyperoxia, heat shock and oxidative stress namely hydrogen peroxide (Table 2.4). HO-1 expression is also augmented by pro-inflammatory cytokines (IL-1, IL-6, IL-10, TNF-α and INF-γ), bacterial endotoxins (e.g. LPS), growth factors (PDGF and TGF-β), tumor promoters and oxidised lipids (Table 2.4) [22, 44]. These agents share the ability to direct or indirectly generate intracellular reactive oxygen species and/or modulate intracellular redox equilibrium. Therefore, the enhancing of HO-1 expression is considered a general indicator of oxidative stress in cells and tissues [66]. After induction, the expression of HO-1 overpowers the protein level of the others isoenzymes, namely in testes, lung and brain [22]. The importance of HO-1 in host defence has been demonstrated in mice since the deletion of HO-1 gene increases the susceptibility of mice to inflammatory conditions associated with atherosclerosis, endotoximia, chronic graft rejection and haem metabolism [67-69].

2.5 Carbon monoxide: signaling pathways and physiological function

Several studies have shown that HO-derived CO has an important role in the cardiac, immune, respiratory, reproductive, gastrointestinal, kidney and liver systems [22]. Such importance is due to the anti-inflammatory, anti-proliferative, anti-apoptotic and vasoregulatory effects of CO [70] (Figure 2.2). CO confers cytoprotection by modulating relatively defined signaling pathways. The CO modulation of the soluble guanylate cyclase (sGC) activity and subsequent stimulation of the cyclic guanosine monophosphate (cGMP) production is the best well known function. Other mechanisms include the modulation of various mitogen-activated protein kinase (MAPK) pathways and the stimulation of large conductance calcium-activated potassium (K_{Ca}) channels activity (Figure 2.2) [70].

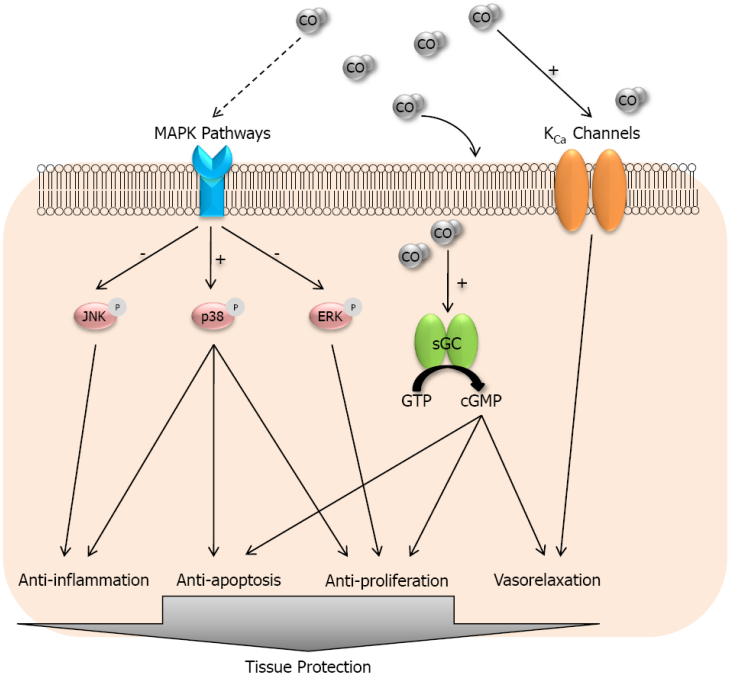

FIGURE 2.2 Signaling pathways modulated by carbon monoxide. CO activates soluble guanylate cyclase (sGC) increasing the levels of cyclic guanosine monophosphate (cGMP). CO also activates large conductance calcium-activated potassium (K_{Ca}) channels and modulates mitogen-activated protein kinase (MAPK) pathways. Adapted from [70].

Soluble guanylate cyclase is a heterodimeric protein consisting of an α and a β subunit and catalyses the conversion of guanosine 5′-triphosphate (GTP) to cGMP and pyrophosphate (PPi) (Figure 2.3). The β subunit contains a haem co-factor where CO binds directly stimulating its enzymatic activity [44].

The binding of CO to the haem of sGC occurs via a hexacoordinate complex and the dissociation of CO from sGC proceeds through a pentacoordinate intermediate and this transition is proposed to be responsible for sGC activation [71]. In contrast, the interaction mode of NO with the haem of sGC occurs through a pentacoordinate complex with concomitant displacement of the axial ligand His [50]. This may be the reason why the activation of sGC by CO is 30-100 times lower than that of NO and that the CO may only activate sGC when the NO concentration is relatively low [72].

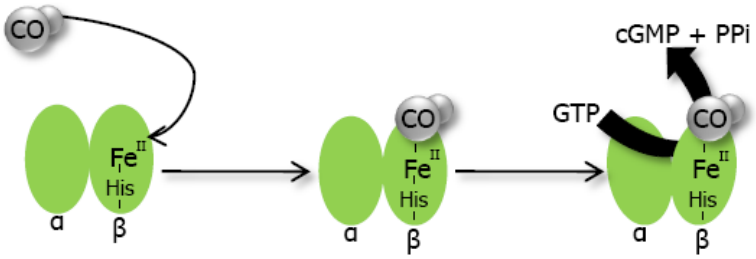

FIGURE 2.3 Schematic representation of soluble guanylate cyclase activity. Binding of CO as the sixth coordinated ligand of the ferrous iron of sGC haem and the displacement of the histidine (His) yields a five-coordinated haem-carbonyl that activates the protein activity [50].

Interestingly, the combination of CO with 1-benzyl-3-(5′-hydroxymethyl-2-furyl)indazole (YC-1), a chemically synthetic benzylindazole compound, that possesses antiplatelet activity by activating sGC, makes CO an activator of sGC as effective as NO [73]. YC-1 interacts with the α-domain of the sGC and induces the displacement of His, producing a five-coordinated iron in the haem-carbonyl complex in a similar way as NO does [50, 74]. On the other hand, at high NO concentration CO blocks the effect of NO on sGC activation [75]. Therefore, the importance of CO on sCG activation is likely to increase not only when the NO level is too low or too high but also when molecules like YC-1 are present in cells or

tissues [50, 75]. By increasing cGMP levels, CO regulates vasorelaxation, neurotransmission and inhibition of platelet aggregation and has anti-proliferative and anti-apoptotic effect in vascular smooth muscle cells and in fibroblasts [17, 76-80].

The mechanism of CO activation of K_{Ca} channels is not completely understood. Wang and co-workers showed that in vascular smooth muscle cells, CO enhances the calcium sensitivity of K_{Ca} channels but does not affect the levels of intracellular calcium, whereas Lin *et al.* reported that CO reduces the intracellular calcium concentrations [81, 82]. A more recent work showed that CO activates K_{Ca} channels by direct interaction with the α-subunit of K_{Ca} channels, lowering their apparent dissociation constant for calcium [83]. It was also reported that haem binds K_{Ca} channels inhibiting transmembranar potassium currents and decreasing the frequency of the channel opening [84]. The inhibition of sGC pathways and K_{Ca} channels completely abolishes the CO-induced vasorelaxation in rat tail artery, while blockers of one of those pathways only partially abolishes the vasodilatation induced by CO. These results showed that CO mediates vasodilatation by activating, in an independent mode, the two pathways [85].

The molecular mechanism by which CO activates/inhibits MAPK pathways remains elusive, especially because these pathways do not involved haemproteins. A potential candidate to which CO may bind is the protein phosphatase 2C, a serine/threonine phosphatase that contains Mn^{2+} at its active site [86]. Nevertheless, several studies have been shown that CO down-regulates extracellular signal-regulated kinase (ERK), which leads to inhibition of proliferation of human airway smooth muscle cells and activates p38 pathways resulting in the reduction of vascular smooth muscle cells and T lymphocytes proliferation and in inhibition of endothelial cells apoptosis [87-91]. The anti-inflammatory effect of CO involves the activation of p38 pathway as well as the inhibition of c-Jun amino-terminal kinases (JNK) as demonstrated in macrophages, in which CO exposure inhibited the expression of pro-inflammatory cytokines (TNF-α, IL-1β, MIP-1β and

IL-6) and increased the expression of anti-inflammatory cytokine interleukin-10 [92-94].

2.6 Carbon monoxide and bacteria

2.6.1 Carbon monoxide as carbon and energy source

Some type of bacteria can use CO as carbon and energy sources. In aerobic and anaerobic bacteria, CO is oxidised to carbon dioxide by CO dehydrogenase (CODH), which also catalyses the reverse reaction (Equation 2.3) [95].

EQUATION 2.3 $CO + H_2O \Rightarrow CO_2 + 2H^+ + 2e^-$

CODH contains as active site a non-haem iron and either molybdenum (in aerobes) or nickel (in anaerobes), and the CO oxidation is coupled to several metabolic pathways that occur in the presence and absence of oxygen [95, 96]. Therefore, the reducing equivalents generated by CO oxidation are received by electron acceptors into a respiratory chain leading to the reduction of the terminal electron acceptor. For example, in aerobic microorganisms, like *P. thermocarboxydovorans*, which possesses a CO-insensitive respiratory chain, the reducing equivalents are used for oxygen reduction or, in some cases, for nitrate reduction [97, 98]. Under anaerobic conditions the respiratory processes that are coupled to CO oxidation distinguish three groups of CO-metabolising bacteria: i) the sulfate reducers that include the desulfuricants (e.g. *Desulfovibrio vulgaris*), in which the oxidation of CO yields carbon dioxide and molecular hydrogen that is subsequently utilised for sulfate reduction (Equation 2.4) [95]; ii) the hydrogenogens such as *Rhodospirillum rubrum* that are able to, in absence of an electron acceptor, oxidise CO and reduce the protons derived from water to form hydrogen (in a reaction analogous to the water-gas-shift reaction) (Equation 2.5) [95]; and iii) the acetogens, which are a diverse group of bacteria that possess a bifunctional CODH/acetyl-coenzyme A (-CoA) synthase that confers the ability to utilise acetyl-CoA as a terminal electron-acceptor. Several bacteria of clostridia

(e.g. *Clostridium aceticum*) contain the bifunctional enzyme and are able to produce acetyl-CoA from a methyl-group, CoA and CO (Equation 2.6) [50, 95, 99].

EQUATION 2.4 $4\ CO + SO_4^{2-} + H^+ \Rightarrow 4\ CO_2 + HS^+$

EQUATION 2.5 $CO + H_2O \Rightarrow CO_2 + H_2$

EQUATION 2.6 $4\ CO + 2\ H_2O \Rightarrow CH_3COO^- + 2\ CO_2 + H^+$

2.6.2 Bacterial haem oxygenase

Iron is essential for bacterial growth and is particular important for pathogens during the infection process. Since the most abundant source of host iron is usually the haem-containing proteins, bacteria have developed efficient haem/iron acquisition mechanisms [100]. Bacterial haem oxygenase are enzymes that cleavage the haem macrocycle, leading to the release of iron, CO and biliverdin in a reaction analogous to that of the human haem oxygenase previously described (2.4; Figure 2.1). In contrast to the eukaryotic counterparts, bacterial haem oxygenases are smaller and soluble enzymes that lack the C-terminal membrane anchor [101].

The first bacterial haem oxygenase was identified in *Corynebacterium diphtheriae* due to the high amino acid sequence similarity with the human HO-1 (70 %) [102]. In *S. aureus* two bacterial haem oxygenases have been identified: IsdG and IsdI, which in spite of showing no significant sequence similarity with human haem oxygenases nor with haem oxygenase of *C. diphtheria* (Table 2.5), possess haem-degrading activity [103]. The two *S. aureus* haem oxygenase enzymes share a high percentage of identity/similarity (63/78 %) and homologues are found in staphylococci species such as *S. epidermidis*, *B.* species (e.g. *B. anthracis*) and *Listeria monocytogenes* (Table 2.5) [104].

In addition to their role in iron acquisition, bacterial haem oxygenases seem also to protect bacterial cells against haem toxicity. In particular, it has been shown that strains of *B. anthracis* and *N. meningitides* deleted in the haem oxygenase genes show growth impairment in the presence of hemin [105, 106].

TABLE 2.5 Amino acid sequence identity/similarity of haem oxygenase with *S. aureus* IsdG and Isd.

Haem oxygenase	IsdG (% I/S)	IsdI (% I/S)
S. epidermidis	30/58	29/58
B. anthracis	33/45	31/45
L. monocytogenes	22/45	23/47
HO-1 (*Homo sapiens*)	5/10	6/12
HO-2 (*Homo sapiens*)	4/12	5/13
C. diphtheria	5/12	4/13

%I/S: Percentage of identity/similarity

Although it is well accepted that the function of HO activity in bacteria is related to iron acquisition, the fate of the other HO-products remains elusive. In eukaryotes, biliverdin is rapidly converted into bilirubin (as described in 2.4), [54-56]. However, due to the lack of genes encoding homologues of mammalian biliverdin reductase it seems unlike that, in bacteria, biliverdin is metabolised to bilirubin. The significance of CO production in bacteria also remains unclear, although the presence of CO receptors in prokaryotes suggest that CO may participate in signaling pathways as it occurs in eukaryotes [101].

2.6.3 Sensors of carbon monoxide

The ability of bacteria to sense small gaseous molecules is an important tool that allows bacteria to adapt to different environments. In general, bacteria possess proteins/sensors that bind CO and regulate the transcription of genes, namely those involved in CO metabolism. The first CO sensor described was CooA from *R. rubrum* [107]. This protein contains haem as prosthetic group that ligates CO as a sixth coordinate ligand, stabilising the dimeric conformation of CooA and allowing the binding of the protein to the DNA target, leading to the activation of the CODH expression [108]. NO also binds to CooA but is unable of inducing DNA binding [109].

Other example of a CO sensor is DosST, a two-component system from *M. tuberculosis* [110]; DosS and DosT bind haem as prosthetic group and are both able to ligate CO forming haem-carbonyl complexes [111]. The binding of CO to DosS or DosT induces the dormancy regulon shifting *M. tuberculosis* to a latent state in which the bacterium remains unresponsive to drug therapy. The deletion of *dosS* severely attenuates the *M. tuberculosis* ability to induce the Dos regulon, while the disruption of *dosT* gene only moderately affect the Dos regulon, suggesting that CO is primarily sensed by DosS. As usually observed for bacterial sensors, DosS and DosT are not specific CO sensors since they are also responsive to dioxygen and NO [110, 111]. The same has been described for the *E. coli* oxygen sensor EcDos that, in addition to oxygen, also binds CO and NO being its activity inhibited by the two gases [112]. It is then expected that other bacterial regulators, particularly those containing haems, might act as CO sensors.

2.7 Carbon monoxide-releasing molecules

The discovery that endogenous production of CO has an essential role in the biological systems triggered the search for compounds that could mimic the action of HO-derived CO. Because the prolonged inhalation of CO gas may compromise the oxygen transport and the delivery to the human body, the use of this gas in a therapeutic context seems unlike. This problem prompted the search and design of a novel class of molecules capable of safely store and release CO into physiological media, the so-called CO-RMs [23]. Such molecules contain a transition metal, like manganese, cobalt or iron, coordinated to carbonyl groups and have the ability of liberating a CO group to myoglobin as indicated by the formation of carboxy-myoglobin [23]. CO-RMs exhibit pharmacological activity and their biological activity is attributed to the release of CO since the addition of reduced myoglobin and/or the substitution of the CO-RM molecule by an inactive form (iCO-RM), in which the CO groups were depleted, impairs the bioactivity [24].

2.7.1 Chemical proprieties and bioactivity of CO-RMS

One of the first metal carbonyl complexes identified and tested for its ability of carrying and releasing CO to myoglobin was the dimanganese decacarbonyl [$Mn_2(CO)_{10}$] molecule, named CORM-1 [34] (Figure 2.4). This compound, insoluble in water, is soluble in dimethyl sulfoxide (DMSO) or ethanol and it only releases CO upon stimulation with light (Table 2.6) [23]. CORM-1 is able to rapidly liberate CO (Table 2.6) in a 1:1 ratio, as quantified by the formation of carboxy-myoglobin [23, 34]. CORM-1 induces vasodilatation of cerebral arterioles and attenuates the vasoconstriction of isolated rat hearts perfused, even when the eNOS enzyme is inhibited, indicating that CORM-1 causes vasorelaxation in a NO-independently way [24, 83, 113]. Studies in mice also show that CORM-1 has anti-inflammatory proprieties [114].

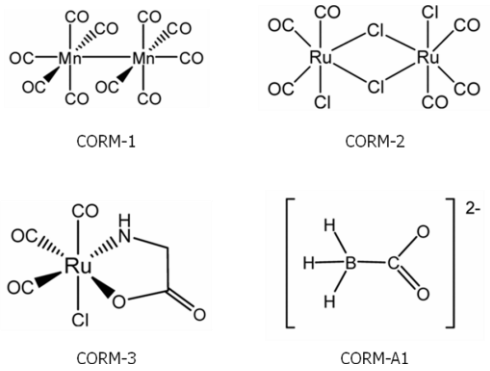

FIGURE 2.4 Chemical structure of several transition metal carbonyls. CORM-1 is a manganese-complex that liberates CO by photodissociation. CORM-2 and CORM-3 are ruthenium-complexes that release CO by ligand substitution. CORM-A1 is the first prototypic of a CO-RM that instead of a transition metal contains a carboxylic group convertible into CO by hydrolysis.

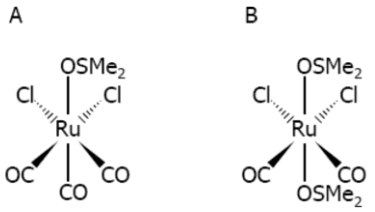

FIGURE 2.5 Chemical structures of the two CORM-2 monomers. The ration between the tri-carbonyl (A) and a di-carbonyl (B) formed upon dissolution in DMSO ($OSMe_2$) is 40:60 [23].

Tricarbonyldichloro ruthenium(II) dimmer ($[Ru(CO)_3Cl_2]_2$), also denominated of CORM-2, is a ruthenium-based carbonyl (Figure 2.4) able to rapidly promote carboxy-myoglobin formation after dissolution in DMSO (Table 2.6), liberating 0.7

moles of CO per molecule [23, 34]. In fact, it has been shown that, after being dissolved in DMSO, CORM-2 does not exist as a dimer but as two distinct monomers, namely a tri-and a di-carbonyl monomer (Figure 2.5) [23].

TABLE 2.6 Proprieties and bioactivity of CO-releasing molecules

CO-RM	Solubility	CO release mode	Physiological role
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 Anti-inflammatory Anti-proliferative Anti-apoptotic Reno-protective Anti-carcinogenic
CORM-3	Water	Ligand substitution $t_{1/2}$ ~ 1min	Vasodilator Anti-inflammatory Anti-proliferative Anti-apoptotic Cardioprotective Reno-protective
CORM-A1	Water	pH dependent $t_{1/2}$ ~ 21min	Vasodilator Anti-apoptotic Reno-protective

*$t_{1/2}$: half-life
Table adapted from [24]

CORM-2 has been used *in vitro* and *in vivo* models and showed to mimic the known physiological functions of CO (Table 2.6). CORM-2 induces vasorelaxation of smooth muscle cells and, even at concentrations as high as 420 μM it does not seem to promote any detectable cytotoxicity. Furthermore, CORM-2 induces the vasodilatation of aortic rings pre-contracted with phenylephrine and the total removal of phenylephrine does not restore the vessel contraction, suggesting that CORM-2 has a prolonged effect. Moreover, the intravenous administration of CORM-2 reduces acute hypertension of rats whereas the iCO-RM ($[RuCl_2(DMSO)_4]$)

does not. Importantly, the administration of CORM-2 to rats (5-20 μmol/Kg) do not changed the levels of oxy-haemoglobin [23].

The anti-inflammatory effect of CORM-2 has been demonstrated in several models. In macrophages, CORM-2 reduces the production of reactive oxygen species and NO, and inhibits the up-regulation of iNOS [115]. In neutrophils, CORM-2 decreases the generation of superoxide suggesting that CO may inhibit the NADPH oxidase [115, 116]. Furthermore, it was shown that CORM-2 impairs the neutrophil adhesion to the human umbilical vein endothelial cells [117, 118]. Treatment with CORM-2 of thermally injured mice also attenuates neutrophils accumulation in kidney, liver and small intestine of burned mice [119-121]. The role of CORM-2 in cell proliferation and apoptosis was also reported, with CORM-2 inhibiting the proliferation of human airway smooth muscle cells, pulmonary artery smooth muscle cells and Jurkat T cells [122-124]. The anti-apoptotic effect of CORM-2 was demonstrated in rat adrenal pheochromocytoma cells (PC12) and in murine fibroblast cells [125, 126]. The biological proprieties of CORM-2 are attributed to CO release since the effects are abolished in the presence of haemoglobin or when using an inactive form [122-124, 126].

To improve the compatibility of CO-RMs with biological systems the first water soluble CO-RM was produced. The tricarbonylchloro(glycinato) ruthenium(II) ($[Ru(CO)_3Cl(glycinate)]$, known as CORM-3 (Figure 2.4), is like CORM-2 a ruthenium-based carbonyl and a fast CO releaser (Table 2.6), and the presence of a glycine coordinated to the metal center confers water solubility to the molecule [23]. The vasorelaxant activity of CORM-3 was first demonstrated in aortic rings [24, 34]. The administration of CORM-3 to rat aortic smooth muscle cells induces vasodilatation in a concentration dependent manner and concentrations up to 500 μM CORM-3 do not cause decrease of cell viability [24]. CORM-3 also has a significant anti-inflammatory role. In particular, in microglia and macrophages cell lines, CORM-3 decreases the level of NO production and reduces the TNF-α release [116, 127, 128]. In both cases, the inhibitory effects are cancelled when using iCORM-3 [116, 127, 128]. Moreover, the intravenous injection of CORM-3 in mice,

lowers the number of neutrophil extravasation, attenuating the acute inflammation [129]. 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 [24].

Sodium boranocarbonate (CORM-A1) is another water-soluble CO releaser (Figure 2.4). This compound was the first CO-RM that does not contain a transition metal but a carboxylic group, which is converted into CO through hydrolysis. Unlike the other molecules described above, CORM-A1 liberates CO at a much slower rate (Table 2.6) and the CO release is pH and temperature dependent; the rate of CO liberation is accelerated by decrease of pH and increase of temperature [130]. The physiological activity of this compound has not been yet intensively explored. Nevertheless, it was reported that CORM-A1 displays vasorelaxation activity, inducing a gradual but profound dilation of isolated aortic rings [130]. More importantly, the administration of CORM-A1 *in vivo* lowers the mean arterial pressure of rats while the inactive form (sodium borate) does not promote vasodilatation *in vitro* or hypotension *in vivo* [24, 130].

2.7.2 Therapeutic applications

The protective role of HO/CO is well established in an array of disease models (Figure 2.6) [22, 52]. Since CO-RMs mitigate the beneficial effects of CO gas (as mention in 2.7), these compounds represent a valid alternative as therapeutic agents to overcome the systemic problem raised by CO inhalation. Unlike CO gas, the CO liberated from CO-RMs is tightly controlled and delivered at a given concentration, and bypass, in a more effective way, the biological trapping by haemoglobin. In particular, the intravenous application of CO-RMs on mice does not increase carboxy-haemoglobin to dangerous levels (COHb levels < 10 %) [131].

The major constraint of the first developed CO-RMs lay on their poor solubility in water, which made their *in vivo* utilisation very limited, but water-soluble CO-RMs compatible with the biological systems are presently available. Hence, CO-RMs

possess all the features required to become CO-based pharmaceuticals to be used to delivery CO to tissues and organs.

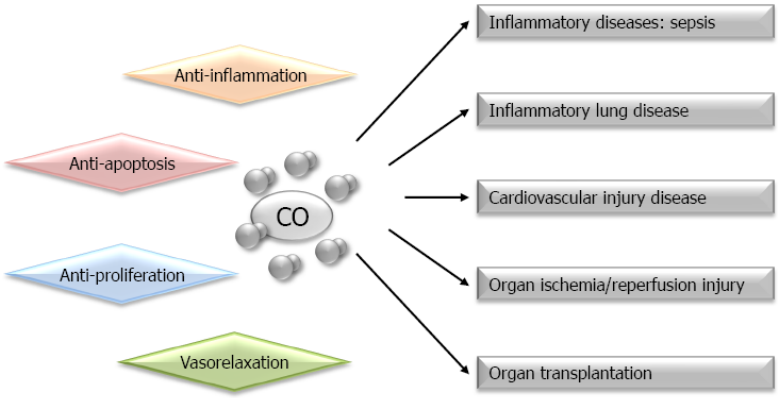

FIGURE 2.6 Physiological function of CO and their possible medical applications. The figure summarises the known physiological functions of HO/CO (left) as well as the potential medical applications (right).

The beneficial effects of the therapeutic application of CO-RMs includes: i) cardioprotection, as ischemic rat hearts reperfused in the presence of CORM-3 are able to recover the perfusion and contractility, and its intravenous administration prolongs the survival of mice after heart transplantation and reduces the infarct size of mice subjected to coronary artery occlusion [34, 132, 133]; ii) renal protection, as the administration of Ru-based CO-RMs decrease the plasma creatinine level and limit renal damage in a mouse model of ischaemia-induced acute renal failure, and the intrarenal administration of CORM-1 and CORM-A1 to rats increase of renal blood flow, glomerular filtration, and urinary cGMP excretion [134-137]; iii) anti-carcinogenic effects since the topical application of CORM-2 in mice skin leads to the reduction of tumor multiplicity, regression of established tumor and impairs the formation of locally invasive tumors [138]; and iv) attenuation of the systemic inflammation causing the administration of CORM-1 to mice the decrease of neutrophils migration and their rolling and adhesion to the endothelium in the inflammation site, while the use of CORM-2 decreases

myeloperoxidase activity in liver of mice with induced polymicrobial sepsis [114, 117].

2.8 Parallelism between carbon monoxide and nitric oxide

There is a parallelism between CO and NO regarding not only the structure and molecular size but also the endogenous production and biological function. Additionally, their regulation seems to be intimately linked. CO and NO are two diatomic molecules that have similar structure, molecular weight and solubility. Furthermore, they share similarity in biochemical reactivity since both bind to the haem iron center of most known haemoproteins [62]. However, while CO only binds ferrous iron, NO may ligate to either ferrous and ferric haem. An important difference between the two gases arises from the radical nature of NO that, in contrast to CO, reacts with other free radicals and assumes several oxidation states, being a more reactive molecule (see Chapter 1).

CO and NO are endogenously produced by HO and NOS, respectively, and both enzymes have constitutive and inducible forms triggered by several common stimulus. Concerning their regulation, NO has been shown to both inhibit and activate the HO activity. As a radical, NO is perhaps the strongest inducer of the HO-1 expression [139]. On the other hand, the binding of NO to the haem of HO prevents the ligation of oxygen and impairs its activity. CO ligates to NOS and inactivates the enzyme [140]. In contrast, it is not clear the effect of CO on NOS expression since it was reported that CO up-regulates iNOS in hepatocytes whereas in other models HO-derived CO inhibits the iNOS expression [22, 141].

Regarding their biological function, both gases are important signaling molecules and their action is mediated by activation of sGC. As stated previously (2.5), the ability to activate the sGC differs between the two gases and the relevance of their action depend on several factors. For example, the enzymatic production of NO is more dependent on oxygen concentration than on CO. Since NOS requires normal oxygen concentrations, as the system becomes anaerobic NOS ceases to work whereas HO continues to produce CO. It is therefore possible that, under anaerobic conditions, CO becomes a more important signaling molecule

than NO [50]. Interestingly, a study conducted in mice hepatocytes has shown that the removal of iNOS/NO from the system results in a loss of protection which can be reversed upon HO-1 induction. However, if HO-1 is removed, in spite of the presence of iNOS/NO, the protection afforded is lost [141].

Since the 19th century, NO-releasing drugs, like amyl nitrite and nitroglycerine have been used to treat angine pectoris [142-144]. Even though the methods for delivering CO to treat human diseases are well behind the knowledge of NO-based compounds, CO-RMs are already envisaged to become relevant pharmaceutical tools.

Although NO has also a established function in pathogen elimination (as discussed in Chapter 1), the role of CO in pathogen eradication has never been explored. In this thesis we have analysed the effect of CO on bacteria survival through the use of CO-RMs and we showed that CO has the ability of killing bacteria, namely *E. coli* and *S. aureus* (Chapter 5).

2.9 References

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

[2] Gréhant, N. (1894) Les gaz du sang. G. Masson, Paris.

[3] Nicloux, M. (1925) L'oxyge de carbone et l'intoxication oxcarbonique. G. Masson, Paris.

[4] Sjostrand, T. (1949) Endogenous formation of carbon monoxide in man. Nature 164, 580.

[5] Sjostrand, T. (1951) Formation of carbon monoxide in connexion with haemoglobin catabolism. Nature 168, 1118-1119.

[6] Sjostrand, T. (1951) Endogenous formation of carbon monoxide; the CO concentration in the inspired and expired air of hospital patients. Acta Physiol. Scand. 22, 137-141.

[7] Engel, R.R., Matsen, J.M., Chapman, S.S. and Schwartz, S. (1972) Carbon monoxide production from haem compounds by bacteria. J. Bacteriol. 112, 1310-1315.

[8] Tenhunen, R., Marver, H.S. and Schmid, R. (1969) The enzymatic conversion of haemoglobin to bilirubin. Trans. Assoc. Am. Physicians 82, 363-371.

[9] Tenhunen, R., Marver, H.S. and Schmid, R. (1968) The enzymatic conversion of haem to bilirubin by microsomal haem oxygenase. Proc. Natl. Acad. Sci. U S A 61, 748-755.

[10] Tenhunen, R. (1976) The enzymatic conversion of haem to bilirubin in vivo. Ann. Clin. Res. 8 Suppl. 17, 2-9.

[11] Barinaga, M. (1993) Carbon monoxide: killer to brain messenger in one step. Science 259, 309.

[12] Snyder, S.H. and Bredt, D.S. (1992) Biological roles of nitric oxide. Sci. Am. 266, 68-71, 74-67.

[13] Moncada, S., Palmer, R.M. and Higgs, E.A. (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. 43, 109-142.

[14] Ignarro, L.J., Buga, G.M., Wood, K.S., Byrns, R.E. and Chaudhuri, G. (1987) Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc. Natl. Acad. Sci. U S A 84, 9265-9269.

[15] Ignarro, L.J., Byrns, R.E., Buga, G.M. and Wood, K.S. (1987) Endothelium-derived relaxing factor from pulmonary artery and vein possesses pharmacologic and chemical properties identical to those of nitric oxide radical. Circ. Res. 61, 866-879.

[16] Patel, R.P., McAndrew, J., Sellak, H., White, C.R., Jo, H., Freeman, B.A. and Darley-Usmar, V.M. (1999) Biological aspects of reactive nitrogen species. Biochim. Biophys. Acta 1411, 385-400.

[17] Verma, A., Hirsch, D.J., Glatt, C.E., Ronnett, G.V. and Snyder, S.H. (1993) Carbon monoxide: a putative neural messenger. Science 259, 381-384.

[18] Rattan, S. and Chakder, S. (1993) Inhibitory effect of CO on internal anal sphincter: haem oxygenase inhibitor inhibits NANC relaxation. Am. J. Physiol. 265, G799-804.

[19] Burstyn, J.N., Yu, A.E., Dierks, E.A., Hawkins, B.K. and Dawson, J.H. (1995) Studies of the haem coordination and ligand binding properties of soluble guanylyl cyclase (sGC): characterization of Fe(II)sGC and Fe(II)sGC(CO) by electronic absorption and magnetic circular dichroism spectroscopies and failure of CO to activate the enzyme. Biochemistry 34, 5896-5903.

[20] Stone, J.R. and Marletta, M.A. (1995) The ferrous haem of soluble guanylate cyclase: formation of hexacoordinate complexes with carbon monoxide and nitrosomethane. Biochemistry 34, 16397-16403.

[21] Christodoulides, N., Durante, W., Kroll, M.H. and Schafer, A.I. (1995) Vascular smooth muscle cell haem oxygenases generate guanylyl cyclase-stimulatory carbon monoxide. Circulation 91, 2306-2309.

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

[23] Motterlini, R., Clark, J.E., Foresti, R., Sarathchandra, P., Mann, B.E. and Green, C.J. (2002) Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. Circ. Res. 90, E17-24.

[24] Motterlini, R., Mann, B.E. and Foresti, R. (2005) Therapeutic applications of carbon monoxide-releasing molecules. Expert Opin. Investig. Drugs 14, 1305-1318.

[25] Motterlini, R. (2007) Carbon monoxide-releasing molecules (CO-RMs): vasodilatory, anti-ischaemic and anti-inflammatory activities. Biochem. Soc. Trans. 35, 1142-1146.

[26] Otterbein, L.E. (2009) The evolution of carbon monoxide into medicine. Respir. Care 54, 925-932.

[27] Li, L., Hsu, A. and Moore, P.K. (2009) Actions and interactions of nitric oxide, carbon monoxide and hydrogen sulphide in the cardiovascular system and in inflammation-a tale of three gases! Pharmacol. Ther. 123, 386-400.

[28] Mancuso, C., Navarra, P. and Preziosi, P. (2010) Roles of nitric oxide, carbon monoxide, and hydrogen sulfide in the regulation of the hypothalamic-pituitary-adrenal axis. J. Neurochem. 113, 563-575.

[29] Li, C., Hossieny, P., Wu, B.J., Qawasmeh, A., Beck, K. and Stocker, R. (2007) Pharmacologic induction of haem oxygenase-1. Antioxid. Redox Signal 9, 2227-2239.

[30] Kajimura, M., Fukuda, R., Bateman, R.M., Yamamoto, T. and Suematsu, M. (2010) Interactions of multiple gas-transducing systems: hallmarks and uncertainties of CO, NO, and H_2S gas biology. Antioxid. Redox Signal 13, 157-192.

[31] Greenwood, N.N. and A., E. (1997) Chemistry of the Elements. Butterworth-Heinemann, Oxford

[32] Green, W. (2008) An introduction to indoor air quality: carbon monoxide (CO). United States Environmental Protection Agency.

[33] Von Berg, R. (1999) Toxicology update. Carbon monoxide. J. Appl. Toxicol 19, 379-386.

[34] Motterlini, R., Mann, B.E., Johnson, T.R., Clark, J.E., Foresti, R. and Green, C.J. (2003) Bioactivity and pharmacological actions of carbon monoxide-releasing molecules. Curr. Pharm. Des. 9, 2525-2539.

[35] Chin, B.Y. and Otterbein, L.E. (2009) Carbon monoxide is a poison... to microbes! CO as a bactericidal molecule. Curr. Opin. Pharmacol. 9, 490-500.

[36] Schlesinger, G. and Miller, S.L. (1983) Prebiotic synthesis in atmospheres containing CH_4, CO, and CO_2. I. Amino acids. J. Mol. Evol. 19, 376-382.

[37] Handa, P.K. and Tai, D.Y. (2005) Carbon monoxide poisoning: a five year review at Tan Tock Seng Hospital, Singapore. Ann. Acad. Med. Singapore 34, 611-614.

[38] Shochat, G.N. and Lucchesi, M. (2009) Toxicity, Carbon Monoxide. emedicine.

[39] Godin, G., Wright, G. and Shephard, R.J. (1972) Urban exposure to carbon monoxide. Arch. Environ. Health 25, 305-313.

[40] Kent, R. and Olson, M.D. (1984) Carbon monoxide poisoning: mechanisms, presentation, and controversies in management. Journal of Emergency Medicine 1 233-243

[41] Ryter, S.W., Morse, D. and Choi, A.M. (2004) Carbon monoxide: to boldly go where NO has gone before. Sci STKE 2004, RE6.

[42] Haldane, J.B. (1927) Carbon Monoxide as a Tissue Poison. Biochem. J. 21, 1068-1075.

[43] Smith, R.P. (1986) Toxic responses of the blood, 223-240. Mc Millan Publishing Company, New York.

[44] Ryter, S.W., Otterbein, L.E., Morse, D. and Choi, A.M. (2002) Haem oxygenase/carbon monoxide signaling pathways: regulation and functional significance. Mol. Cell Biochem. 234-235, 249-263.

[45] Varon, J., Marik, P.E., Fromm, R.E., Jr. and Gueler, A. (1999) Carbon monoxide poisoning: a review for clinicians. J. Emerg. Med. 17, 87-93.

[46] Ryter, S.W. and Otterbein, L.E. (2004) Carbon monoxide in biology and medicine. Bioessays 26, 270-280.

[47] Gorman, D., Drewry, A., Huang, Y.L. and Sames, C. (2003) The clinical toxicology of carbon monoxide. Toxicology 187, 25-38.

[48] Rodgers, P.A., Vreman, H.J., Dennery, P.A. and Stevenson, D.K. (1994) Sources of carbon monoxide (CO) in biological systems and applications of CO detection technologies. Semin. Perinatol. 18, 2-10.

[49] Coburn, R.F., Blakemore, W.S. and Forster, R.E. (1963) Endogenous carbon monoxide production in man. J. Clin. Invest. 42, 1172-1178.
[50] Mann, B.E. and Motterlini, R. (2007) CO and NO in medicine. Chem Commun (Camb) 4197-4208.
[51] Tenhunen, R., Marver, H.S. and Schmid, R. (1970) The enzymatic catabolism of haemoglobin: stimulation of microsomal haem oxygenase by hemin. J. Lab. Clin. Med. 75, 410-421.
[52] Wagener, F.A., Volk, H.D., Willis, D., Abraham, N.G., Soares, M.P., Adema, G.J. and Figdor, C.G. (2003) Different faces of the haem-haem oxygenase system in inflammation. Pharmacol. Rev. 55, 551-571.
[53] Vile, G.F., Basu-Modak, S., Waltner, C. and Tyrrell, R.M. (1994) Haem oxygenase 1 mediates an adaptive response to oxidative stress in human skin fibroblasts. Proc. Natl. Acad. Sci. U S A 91, 2607-2610.
[54] Kutty, R.K. and Maines, M.D. (1981) Purification and characterization of biliverdin reductase from rat liver. J. Biol. Chem. 256, 3956-3962.
[55] McCoubrey, W.K., Jr., Cooklis, M.A. and Maines, M.D. (1995) The structure, organization and differential expression of the rat gene encoding biliverdin reductase. Gene 160, 235-240.
[56] Baranano, D.E., Rao, M., Ferris, C.D. and Snyder, S.H. (2002) Biliverdin reductase: a major physiologic cytoprotectant. Proc. Natl. Acad. Sci. U S A 99, 16093-16098.
[57] Yoshida, T., Takahashi, S. and Kikuchi, G. (1974) Partial purification and reconstitution of the haem oxygenase system from pig spleen microsomes. J. Biochem. 75, 1187-1191.
[58] Maines, M.D. and Kappas, A. (1974) Cobalt induction of hepatic haem oxygenase; with evidence that cytochrome P-450 is not essential for this enzyme activity. Proc. Natl. Acad. Sci. U S A 71, 4293-4297.
[59] Maines, M.D., Trakshel, G.M. and Kutty, R.K. (1986) Characterization of two constitutive forms of rat liver microsomal haem oxygenase. Only one molecular species of the enzyme is inducible. J. Biol. Chem. 261, 411-419.
[60] Trakshel, G.M., Kutty, R.K. and Maines, M.D. (1986) Purification and characterization of the major constitutive form of testicular haem oxygenase. The noninducible isoform. J. Biol. Chem. 261, 11131-11137.
[61] McCoubrey, W.K., Jr., Huang, T.J. and Maines, M.D. (1997) Isolation and characterization of a cDNA from the rat brain that encodes haemoprotein haem oxygenase-3. Eur. J. Biochem. 247, 725-732.
[62] Maines, M.D. (1997) The haem oxygenase system: a regulator of second messenger gases. Annu. Rev. Pharmacol. Toxicol. 37, 517-554.
[63] Braggins, P.E., Trakshel, G.M., Kutty, R.K. and Maines, M.D. (1986) Characterization of two haem oxygenase isoforms in rat spleen: comparison with the hematin-induced and constitutive isoforms of the liver. Biochem. Biophys. Res. Commun. 141, 528-533.
[64] Otterbein, L.E. and Choi, A.M. (2000) Haem oxygenase: colors of defense against cellular stress. Am. J. Physiol. Lung. Cell Mol. Physiol. 279, L1029-1037.

[65] Scapagnini, G., Foresti, R., Calabrese, V., Giuffrida Stella, A.M., Green, C.J. and Motterlini, R. (2002) Caffeic acid phenethyl ester and curcumin: a novel class of haem oxygenase-1 inducers. Mol. Pharmacol. 61, 554-561.

[66] Gozzelino, R., Jeney, V. and Soares, M.P. (2010) Mechanisms of cell protection by haem oxygenase-1. Annu. Rev. Pharmacol. Toxicol. 50, 323-354.

[67] Duckers, H.J., Boehm, M., True, A.L., Yet, S.F., San, H., Park, J.L., Clinton Webb, R., Lee, M.E., Nabel, G.J. and Nabel, E.G. (2001) Haem oxygenase-1 protects against vascular constriction and proliferation. Nat. Med. 7, 693-698.

[68] Fujita, T., Toda, K., Karimova, A., Yan, S.F., Naka, Y., Yet, S.F. and Pinsky, D.J. (2001) Paradoxical rescue from ischemic lung injury by inhaled carbon monoxide driven by derepression of fibrinolysis. Nat. Med. 7, 598-604.

[69] Soares, M.P., Lin, Y., Anrather, J., Csizmadia, E., Takigami, K., Sato, K., Grey, S.T., Colvin, R.B., Choi, A.M., Poss, K.D. and Bach, F.H. (1998) Expression of haem oxygenase-1 can determine cardiac xenograft survival. Nat. Med. 4, 1073-1077.

[70] Ryter, S.W., Alam, J. and Choi, A.M. (2006) Haem oxygenase-1/carbon monoxide: from basic science to therapeutic applications. Physiol. Rev. 86, 583-650.

[71] Kharitonov, V.G., Sharma, V.S., Pilz, R.B., Magde, D. and Koesling, D. (1995) Basis of guanylate cyclase activation by carbon monoxide. Proc. Natl. Acad. Sci. U S A 92, 2568-2571.

[72] Furchgott, R.F. and Jothianandan, D. (1991) Endothelium-dependent and -independent vasodilation involving cyclic GMP: relaxation induced by nitric oxide, carbon monoxide and light. Blood Vessels 28, 52-61.

[73] Ko, F.N., Wu, C.C., Kuo, S.C., Lee, F.Y. and Teng, C.M. (1994) YC-1, a novel activator of platelet guanylate cyclase. Blood 84, 4226-4233.

[74] Ibrahim, M., Derbyshire, E.R., Marletta, M.A. and Spiro, T.G. Probing soluble guanylate cyclase activation by CO and YC-1 using resonance Raman spectroscopy. Biochemistry 49, 3815-3823.

[75] Li, L. and Moore, P.K. (2007) An overview of the biological significance of endogenous gases: new roles for old molecules. Biochem. Soc. Trans. 35, 1138-1141.

[76] Morita, T., Mitsialis, S.A., Koike, H., Liu, Y. and Kourembanas, S. (1997) Carbon monoxide controls the proliferation of hypoxic vascular smooth muscle cells. J. Biol. Chem. 272, 32804-32809.

[77] Brune, B. and Ullrich, V. (1987) Inhibition of platelet aggregation by carbon monoxide is mediated by activation of guanylate cyclase. Mol. Pharmacol. 32, 497-504.

[78] Morita, T. and Kourembanas, S. (1995) Endothelial cell expression of vasoconstrictors and growth factors is regulated by smooth muscle cell-derived carbon monoxide. J. Clin. Invest. 96, 2676-2682.

[79] Liu, J.P., Schlosser, R., Ma, W.Y., Dong, Z., Feng, H., Lui, L., Huang, X.Q., Liu, Y. and Li, D.W. (2004) Human alphaA- and alphaB-crystallins prevent UVA-induced apoptosis through regulation of PKCalpha, RAF/MEK/ERK and AKT signaling pathways. Exp. Eye Res. 79, 393-403.

[80] Petrache, I., Otterbein, L.E., Alam, J., Wiegand, G.W. and Choi, A.M. (2000) Haem oxygenase-1 inhibits TNF-alpha-induced apoptosis in cultured fibroblasts. Am. J. Physiol. Lung Cell Mol. Physiol. 278, L312-319.

[81] Wang, R., Wu, L. and Wang, Z. (1997) The direct effect of carbon monoxide on K_{Ca} channels in vascular smooth muscle cells. Pflugers Arch. 434, 285-291.

[82] Lin, H. and McGrath, J.J. (1988) Carbon monoxide effects on calcium levels in vascular smooth muscle. Life Sci. 43, 1813-1816.

[83] Xi, Q., Tcheranova, D., Parfenova, H., Horowitz, B., Leffler, C.W. and Jaggar, J.H. (2004) Carbon monoxide activates K_{Ca} channels in newborn arteriole smooth muscle cells by increasing apparent Ca^{2+} sensitivity of alpha-subunits. Am. J. Physiol. Heart Circ. Physiol. 286, H610-618.

[84] Tang, X.D., Xu, R., Reynolds, M.F., Garcia, M.L., Heinemann, S.H. and Hoshi, T. (2003) Haem can bind to and inhibit mammalian calcium-dependent Slo1 BK channels. Nature 425, 531-535.

[85] Wang, R., Wang, Z. and Wu, L. (1997) Carbon monoxide-induced vasorelaxation and the underlying mechanisms. Br. J. Pharmacol. 121, 927-934.

[86] Boczkowski, J., Poderoso, J.J. and Motterlini, R. (2006) CO-metal interaction: vital signaling from a lethal gas. Trends Biochem. Sci. 31, 614-621.

[87] Song, R., Mahidhara, R.S., Liu, F., Ning, W., Otterbein, L.E. and Choi, A.M. (2002) Carbon monoxide inhibits human airway smooth muscle cell proliferation via mitogen-activated protein kinase pathway. Am. J. Respir. Cell Mol. Biol. 27, 603-610.

[88] Otterbein, L.E., Zuckerbraun, B.S., Haga, M., Liu, F., Song, R., Usheva, A., Stachulak, C., Bodyak, N., Smith, R.N., Csizmadia, E., Tyagi, S., Akamatsu, Y., Flavell, R.J., Billiar, T.R., Tzeng, E., Bach, F.H., Choi, A.M. and Soares, M.P. (2003) Carbon monoxide suppresses arteriosclerotic lesions associated with chronic graft rejection and with balloon injury. Nat. Med. 9, 183-190.

[89] Song, R., Mahidhara, R.S., Zhou, Z., Hoffman, R.A., Seol, D.W., Flavell, R.A., Billiar, T.R., Otterbein, L.E. and Choi, A.M. (2004) Carbon monoxide inhibits T lymphocyte proliferation via caspase-dependent pathway. J. Immunol. 172, 1220-1226.

[90] Zhang, X., Shan, P., Otterbein, L.E., Alam, J., Flavell, R.A., Davis, R.J., Choi, A.M. and Lee, P.J. (2003) Carbon monoxide inhibition of apoptosis during ischemia-reperfusion lung injury is dependent on the p38 mitogen-activated protein kinase pathway and involves caspase 3. J. Biol. Chem. 278, 1248-1258.

[91] Zhang, X., Shan, P., Alam, J., Davis, R.J., Flavell, R.A. and Lee, P.J. (2003) Carbon monoxide modulates Fas/Fas ligand, caspases, and Bcl-2 family proteins via the p38alpha mitogen-activated protein kinase pathway during ischemia-reperfusion lung injury. J. Biol. Chem. 278, 22061-22070.

[92] Kim, H.P., Ryter, S.W. and Choi, A.M. (2006) CO as a cellular signaling molecule. Annu. Rev. Pharmacol. Toxicol. 46, 411-449.

[93] Otterbein, L.E., Bach, F.H., Alam, J., Soares, M., Tao Lu, H., Wysk, M., Davis, R.J., Flavell, R.A. and Choi, A.M. (2000) Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat. Med. 6, 422-428.

[94] Morse, D., Pischke, S.E., Zhou, Z., Davis, R.J., Flavell, R.A., Loop, T., Otterbein, S.L., Otterbein, L.E. and Choi, A.M. (2003) Suppression of inflammatory cytokine production by carbon monoxide involves the JNK pathway and AP-1. J. Biol. Chem. 278, 36993-36998.

[95] Oelgeschlager, E. and Rother, M. (2008) Carbon monoxide-dependent energy metabolism in anaerobic bacteria and archaea. Arch. Microbiol. 190, 257-269.

[96] King, G.M. and Weber, C.F. (2007) Distribution, diversity and ecology of aerobic CO-oxidizing bacteria. Nat. Rev. Microbiol. 5, 107-118.

[97] Jacobitz, S. and 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.

[98] Frunzke, K. and Meyer, O. (1990) Nitrate respiration, denitrification, and utilization of nitrogen sources by aerobic carbon monoxide-oxidizing bacteria. Arch. Microbiol. 154, 168-174.

[99] Drake, H.L., Gossner, A.S. and Daniel, S.L. (2008) Old acetogens, new light. Ann. N. Y. Acad. Sci. 1125, 100-128.

[100] Bossche, H.V., Lauwers, W., Willemsens, G., Marichal, P., Cornelissen, F. and Cools, W. (2006) Molecular basis for the antimycotic and antibacterial activity of N-substituted imidazoles and triazoles: The inhibition of isoprenoid biosynthesis Pesticide Science 15, 188-198.

[101] Frankenberg-Dinkel, N. (2004) Bacterial haem oxygenases. Antioxid Redox Signal 6, 825-834.

[102] Schmitt, M.P. (1997) Utilization of host iron sources by *Corynebacterium diphtheriae*: identification of a gene whose product is homologous to eukaryotic haem oxygenases and is required for acquisition of iron from haem and haemoglobin. J. Bacteriol. 179, 838-845.

[103] Skaar, E.P., Gaspar, A.H. and Schneewind, O. (2004) IsdG and IsdI, haem-degrading enzymes in the cytoplasm of *Staphylococcus aureus*. J. Biol. Chem. 279, 436-443.

[104] Wu, R., Skaar, E.P., Zhang, R., Joachimiak, G., Gornicki, P., Schneewind, O. and Joachimiak, A. (2005) Staphylococcus aureus IsdG and IsdI, haem-degrading enzymes with structural similarity to monooxygenases. J. Biol. Chem. 280, 2840-2846.

[105] Skaar, E.P., Gaspar, A.H. and Schneewind, O. (2006) *Bacillus anthracis* IsdG, a haem-degrading monooxygenase. J. Bacteriol. 188, 1071-1080.

[106] Zhu, W., Hunt, D.J., Richardson, A.R. and Stojiljkovic, I. (2000) Use of haem compounds as iron sources by pathogenic neisseriae requires the product of the *hemO* gene. J. Bacteriol. 182, 439-447.

[107] Bonam, D., Lehman, L., Roberts, G.P. and Ludden, P.W. (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.

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

[109] Reynolds, M.F., Parks, R.B., Burstyn, J.N., Shelver, D., Thorsteinsson, M.V., Kerby, R.L., Roberts, G.P., Vogel, K.M. and Spiro, T.G. (2000) Electronic absorption, EPR, and resonance raman spectroscopy of CooA, a CO-sensing transcription activator from *R. rubrum*, reveals a five-coordinate NO-haem. Biochemistry 39, 388-396.

[110] Kumar, A., Deshane, J.S., Crossman, D.K., Bolisetty, S., Yan, B.S., Kramnik, I., Agarwal, A. and Steyn, A.J. (2008) Haem oxygenase-1-derived carbon monoxide induces the *Mycobacterium tuberculosis* dormancy regulon. J. Biol. Chem. 283, 18032-18039.

[111] Kumar, A., Toledo, J.C., Patel, R.P., Lancaster, J.R., Jr. and Steyn, A.J. (2007) *Mycobacterium tuberculosis* DosS is a redox sensor and DosT is a hypoxia sensor. Proc. Natl. Acad. Sci. U S A 104, 11568-11573.

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

[113] Fiumana, E., Parfenova, H., Jaggar, J.H. and Leffler, C.W. (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.

[114] Freitas, A., Alves-Filho, J.C., Secco, D.D., Neto, A.F., Ferreira, S.H., Barja-Fidalgo, C. and Cunha, F.Q. (2006) Haem oxygenase/carbon monoxide-biliverdin pathway down regulates neutrophil rolling, adhesion and migration in acute inflammation. Br. J. Pharmacol. 149, 345-354.

[115] Srisook, K., Han, S.S., Choi, H.S., Li, M.H., Ueda, H., Kim, C. and Cha, Y.N. (2006) CO from enhanced HO activity or from CORM-2 inhibits both O_2- and NO production and downregulates HO-1 expression in LPS-stimulated macrophages. Biochem. Pharmacol. 71, 307-318.

[116] Sawle, P., Foresti, R., Mann, B.E., Johnson, T.R., Green, C.J. and 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.

[117] Cepinskas, G., Katada, K., Bihari, A. and Potter, R.F. (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.

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

[119] Sun, B., Sun, Z., Jin, Q. and Chen, X. (2008) 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.

[120] Sun, B.W., Chen, Z.Y., Chen, X. and 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.

[121] Liu, D.M., Sun, B.W., Sun, Z.W., Jin, Q., Sun, Y. and 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.

[122] Taille, C., El-Benna, J., Lanone, S., Boczkowski, J. and 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.

[123] Stanford, S.J., Walters, M.J., Hislop, A.A., Haworth, S.G., Evans, T.W., Mann, B.E., Motterlini, R. and Mitchell, J.A. (2003) Haem oxygenase is expressed in human pulmonary artery smooth muscle where carbon monoxide has an anti-proliferative role. Eur. J. Pharmacol. 473, 135-141.

[124] Pae, H.O., Choi, B.M., Oh, G.S., Lee, M.S., Ryu, D.G., Rhew, H.Y., Kim, Y.M. and Chung, H.T. (2004) Roles of haem oxygenase-1 in the antiproliferative and antiapoptotic effects of nitric oxide on Jurkat T cells. Mol. Pharmacol. 66, 122-128.

[125] Li, M.H., Cha, Y.N. and Surh, Y.J. (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.

[126] Choi, B.M., Pae, H.O. and Chung, H.T. (2003) Nitric oxide priming protects nitric oxide-mediated apoptosis via haem oxygenase-1 induction. Free Radic. Biol. Med. 34, 1136-1145.

[127] Bani-Hani, M.G., Greenstein, D., Mann, B.E., Green, C.J. and Motterlini, R. (2006) A carbon monoxide-releasing molecule (CORM-3) attenuates lipopolysaccharide- and interferon-gamma-induced inflammation in microglia. Pharmacol. Rep. 58 Suppl., 132-144.

[128] Bani-Hani, M.G., Greenstein, D., Mann, B.E., Green, C.J. and Motterlini, R. (2006) Modulation of thrombin-induced neuroinflammation in BV-2 microglia by carbon monoxide-releasing molecule 3. J. Pharmacol. Exp. Ther. 318, 1315-1322.

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

[130] Motterlini, R., Sawle, P., Hammad, J., Bains, S., Alberto, R., Foresti, R. and Green, C.J. (2005) CORM-A1: a new pharmacologically active carbon monoxide-releasing molecule. Faseb J. 19, 284-286.

[131] Foresti, R., Bani-Hani, M.G. and Motterlini, R. (2008) Use of carbon monoxide as a therapeutic agent: promises and challenges. Intensive Care Med. 34, 649-658.

[132] Clark, J.E., Naughton, P., Shurey, S., Green, C.J., Johnson, T.R., Mann, B.E., Foresti, R. and Motterlini, R. (2003) Cardioprotective actions by a water-soluble carbon monoxide-releasing molecule. Circ. Res. 93, e2-8.

[133] Guo, Y., Stein, A.B., Wu, W.J., Tan, W., Zhu, X., Li, Q.H., Dawn, B., Motterlini, R. and 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.

[134] Vera, T., Henegar, J.R., Drummond, H.A., Rimoldi, J.M. and Stec, D.E. (2005) Protective effect of carbon monoxide-releasing compounds in ischemia-induced acute renal failure. J. Am. Soc. Nephrol. 16, 950-958.

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

[136] Ryan, M.J., Jernigan, N.L., Drummond, H.A., McLemore, G.R., Jr., Rimoldi, J.M., Poreddy, S.R., Gadepalli, R.S. and Stec, D.E. (2006) Renal vascular responses to CORM-A1 in the mouse. Pharmacol. Res. 54, 24-29.

[137] Sandouka, A., Fuller, B.J., Mann, B.E., Green, C.J., Foresti, R. and Motterlini, R. (2006) Treatment with CO-RMs during cold storage improves renal function at reperfusion. Kidney Int. 69, 239-247.

[138] Allanson, M. and Reeve, V.E. (2007) Carbon monoxide signalling reduces photocarcinogenesis in the hairless mouse. Cancer Immunol. Immunother. 56, 1807-1815.

[139] Motterlini, R., Foresti, R., Intaglietta, M. and Winslow, R.M. (1996) NO-mediated activation of haem oxygenase: endogenous cytoprotection against oxidative stress to endothelium. Am. J. Physiol. 270, H107-114.

[140] Ding, Y., McCoubrey, W.K., Jr. and Maines, M.D. (1999) Interaction of haem oxygenase-2 with nitric oxide donors. Is the oxygenase an intracellular 'sink' for NO? Eur. J. Biochem. 264, 854-861.

[141] Zuckerbraun, B.S., Billiar, T.R., Otterbein, S.L., Kim, P.K., Liu, F., Choi, A.M., Bach, F.H. and Otterbein, L.E. (2003) Carbon monoxide protects against liver failure through nitric oxide-induced haem oxygenase 1. J. Exp. Med. 198, 1707-1716.

[142] Brunton, T.L. (1867) Lancet. 90, 97.

[143] Murrell, W. (1879) Lancet. 113, 80.

[144] Marsh, N. and Marsh, A. (2000) A short history of nitroglycerine and nitric oxide in pharmacology and physiology. Clin. Exp. Pharmacol. Physiol. 27, 313-319.

Results

CHAPTER 3

Flavohaemoglobin requires microaerophilic conditions for nitrosative protection of *Staphylococcus aureus*

Summary

Flavohaemoglobins and flavodiiron proteins are two families of enzymes involved in nitrosative detoxification. However, the physiological oxygen-related conditions under which they work, and their relative role are still a matter of debate. To address this question we analysed the function of the putative flavohaemoprotein of *Staphylococcus aureus*, an organism that lacks a flavodiiron-like gene. In this report we show that the recombinant protein contains all features typical of canonical flavohaemoglobins and that the transcription of

flavohaemoglobin gene was up-regulated by nitrosative stress in an oxygen-dependent manner. However, and in contrast to other bacterial flavohaemoglobins, the *S. aureus* protein has no apparent role in aerobic nitrosative protection, being only beneficial when cells of *S. aureus* are submitted to nitrosative stress in a microaerophilic environment. The *in vivo* data corroborates the proposal that flavohaemoglobin acts physiologically as a denitrosylase.

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3.1 Introduction

The prokaryotic defence systems against nitrosative stress involve at least two families of enzymes that directly detoxify nitric oxide or *S*-nitrosothiols: the flavodiiron NO reductases and the flavohaemoglobins [1, 2]. Flavohaemoglobins, which have a two-domain structure formed by a haemoglobin-like domain (containing a single *b*-type haem) and a $NADP^+$:ferredoxin oxidoreductase-like domain (harbouring a FAD moiety and a NAD(P)H binding motif) are able to function as NO scavengers. In the presence of oxygen, Hmp oxidises NO to nitrate, with a range of activities that varies from 7.4 to 128 s^{-1}, at 20 °C [3-5]. Anaerobically, Hmp is also able to reduce NO to nitrous oxide, but with a much lower activity of 0.14–0.5 s^{-1} [2, 6]. Both the oxidation of NO to nitrate as well as the reduction of NO to nitrous oxide are proposed to occur through a common NO-(nitroxyl anion-bound haem) intermediate. Since the former reaction can operate very efficiently under microaerobic conditions [3, 4], Hmp may be adapted mainly to protect under microaerobic conditions, which are typical *in vivo*.

The beneficial role of Hmp in protection from nitrosative stress under aerobic and anaerobic conditions has been demonstrated for several microorganisms [2-9]. In *E. coli*, both under microaerobic and anaerobic growth conditions, flavohaemoglobin was found to confer a degree of NO protection similar to that of the flavodiiron NO reductase [7, 8, 10]. It was also observed that, when compared with flavorubredoxin, the higher levels of Hmp expression are reached at longer times after the addition of the nitrosative stress, and that the lack of flavohaemoglobin production was not compensated by an increase in the expression of flavorubredoxin and vice-versa [10]. These results strongly suggest that in *E. coli* the two enzymes play different roles in nitrosative cell protection. Flavodiiron NO reductases and flavohaemoglobin-like proteins are widely distributed among bacteria, fungi and protozoa and, as in *E. coli*, in a large number of genomes the two genes are present in the same organism. On the contrary, in the *S. aureus* genome sequence only the gene encoding for a putative flavohaemoprotein is present, making *S. aureus* a good system to study the role of Hmp *per se*. In addition, *S. aureus* is a major human pathogen capable of causing

from mild to life-threatening systemic diseases, which results from the ability to colonise different environmental niches, i.e., to survive a diverse range of stresses. Furthermore, the spread of antibiotic-resistance of *S. aureus* strains constitutes a major worldwide concern [11, 12]. In spite of its clinical importance the *S. aureus* cellular components involved in the response to reactive nitrogen species, that constitutes a major mammalian defence mechanism against pathogens, remains almost unknown.

In the present work, we have cloned and produced the recombinant *S. aureus* Hmp and performed its biochemical characterisation. To elucidate its physiological function the *S. aureus hmp* gene was disrupted and the resistance to the nitrosative stress of the mutant was analysed.

3.2 Materials and methods

Bacterial strains, plasmids, media and growth conditions

Strains *E. coli* XL2-Blue, *E. coli* BL21Gold(DE3), *S. aureus* NCTC 8325 and its derivative *S. aureus* RN4220, and plasmids pET-28a (Novagen) and pSP64E [13, 14] were used in this work. *S. aureus* strains and pSP64E were a kind gift of Prof. H. de Lencastre. *S. aureus* cells were grown, in tryptic soy broth (TSB) or Luria-Bertani (LB) media, at pH 7, aerobically in flasks filled with 1/5 of its volume, microaerophilically in closed flasks completely filled or anaerobically in rubber seal capped flasks that, once filled with media and closed, were extensively bubbled with nitrogen. Cells were cultivated at 37 °C and only the aerobically grown cultures were shaken at 180 rpm.

Cloning, expression and purification of *S. aureus* recombinant Hmp

To clone the *hmp gene*, primers based on flaking sequences that generated *Nco*I and *Eco*RI restriction sites were used to amplify a 1.2-kb fragment from the *S. aureus* NCTC 8325 genomic DNA [15], isolated as described in [16]. The gene was ligated into pET28a (Novagen), yielding pETHmp, and introduced into *E. coli* XL2-Blue. Positive recombinant plasmids were selected from kanamycin-resistant

colonies and DNA sequenced. Over-expression of the recombinant protein was achieved in pETHmp containing *E. coli* Bl21Gold(DE3) cells, grown aerobically in LB supplemented with 3 μM $FeCl_3$, 100 μM riboflavin and 30 μg/mL kanamycin. When cells reached OD_{600} of 0.4, 500 μM isopropyl-1-thio-β-d-galactopyranoside and 50 μM aminolevulinic acid were added and growth continued for another 6 h. Cells were disrupted and the soluble extract loaded into a Q-Sepharose High Performance column, previously equilibrated with buffer A (Tris–HCl 10 mM, pH 7.6, and glycerol 20 %). Hmp eluted at ~ 240 mM NaCl, was applied to a Superdex S-75 gel filtration column equilibrated with buffer A + 150 mM NaCl. The protein was then reloaded on the Q-Sepharose column and the protein eluted at ~ 170 mM NaCl was found to be pure, as judged by SDS–PAGE. Protein concentration was assayed by the bicinchoninic acid method with BSA as the standard [17], flavin content was quantified after acid extraction with trichloroacetic acid [18], and haem content assayed using the haemochromopyridine method [19].

EPR, UV–Vis spectroscopy and redox titration

EPR spectra were obtained on a Bruker ESP 380 spectrometer, equipped with an Oxford Instruments continuous flow helium cryostat. UV–Vis absorption spectra of *S. aureus* Hmp were acquired using a Shimadzu UV-1603 spectrophotometer, at room temperature. *S. aureus* Hmp (~ 11 μM) was titrated anaerobically as described in [20].

Construction of *S. aureus hmp* deletion strain and complementation analysis

To disrupt the *hmp* gene, an internal fragment (800 bp) of the *S. aureus* NCTC 8325 *hmp* was PCR amplified, using oligonucleotides (SAHECO:5′-GAAAGGGACAGAATTCACGTCAATC-3′ and SAHBAM: 5′-GTTGTCATGATGGATCCCGATACT-3′), and ligated into pSP64E. The resulting pSPHmp, was electroporated into *S. aureus* RN4220 [21, 22], and transformants were selected on TSB-agar (TSA) plates containing erythromycin (10 μg/ml).

Chromosomal DNA isolated from single colonies and PCR analysis was used to confirm the correct integration of pSPHmp into the chromosome of RN4220. One of such colonies was designated LMS800 and used in subsequent studies.

For the complementation analysis, the plasmids pETHmp and pET28a were individually transformed into LMS2710 strain (*E. coli norV* mutant) [10]. Single colonies were grown overnight and used to inoculate minimal salt medium [10] containing chloramphenicol and kanamycin. The growth was performed under anaerobic conditions and monitored at 600 nm.

RNA extraction and RT-PCR analysis

Total RNA was isolated from cells grown under the indicated conditions using the hot-phenol method, and treated with DNaseI. The forward and reverse primers used to create the disruption of the *hmp* gene were also utilised in the RT-PCR assays, performed with USB Reverse Transcriptase Kit. After confirming the absence of any residual DNA, RT-PCR reactions were performed with 150 ng of RNA. The 16S rRNA gene was used to guarantee that equal amounts of RNA were compared.

NADH oxidase, NO denitrosylase and NO reductase activities of *S. aureus* Hmp

The kinetic experiments were recorded in a Shimadzu UV-1603 spectrophotometer and performed at 25 °C. The NADH oxidase activity of *S. aureus* Hmp (30 nM) was measured by monitoring the anaerobic NADH consumption (200 μM), using potassium ferricyanide ($K_3Fe(CN)_6$) (500 μM) as artificial electron acceptor, and following the absorbance decrease at 420 nm ($\varepsilon_{Fe(CN)6}$ = 1020 M^{-1} cm^{-1}). The NO denitrosylase activity of Hmp (36 nM) was measured aerobically by following the NADH oxidation (200 μM) ($\varepsilon_{340\ nm}$ = 6200 M^{-1} cm^{-1}) upon addition of aliquots of a saturated NO solution [10]. The NADH:NO oxidoreductase activity was determined by anaerobically incubating NADH (200 μM) with Hmp (365 nM), and monitoring the NADH oxidation by NO. Activities are reported in terms of NO consumption, using the proposed stoichiometry of 2 NO

molecules per NADH molecule, for both the denitrosylase and reductase reactions [23]. All activities were calculated taking into account the stoichiometric haem and flavin content of the as purified protein.

3.3 Results and discussion

S. aureus flavohaemoprotein is a canonical flavohaemoglobin

In all known genome sequences of *S. aureus* strains it is present a gene predicted to encode a flavohaemoprotein that shares ~ 30 % sequence similarity with the *E. coli* Hmp. To assess the function of the *S. aureus* Hmp, the recombinant protein was produced and characterised. Upon purification, *S. aureus* Hmp exhibited a molecular mass of 44 kDa that corresponds to a monomeric protein, and contained 0.7 mol flavin and 1 mol haem. The UV–Vis spectrum of *S. aureus* Hmp displayed the features typical of the canonical Hmps in the oxidised, reduced and CO bound states (Figure 3.1A). Also, the EPR spectrum is characteristic of high-spin ferric iron (data not shown). A reduction potential of −170 mV was measured for the haem center and two identical reduction potentials of −190 mV were measured for the FAD center (Figure 3.1B). The NADH oxidase activity of *S. aureus* Hmp was determined to be 34 s^{-1}, using potassium ferricyanide as the electron acceptor. Hmp exhibited an NO denitrosylase activity of 66 s^{-1}, measured upon addition of 20 μM NO, and a NADH:NO oxidoreductase activity of 0.7 s^{-1}. These values are within the range usually reported for homologous enzymes [23].

S. aureus hmp transcription is mainly regulated by oxygen limitation

The mRNA level of *hmp* under nitrosative stress conditions in aerobic and oxygen-limiting conditions was evaluated in RT-PCR experiments (Figure 3.2). The transcription of *hmp* was found to be low under aerobic conditions and to increase considerably on switching to oxygen-limited growth conditions. *S*-nitrosoglutathione (GSNO) caused an increase in the *hmp* transcription level under aerobic conditions, and no major variations were observed in microaerophilic or

anaerobic grown conditions. Hence, the results indicate that the major trigger for *hmp* induction is oxygen limitation, thus suggesting a physiological function under oxygen limited conditions. In general, *hmp* exhibit oxygen-dependent gene expression via the oxygen sensor-regulator Fnr [2], but analysis of the upstream sequence of the *S. aureus hmp* coding region did not allow identification of any obvious Fnr binding motif.

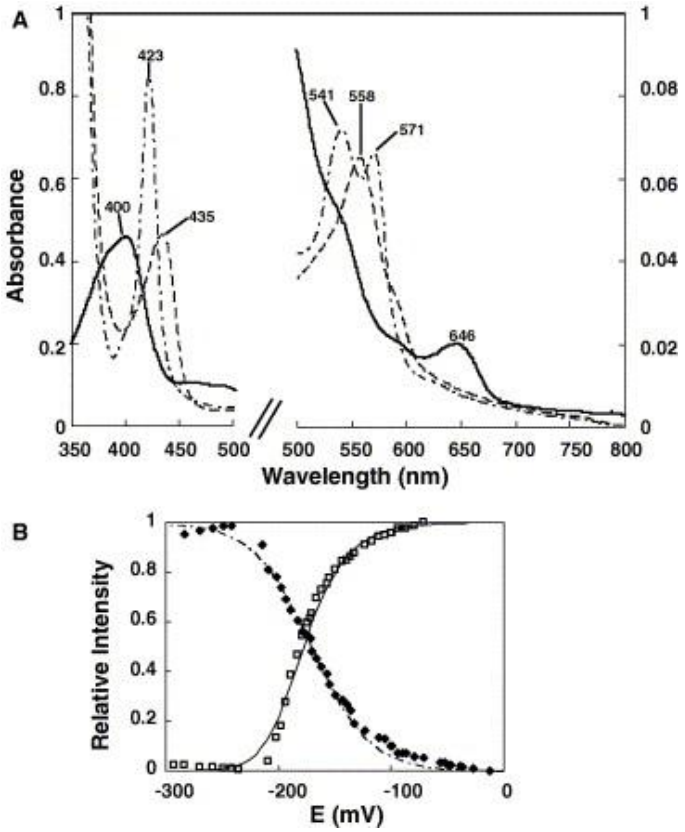

FIGURE 3.1 Characterization of the as-purified *S. aureus* Hmp. (A) UV–Vis spectra of Hmp oxidized (—), reduced with sodium dithionite (– – –), and reduced and CO ligated (–·–·–). (B) Redox titration of the haem centre (♦) and of the FAD centre (□) of *S. aureus* Hmp. Full lines were calculated with the Nernst equation for a monoelectronic (reduction potential of −170 mV) and two monoelectronic consecutive processes (identical reduction potentials of −190 mV), for the haem and FAD centres, respectively.

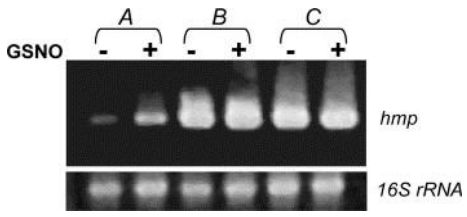

FIGURE 3.2 *S. aureus hmp* expression increases upon oxygen limitation. RNAs isolated from *S. aureus* grown aerobically (A), microaerophilic (B) or anaerobically (C) in LB for 4 h in the absence (−) or in the presence (+) of 200 µM (A,B) or 50 µM (C) GSNO. The equal loading of total RNA was confirmed by the same intensity of the 16S rRNA band (lower panel). Data are representative of reactions performed with two independent RNA samples.

Protection of *S. aureus* by flavohaemoglobin in response to GSNO depends on the oxygen-related conditions

To infer the role for Hmp in nitrosative protection, the physiological effects of various concentrations of GSNO and oxygen on *S. aureus hmp* mutant and wild type strains were analysed (Figure 3.3). Under aerobic conditions, GSNO concentrations up to 100 µM did not cause growth inhibition of wild type *S. aureus*, while 200 µM GSNO induced a severe growth arrestment (Figure 3.3A). Under microaerophilic conditions, all tested concentrations of GSNO ranging from 50 to 200 µM only caused a negligible effect on growth of wild type *S. aureus* (Figure 3.3C). However, when wild type *S. aureus* was grown under anoxic conditions the effect of similar concentrations of GSNO was more pronounced, as judged by the significant degree of growth impairment observed with 50 µM GSNO (Figure 3.3E). Therefore, no additional work was done with GSNO and the *hmp* mutant under anaerobic conditions.

In the absence of GSNO, the *S. aureus hmp* mutant displayed an oxygen-dependent growth behaviour similar to that of wild type RN4220 strain (Figure 3.3B,D). Under aerobic conditions, and for all the concentrations of GSNO tested, there was no discernible difference in the rates and extents of growth, as measured by the OD_{600} between the parent and mutant strain (Figure 3.3B). However, under microaerophilic conditions exposure of the wild-type *S. aureus* to 200 µM GSNO caused a decrease of the growth rate of ~ 7 %, whereas the Δ*hmp* strain exhibited a lag in growth of ~ 50 % (Figure 3.3D). A similar behaviour was

also observed for the *S. aureus hmp* mutant in the presence of 50 µM of NO gas (data not shown). Hence, Hmp seems to be able to protect *S. aureus* submitted to a strong nitrosative stress only under microaerophilic conditions.

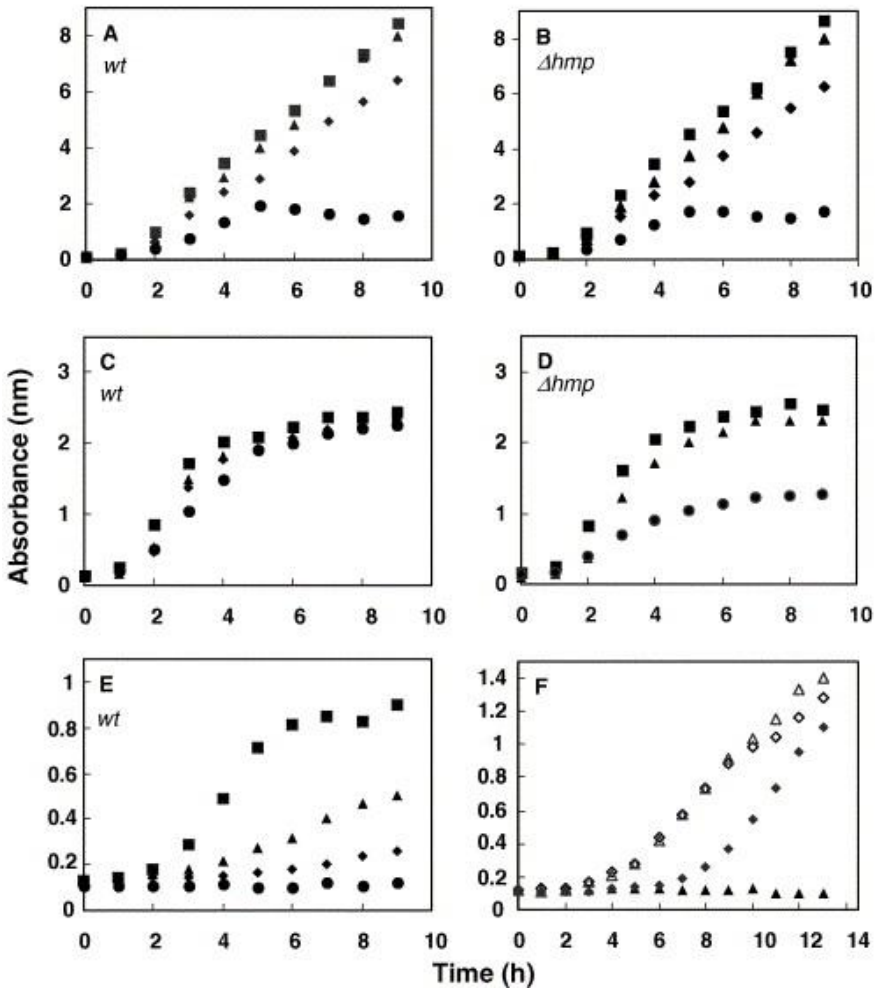

FIGURE 3.3 Microaerophilic GSNO-protection conferred by flavohaemoglobin to *S. aureus*. *S. aureus* wild type strain RN4220 (wt) and mutant strain LMS800 (Δ*hmp*), were grown aerobically (A,B), microaerophilically (C,D) or anaerobically (E) in LB medium and left untreated (■) or treated with 50 µM GSNO (▲), 100 µM GSNO (♦) and 200 µM GSNO (•). Each growth curve represents the average of at least three independent cultures. Panel (F) Analysis of complementation of *E. coli* LMS2710 (Δ*norV*) with *S. aureus* Hmp. Growth curves acquired under anaerobic conditions in minimal medium for the *E. coli* flavorubredoxin mutant strain LMS2710 harbouring either vector alone (pET) (Δ) and with pETHmp expressing *S. aureus* Hmp (◇) without addition of GSNO or in the presence of 50 µM GSNO: LMS2710 (pET) (▲) and LMS2710 (pETHmp) (♦).

***S. aureus* Hmp attenuates the anaerobic NO damage in *E. coli* flavorubredoxin mutant**

Our previous work showed that deletion of flavorubredoxin gene (*norV*) in *E. coli*, strain LMS2710, resulted in a mutant with increased sensitivity to anaerobically added NO [10]. Complementation studies were conducted to test whether *S. aureus* Hmp could perform the role of flavorubredoxin in *E. coli*. The results showed that expression of *S. aureus hmp* in the *E. coli norV* mutant, LMS2710, lead to a significant increase in the anaerobic GSNO resistance of the mutant strain (Figure 3.3F), indicating that *S. aureus* Hmp has the ability to perform anaerobic nitrosative detoxification. In fact, this ability could not be analysed in *S. aureus* since the wild type strain is itself highly sensitive to GSNO under anaerobic conditions. Nevertheless, the possibility that the low NO reductase activity of *S. aureus* Hmp was compensated by the presence of a large amount of protein generated by overexpression cannot be excluded. Since only over-expression of *S. aureus hmp* could improve nitrosative protection, the level of anoxic expression *S. aureus hmp* and/or lower NO reductase activity seems to be insufficient to protect *S. aureus* anaerobically. Furthermore, it is already well documented in other organisms that, besides flavorubredoxin and Hmp, there are many other factors involved in the response to nitrosative stress, e.g. [10], which are so far unknown in *S. aureus* and may be also responsible for the high sensitivity of *S. aureus* to anaerobic nitrosative stress.

3.4 Conclusion

S. aureus infection is related to the versatility of the pathogen to grow in different and often hostile environmental niches. In this study we observed that wild type *S. aureus* grows, although differently, under aerobic and anaerobic conditions and also in the presence of GSNO. However, GSNO resistance is fully dependent on the degree of oxygenation. While, in anaerobic conditions *S. aureus* is very sensitive to GSNO since slow growth is observed in any of the tested concentrations, in microaerophilic *S. aureus* sustains growth up to 200 µM GSNO. Interestingly, Hmp confers *S. aureus* protection against nitrosative challenge only

under microaerophilic conditions. This suggests that *in vivo* Hmp acts as a denitrosylase, i.e., under low oxygen concentration and high NO concentration the denitrosylase mechanism is operative, as previously proposed by Hausladen and co-workers [4]. Last but not least, it is quite interesting that recent studies on *S. aureus* indicated that the pathogenesis of this microbe, and in particular the production of virulence factors, is also dependent on the oxygen concentration [24, 25].

3.5 References

[1] Saraiva, L.M., Vicente, J.B. and Teixeira, M. (2004) The Role of Flavodiiron Proteins in Nitric Oxide Detoxification. Advances in Microbial Physiology *49*, 77-130.

[2] Frey, A.D. and Kallio, P.T. (2003) Bacterial haemoglobins and flavohaemoglobins: versatile proteins and their impact on microbiology and biotechnology. FEMS Microbiol. Rev. 27, 525-545.

[3] Hausladen, A., Gow, A.J. and Stamler, J.S. (1998) Nitrosative stress: metabolic pathway involving the flavohaemoglobin. Proc. Natl. Acad. Sci. U S A 95, 14100-14105.

[4] Hausladen, A., Gow, A. and Stamler, J.S. (2001) Flavohaemoglobin denitrosylase catalyzes the reaction of a nitroxyl equivalent with molecular oxygen. Proc. Natl. Acad. Sci. U S A 98, 10108-10112.

[5] Gardner, P.R., Gardner, A.M., Martin, L.A. and Salzman, A.L. (1998) Nitric oxide dioxygenase: an enzymic function for flavohaemoglobin. Proc. Natl. Acad. Sci. U S A 95, 10378-10383.

[6] Kim, S.O., Orii, Y., Lloyd, D., Hughes, M.N. and Poole, R.K. (1999) Anoxic function for the *Escherichia coli* flavohaemoglobin (Hmp): reversible binding of nitric oxide and reduction to nitrous oxide. FEBS Lett. 445, 389-394.

[7] Wu, G., Wainwright, L.M., Membrillo-Hernandez, J. and Poole, R.K. (2004) Bacterial haemoglobins: old proteins with new functions? Roles in respiratory and nitric oxide metabolism. Respiration in Archaea and Bacteria Diversity of Prokaryotic Electron Transport Carriers, ed Davide Zanoni 251-284.

[8] Gardner, A.M. and Gardner, P.R. (2002) Flavohaemoglobin detoxifies nitric oxide in aerobic, but not anaerobic, *Escherichia coli*. Evidence for a novel inducible anaerobic nitric oxide-scavenging activity. J. Biol. Chem. 277, 8166-8171.

[9] Liu, L., Zeng, M., Hausladen, A., Heitman, J. and Stamler, J.S. (2000) Protection from nitrosative stress by yeast flavohaemoglobin. Proc. Natl. Acad. Sci. U S A 97, 4672-4676.

[10] Justino, M.C., Vicente, J.B., Teixeira, M. and Saraiva, L.M. (2005) New genes implicated in the protection of anaerobically grown *Escherichia coli* against nitric oxide. J. Biol. Chem. 280, 2636-2643.

[11] Lowy, F.D. (1998) *Staphylococcus aureus* infections. N. Engl. J. Med. 339, 520-532.

[12] Lowy, F.D. (2003) Antimicrobial resistance: the example of *Staphylococcus aureus*. J. Clin. Invest. 111, 1265-1273.

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

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

[15] Iandolo, J.J., Worrell, V., Groicher, K.H., Qian, Y., Tian, R., Kenton, S., Dorman, A., Ji, H., Lin, S., Loh, P., Qi, S., Zhu, H. and Roe, B.A. (2002) Comparative analysis of the genomes of the temperate bacteriophages phi 11, phi 12 and phi 13 of *Staphylococcus aureus* 8325. Gene 289, 109-118.

[16] Aires de Sousa, M., Sanches, I.S., van Belkum, A., van Leeuwen, W., Verbrugh, H. and de Lencastre, H. (1996) Characterization of methicillin-resistant *Staphylococcus aureus* isolates from Portuguese hospitals by multiple genotyping methods. Microb. Drug Resist. 2, 331-341.

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

[18] Susin, S., Abian, J., Sanchez-Baeza, F., Peleato, M.L., Abadia, A., Gelpi, E. and Abadia, J. (1993) Riboflavin 3'- and 5'-sulfate, two novel flavins accumulating in the roots of iron-deficient sugar beet (Beta vulgaris). J. Biol. Chem. 268, 20958-20965.

[19] Berry, E.A. and Trumpower, B.L. (1987) Simultaneous determination of haems *a*, *b*, and *c* from pyridine haemochrome spectra. Anal. Biochem. 161, 1-15.

[20] Gomes, C.M., Vicente, J.B., Wasserfallen, A. and Teixeira, M. (2000) Spectroscopic studies and characterization of a novel electron-transfer chain from *Escherichia coli* involving a flavorubredoxin and its flavoprotein reductase partner. Biochemistry 39, 16230-16237.

[21] Novick, R., Kornblum, J., Kreiswirth, B., Projan, S. and Ross, H. (1990) Regulation of post-exponential-phase exoprotein synthesis in *Staphylococcus aureus* in: Microbial determinants of virulence and host response (Henry, T. J., Ed.). American Society for Microbiology 3-18.

[22] Kraemer, G.R. and Iandolo, J.J. (1990) High-frequency transformation of *Staphylococcus aureus* by electroporation. Curr. Microbiol. 21, 373-376.

[23] Gardner, P.R. (2005) Nitric oxide dioxygenase function and mechanism of flavohaemoglobin, haemoglobin, myoglobin and their associated reductases. J. Inorg. Biochem. 99, 247-266.

[24] Yarwood, J.M., McCormick, J.K. and Schlievert, P.M. (2001) Identification of a novel two-component regulatory system that acts in global regulation of virulence factors of *Staphylococcus aureus*. J. Bacteriol. 183, 1113-1123.

[25] Ross, R.A. and Onderdonk, A.B. (2000) Production of toxic shock syndrome toxin 1 by *Staphylococcus aureus* requires both oxygen and carbon dioxide. Infect. Immun. 68, 5205-5209.

CHAPTER 4

Binding of azole antibiotics to *Staphylococcus aureus* flavohaemoglobin increases intracellular oxidative stress

Summary

In this work we report that flavohaemoglobin contributes to the azole susceptibility of *Staphylococcus aureus*. We first observed that deletion of the flavohaemoglobin gene leads to a viability increase of imidazole-treated *S. aureus* cells and that reversion to the wild-type phenotype occurs upon expression of flavohaemoglobin from a multicopy plasmid. Further spectroscopic analyses showed that miconazole, the most efficient azole antibiotic against *S. aureus*,

ligates to haem of both oxidised and reduced flavohaemoglobin. The binding of miconazole to oxidised flavohaemoglobin, with an association constant of 1.7×10^6 M^{-1}, typical of a tight, specific binding equilibrium, results in augmentation of the superoxide production by the enzyme. These results are corroborated by *in vivo* studies showing that imidazole-treated *S. aureus* cells expressing flavohaemoglobin contain a higher amount of reactive oxygen species. Moreover, it was observed that the survival of miconazole-treated *S. aureus* internalized by murine macrophages is higher for cells lacking flavohaemoglobin. Altogether, the present data revealed that in *S. aureus* flavohaemoglobin enhances the antimicrobial activity of imidazoles via an increase of intracellular oxidative stress.

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4.1 Introduction

S. aureus is an opportunistic pathogen responsible for a large number of human infections that cause systemic diseases of mild to life-threatening character. The increasing incidence of methicillin-resistant *S. aureus* strains observed in the past few years make *S. aureus* infections a leading threat to the public health, causing in the United States and Europe more deaths than the human immunodeficiency virus [1]. Like other Gram-positive bacteria, staphylococci are sensitive to imidazoles [2]. Imidazoles (such as clotrimazole, miconazole, ketoconazole, sulconazole, Figure 4.1) represent one of the major classes of azole antifungal that are useful in the treatment of infections, including cutaneous and vaginal candidiasis [3]. The activity of these antifungal drugs derives primarily from inhibition of the biosynthesis of ergosterol, an essential component of the fungal plasma membrane, at the level of lanosterol 14-α demethylase. Furthermore, in fungi and yeast, azole treatment leads to an increase in the endogenous production of reactive oxygen species [4, 5]. For example, in *C. albicans* and *S. cerevisiae* the miconazole inhibition of cytochrome *c* oxidase, peroxidase and catalase has been reported to be responsible for a high level of ROS production [6, 7]. It has also been reported that clotrimazole inhibition of *P. falciparum* haemoperoxidase leads to ROS accumulation in this protozoan pathogen [8]. For *S. cerevisiae*, *C. albicans* and *E. coli* the action of imidazoles was also correlated with the inhibition of the nitric oxide scavenger activity of flavohaemoglobin [9].

Flavohaemoglobins are widespread among bacteria and yeast, and contain three domains: C-terminal NAD- and FAD-binding domains that together constitute a ferredoxin-$NADP^+$ oxidoreductase-like domain, and an N-terminal globin domain, that harbours a single *b*-type haem. The high-spin haem contains one axial histidine and binds small molecules like NO, CO and O_2. The haem can also bind bulky aromatic bases, since it is inserted in a large hydrophobic pocket [9]. We observed that the binding of imidazoles to *S. aureus* flavohaemoglobin results in an increase of the amount of deleterious reactive oxygen species produced by flavohaemoglobin that contributes to the bactericidal effect of azoles antibiotics towards *S. aureus*.

4.2 Materials and methods

Reagents

Miconazole, sulconazole, clotrimazole, ketoconazole and 2',7'-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma, and 5-tert-butoxycarbonyl 5-methyl-1-pyrroline N-oxide (BMPO) from Northwest. All the reagents were dissolved in DMSO, except for ketoconazole and BMPO, which were prepared in methanol and water, respectively. For the UV-Visible and Resonance Raman spectroscopic studies, a water-saturated solution of miconazole was used, to avoid the effect of DMSO on the reduced form of flavohaemoglobin.

Bacterial strains, culture conditions and viability assays

Overnight cultures of *S. aureus* wild-type (RN4220) and *S. aureus* Δ*hmp* (LMS800) [10] grown in TSB medium were used to inoculate, to an optical density at 600 nm (OD_{600nm}) of 0.1, LB medium supplemented with the appropriate antibiotics (2 μM miconazole, 5 μM sulconazole, 12 μM clotrimazole and 120 μM ketoconazole) and contained in closed flasks. For control purposes, untreated cultures, in which an equal volume of correspondent antibiotic's solvent was added, were also analysed. *S. aureus* viability was then evaluated after 5 h treatment of the liquid cultures with antibiotic, and the number of viable cells was determined by measuring the colony forming units (CFU) per milliliter upon plating 5 μl of each dilution on agar and counting the isolated colonies formed after overnight incubation. The percentage of survival was calculated as the number of cells originated by the treated cultures divided by the number of colonies formed after plating the control cultures.

Minimal inhibitory concentration (MIC) of the azole antibiotics were determined on 24-well microtitre plates as previously described [11]. Assays were conducted in LB, at 37 ºC, performed in triplicate, and repeated at least twice.

Complementation studies

For the complementation analysis, a vector expressing *S. aureus hmp* was constructed. To this end, a fragment containing the complete *S. aureus hmp* gene was amplified, using oligonucleotides SAHmpFw (5'-TCACATTTTTATTATCATGTTTACTTTTTTCTAGGA-3') and SAHmpEcoRI (5'-CGTTGATTAAGTTTCATATGAGCACTAATTCTCTTT-3') and ligated to pMK4 [12]. The resulting vector (pHmp) and the empty vector (pMK4) were electroporated into *S. aureus* Δ*hmp* and wild-type (RN4220) strains. For the mutant strain the positive transformants were selected on TSA medium containing 10 µg/ml erythromycin plus 5 µg/ml chloramphenicol, while selection of the wild-type was achieved using only chloramphenicol. Cell growth was performed in liquid medium treated with miconazole for 5 h, as above described, and analysed by serial dilutions plated on agar.

Spectroscopic studies: UV-Visible, Resonance Raman and EPR

S. aureus flavohaemoglobin was cloned, expressed and purified as described previously [13]. UV-Visible spectra were recorded on a Shimadzu UV-1700 spectrophotometer, using 10 µM flavohaemoglobin in 10 mM Tris-HCl, pH 7.6 buffer containing 9 % glycerol.

EPR spectra were obtained on a Bruker EMX spectrometer equipped with an Oxford Instruments continuous flow helium cryostat. BMPO was used as a spin-trap for the detection of reactive oxygen species, which allows to distinguish between superoxide anion and the hydroxyl radical [14]. These experiments were performed using 10 µM *S. aureus* flavohaemoglobin, 200 µM NADH, 25 mM BMPO, and 50 µM miconazole, in a quartz flat cell, at room temperature.

Resonance Raman (RR) spectra were measured using a confocal microscope coupled to a Raman spectrometer (Jobin Yvon U1000) equipped with 1200 l/mm grating and a liquid-nitrogen-cooled back-illuminated CCD detector. Samples of flavohaemoglobin (20 µM) were placed in a quartz rotating cell and excited with the 413 nm line of a krypton ion laser (Coherent Innova 302), with a laser power of 2-4 mW and accumulation times of 60 s. After polynomial background

subtraction, the positions and line-widths of the Raman bands were determined by component analysis using in-house software.

Enzymatic studies

The equilibrium constant for miconazole binding was determined by titrating a fixed amount of flavohaemoglobin with increasing quantities of the antibiotic, and monitoring the changes in absorbance in the visible region. The amount of miconazole-protein complex was calculated using a differential absortivity at 418-500 nm, determined from the difference of a spectrum of a solution having excess antibiotic (thus assuring full complex formation) and the spectrum of the oxidised, isolated protein. A value of $\Delta\varepsilon$ (418-500) = 69565 $M^{-1}cm^{-1}$ was obtained; from this value, the amount of complex at each solution could be determined, and by using the appropriate mass balance equations, the concentrations of free antibiotic and free protein were also calculated. The number of binding sites and the equilibrium constant were then determined by a Scatchard equation [15]. The same procedure could not be applied with precision to the reduced protein, due to the interference of DMSO; nevertheless, a lower limit for the binding constant could be determined using a differential absortivity at 426-390 nm. The assays were performed with 5 μM *S. aureus* flavohaemoglobin and the miconazole concentrations varied between 2 μM and 60 μM. The percentage of DMSO used in all the assays was 0.4 % v/v.

Measurement of endogenous ROS production

Endogenous ROS production was determined by a fluorometric assay according to the method described previously [4]. Cells of wild-type *S. aureus* (RN4220) and Δ*hmp* (LMS800) mutant were grown for 5 h, in the absence or presence of azoles. Cells were then collected by centrifugation, washed, and resuspended in phosphate buffer (PBS), followed by the addition of 10 μM DCFH-DA. The fluorescence intensities (FI) were measured on a Varian Eclipse 96-well spectrofluorimeter (excitation at 485 nm and emission at 538 nm). The FI's were normalized in relation to the final OD_{600nm} of each culture. To assess the variation of ROS, the FI of control cultures and the FI of azole-treated cultures were subtracted.

Quantitative real-time RT-PCR

For real-time RT-PCR experiments, 2.0 µg of *S. aureus* total RNA derived from samples grown in LB and treated with 2 µM miconazole for 5 h was used to synthesise cDNA, according to the Transcriptor High Fidelity cDNA Synthesis kit protocol (Roche Applied Science). Real-time PCRs were performed in a LightCycler Instrument using LightCycler FastStart DNA Master SYBER Green I Kit according to the manufacturer's instructions (Roche Applied Science). The amplification reactions were carried out with equal amounts of cDNA (100 ng) as initial template, and each reaction contained 0.5 µM of specific primers, 2 mM of $MgCl_2$, and the hot-start PCR reaction mix from Roche Applied Science. The expression ratio of the target gene was determined relatively to a reference gene, the *S. aureus* 16S rRNA whose transcription abundance remains invariant under the tested conditions. The samples were assayed in triplicate.

Assay of intracellular *S. aureus* viability in J774A.1 macrophages

Murine macrophages J774A.1 (LGC Promochem) were inoculated with 5 x 10^5 cells/ml and cultured for 2 days, at 37 ºC in a 5 % CO_2/air atmosphere, in 24-well plates containing Dulbecco's Modified Eagle medium (DMEM) supplemented with 4.5 g/l glucose, 110 mg/ml sodium pyruvate (DMEM Glutamax), 10 % fetal bovine serum, 100 µM non-essential amino acids, 50 U/ml penicillin and 50 µg/ml streptomycin, all from GIBCO. Prior to infection, macrophages were activated for 5 h with 1 µg/ml interferon-γ (Sigma) and 5 µg/ml lipopolysaccharides (Sigma). When required, 800 µM NG-Monomethyl-L-arginine acetate salt (L-NMMA; Sigma) was also added to achieve inhibition of the murine macrophage inducible NO synthase. *S. aureus* wild-type and Δ*hmp* were grown for 5 h in the presence or absence of miconazole (2 µM), washed three times with PBS and resuspended in DMEM, to obtain for all cultures an initial bacterial concentration of 10^7 cfu/ml. Macrophages were then infected with these bacterial suspensions, at a multiplicity of infection (MOI) of at least 16, for 30 min at 37º C. The supernatants were then collected to determine the number of bacteria not internalized. Extracellular bacteria were killed by incubation in DMEM supplemented with 50 U/ml penicillin

and 50 µg/ml streptomycin, for 5 min, and the wells were washed three times with PBS. After that, macrophages were lysed with 2 % saponin, and the number of intracellular bacteria determined by CFU counting of viable bacteria.

4.3 Results

S. aureus is susceptible to azole antibiotics

The susceptibility of *S. aureus* to several azole antibiotics was analysed. For *S. aureus* RN4220, the minimal inhibitory concentrations were of the same order of magnitude for miconazole (15 ± 2 µM), sulconazole (20 ± 0 µM), and clotrimazole (30 ± 3 µM), while a much higher value was determined for ketoconazole (500 ± 70 µM). In previous work, *S. aureus* viability was also reported to decrease significantly with miconazole, while essentially no effect (> 200 µM) was observed with ketoconazole [16]. Additionally, we observed that *S. aureus* is resistant to concentrations of the triazole antibiotics fluconazole and itraconazole (Figure 4.1) up to 2 mM.

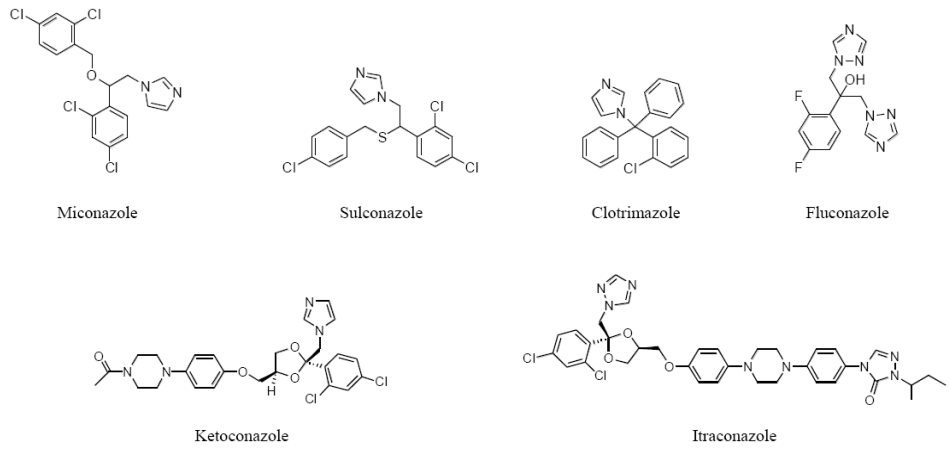

FIGURE 4.1 Structures of the azole (imidazole; 1,2,4-triazole) antibiotics investigated.

S. aureus susceptibility to azoles involves flavohaemoglobin

We next investigated the possible role of flavohaemoglobin in the sensitivity of *S. aureus* to imidazoles. To this end, we compared the viability of *S. aureus* wild-

type and Δ*hmp* mutant cells upon treatment with several azole antibiotics. The results showed that inactivation of flavohaemoglobin caused an increase in the resistance of *S. aureus* to imidazoles, i.e., the mutant strain produced higher number of viable cells, a result which was observed for all imidazoles tested (Figure 4.2A-B).

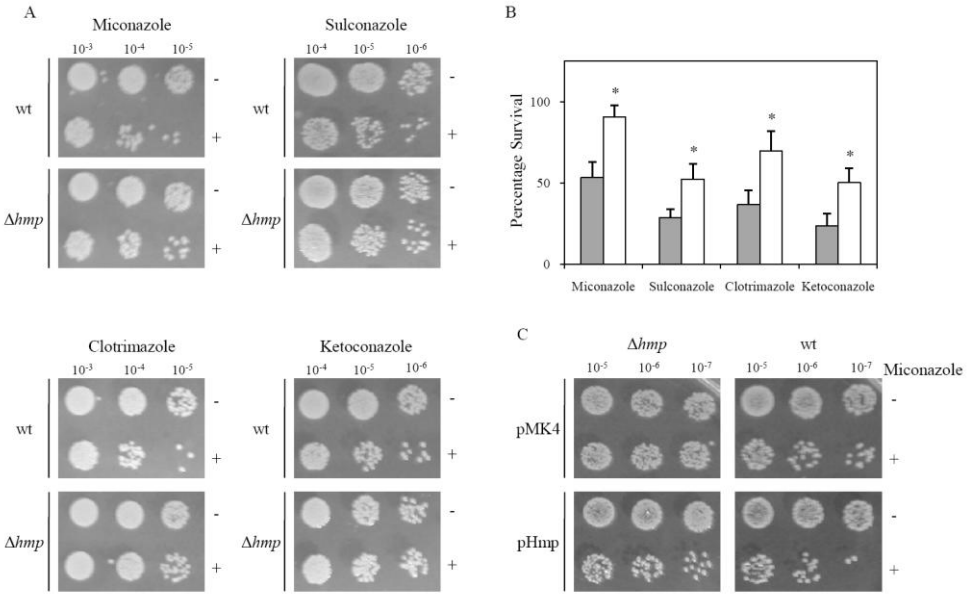

FIGURE 4.2 Flavohaemoglobin contributes to imidazole-sensitivity of *S. aureus*. (A) Cell viability of *S. aureus* wild-type and Δ*hmp* in the absence (-) and in the presence (+) of 2 μM miconazole, 5 μM sulconazole, 12 μM clotrimazole and 120 μM ketoconazole. (B) The number of *S. aureus* wild type (gray bars) and Δ*hmp* (white bars) cells was evaluated by measurement of CFU per milliliter, and the percentage of survival was calculated dividing the number of colonies of treated cultures by that of control cultures (**P*<0.05). (C) Viability of *S. aureus* Δ*hmp* and wild-type cells transformed with pHmp and pMK4, untreated (-) and treated with miconazole (2 μM) (+).

To confirm that imidazole resistance was a specific consequence of flavohaemoglobin gene deletion, the viability of the Δ*hmp* strain expressing flavohaemoglobin (from a multicopy plasmid) treated with miconazole was evaluated. As expected, upon complementation, reversion to the wild-type phenotype was observed (Figure 4.2C). Furthermore, the over-expression of *S. aureus* flavohaemoglobin in the wild-type strain led to an increase in the sensitivity

to imidazoles (Figure 4.2C). These results show that flavohaemoglobin contributes to the activity of imidazoles against *S. aureus*, independently of any other stress agent.

To understand the mechanism by which flavohaemoglobin affects the susceptibility of *S. aureus* to imidazoles, we analysed the binding of miconazole, the most active commercially available azole antibiotic against *S. aureus*, to flavohaemoglobin by UV–Visible, EPR and Resonance Raman (RR) spectroscopies.

The UV–Visible absorption spectra of *S. aureus* flavohaemoglobin in the oxidised and reduced states displayed the characteristic features of high-spin ferric and ferrous *b*-haems, respectively (Figure 4.3A). Upon addition of miconazole, the spectra of both redox forms changed considerably, indicating binding of the antibiotic to the haem and formation of six-coordinated, low-spin forms. Miconazole shifts the Soret band of the oxidised form to 414 nm, and a broad band at 538 nm appears; also, the charge-transfer band at 645 nm, characteristic of high-spin haems, bleaches completely. In the reduced form, the Soret band shifts to 427 nm and bands at 531 and 560 nm are clearly distinguished (Figure 4.3A).

The binding of the imidazole antibiotic to *S. aureus* flavohaemoglobin was also studied by EPR spectroscopy. In the absence of the antibiotic, the EPR spectrum of oxidised flavohaemoglobin exhibits resonances characteristic of a high-spin ferric haem, with g= 6.34, 5.31 and 2.0. Upon addition of miconazole, the high-spin signature almost disappears, being substituted by a set of resonances indicative of the formation of a low-spin ferric haem in a rhombic ligand field, with g-values at 2.85, 2.27 and 1.59, compatible with a ligation by two imidazoles from the axial histidine and from miconazole (Figure 4.3B). A lower intensity signal is also observed at g_{max}=3.25, which suggests the presence of a second low-spin ferric haem configuration, with a more axial ligand field. The rhombic species (the one with g_{max}=2.85) corresponds to a geometry where the two imidazole planes are essentially parallel, while the more axial one (g_{max}=3.25) reflects a situation where the dihedral angle between those two planes is higher (e.g., [17]). The UV-Visible and EPR data show that miconazole binds to the oxidised flavohaemoglobin haem, yielding a low-spin ferric species, as expected for ligation through imidazole

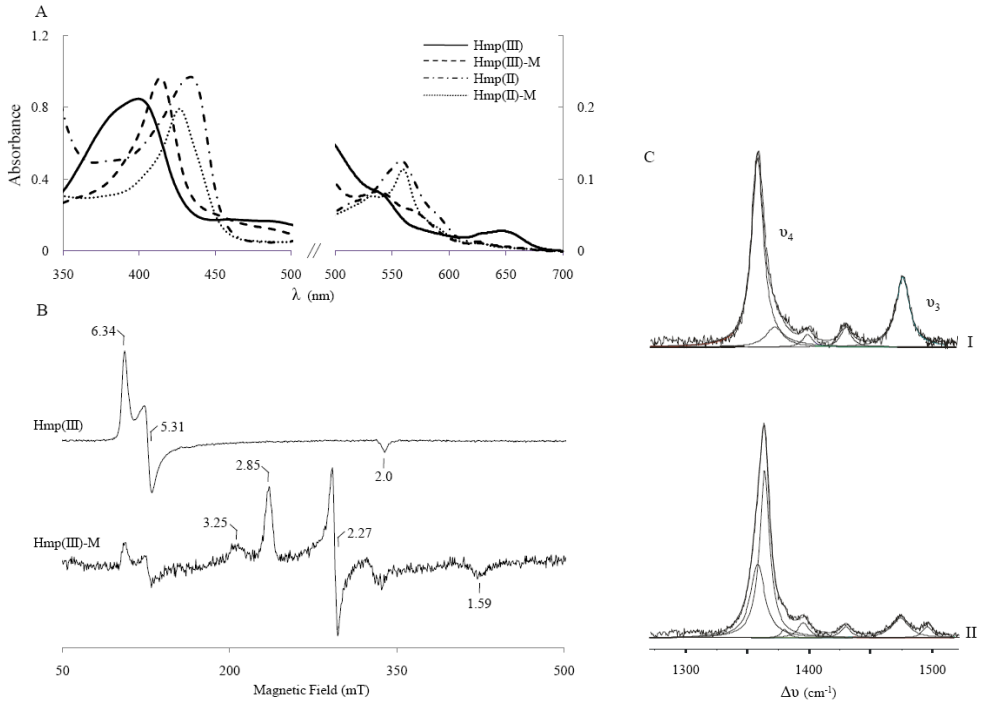

FIGURE 4.3 Spectroscopic analysis of miconazole binding to *S. aureus* flavohaemoglobin. (A) UV-Visible spectra of 10 μM oxidised Hmp (Hmp(III)), after the binding of miconazole (M) to the ferric protein binding of miconazole (M) to the ferric protein (Hmp(III)-M), of dithionite reduced Hmp (Hmp(II)) and upon addition of miconazole to ferrous Hmp (Hmp(II)-M). (B) EPR spectra of *S. aureus* Hmp (Hmp(III)) and of flavohaemoglobin treated with miconazole (Hmp(III)-M). At 16 K, spectra were obtained at 9.4 GHz, 2.0 mW microwave power, 1 mT modulation amplitude and 100 kHz modulation frequency. The spectrum of Hmp(III) showed was divided by 5. (C) RR spectra of flavohaemoglobin. (I) reduced flavohaemoglobin; (II) reduced flavohaemoglobin upon addition of miconazole. All RR spectra were measured with 413 nm excitation, 20 μM flavohaemoglobin (in 10 mM Tris HCl, pH 7.6) in the presence or absence of miconazole, at ambient temperature with a laser power of 2 mW and accumulation times of 60 s.

nitrogens. These results were confirmed by RR spectroscopy since the spectra of haems include marker bands that are sensitive to the oxidation, coordination and spin state of the haem iron [18-20], for both paramagnetic and diamagnetic species. We first investigated the ferric protein, in the presence/absence of

miconazole. RR spectra of oxidised flavohaemoglobin are characteristic of a five-coordinated high-spin (5cHS) configuration, with the ν_4 and ν_3 vibrational modes at 1370 cm^{-1} and 1494 cm^{-1}, respectively. This form undergoes a change of spin state upon miconazole binding, as revealed by the shift of the ν_3 band to 1505 cm^{-1}, characteristic of a six-coordinated low-spin (6cLS) ferric haem (Table 4.1). The reduced *S. aureus* flavohaemoglobin is in a 5cHS configuration, with ν_4 at 1357 cm^{-1} and ν_3 at 1474 cm^{-1} (Figure 4.3C, spectrum I).

TABLE 4.1 Positions of the marker bands ν_4 and ν_3 (cm^{-1}) in the Resonance Raman spectra of oxidised (Hmp (III)) and reduced (Hmp(II)) flavohaemoglobin, unbound and bound to miconazole (M).

Species	5cHS (ox)		5cHS (red)		6cLS (ox)		6cLS (red)	
	ν4	ν3	ν4	ν3	ν4	ν3	ν4	ν3
Hmp(III)	1370	1494						
Hmp(III)-M					1372	1505		
Hmp(II)			1357	1474				
Hmp(II)-M							1364	1494

Component analysis of the spectrum obtained upon addition of miconazole, revealed the presence of two species: five-coordinated ferrous haem, as in the pure protein, and a major component with ν_4 up shifted to 1364 cm^{-1} (Figure 4.3C, spectrum II, Table 4.1), indicating the formation of a six-coordinated low-spin ferrous form. Moreover, since the ν_4 band up-shifts by 7 cm^{-1} (in comparison with the ν_4 of the unbound ferrous flavohaemoglobin), it is apparent that delocalization of the electron cloud from the haem to the miconazole takes place upon binding of the antibiotic [19]. We have also observed, both by RR and UV-Visible spectroscopies that DMSO binds to the ferrous haem, resulting in a distinct low-spin form of flavohaemoglobin. In summary, the spectroscopic results show that miconazole coordinates to the haem moiety of *S. aureus* flavohaemoglobin in the oxidised as well as in the reduced state.

The binding of miconazole to *S. aureus* flavohaemoglobin was also studied by titrating the protein with different antibiotic concentrations and following the

absorption change of the Soret band of the flavohaemoglobin after ligation of the antibiotic. Following the procedure described in the methods section, we determined an equilibrium association constant of 1.7 x 10^6 M^{-1}, indicating that one molecule of miconazole binds tightly to oxidised flavohaemoglobin. A lower limit for the association constant for the reduced form was determined to be 1.2 x 10^5 M^{-1} (data not shown).

Imidazoles increase the ROS production by flavohaemoglobin

Since, as mentioned before, we detected an increase in imidazole resistance upon inactivation of *S. aureus hmp* (Figure 4.2), we next addressed the origin of this behavior. We first observed that the level of endogenously produced reactive oxygen species augmented in cells of wild-type *S. aureus* treated with miconazole or ketoconazole (Figure 4.4A). More importantly, when the ROS production was evaluated in the Δ*hmp* mutant treated with imidazoles the ROS levels were lower, indicating that the presence of flavohaemoglobin contributes to the imidazole-associated ROS generation (Figure 4.4A).

It has been reported that, under certain conditions, *E. coli* flavohaemoglobin produces superoxide ions [21, 22]. To investigate the effect of antibiotic binding to flavohaemoglobin on radical formation, we used the spin trap BMPO, in EPR experiments. In the absence of the antibiotic, when using NADH and under aerobic conditions, the BMPO-OOH adduct was observed [14] being indicative of the formation of superoxide by flavohaemoglobin (Figure 4.4B, upper line). In the presence of miconazole, the same EPR species was detected but at a significantly higher concentration (Figure 4.4B, lower line). By comparing the intensities of the spectra of the BMPO-OOH adduct after 15 min (the time determined to yield the maximum concentration of this adduct), we determined the concentration of BMPO-OOH to be ca. 3-fold higher, in samples with miconazole. We thus conclude that not only is superoxide formed by *S. aureus* flavohaemoglobin, since its EPR spectrum is identical to that of the superoxide–BMPO adduct [14], but also that this production increases upon the binding of miconazole to flavohaemoglobin. Furthermore, we detected, by mass spectrometry, that the integrity of the

imidazole was maintained since no changes occurred in the mass and intensity of the peak of the antibiotic before and after incubation of miconazole with flavohaemoglobin in the presence of NADH (data not shown).

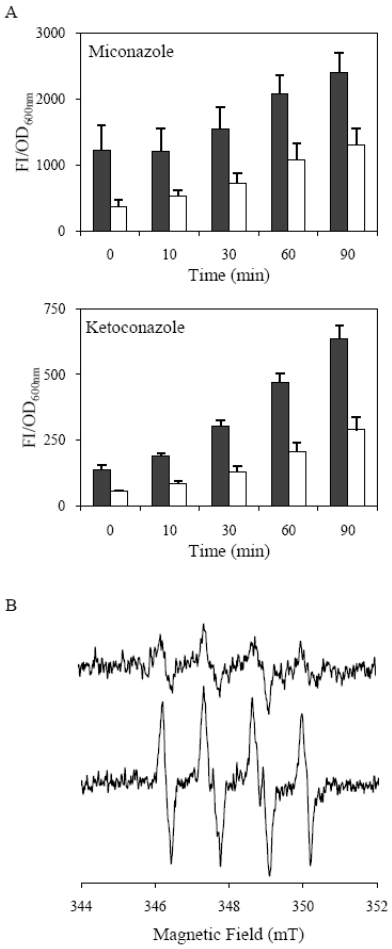

FIGURE 4.4 Imidazoles increase the ROS production by flavohaemoglobin. (A) Evaluation of endogenous ROS production of *S. aureus* wild-type (black bar) and Δ*hmp* (white bar) after treatment with 2 μM miconazole and 120 μM ketoconazole. Each data point represents the average of three independent measurements with the correspondent standard errors. (B) EPR spectra of BMPO-OOH obtained for a sample containing 10 μM Hmp, 200 μM NADH, 25 mM BMPO and 50 μM miconazole (lower line) and for a sample in which the antibiotic was replaced by an equivalent amount of the antibiotic's solvent (upper line). The spectra were recorded at ambient temperature, at 9.8 GHz, microwave power of 10 mW and modulation amplitude of 0.1 mT, after 15 min of incubation.

Real time RT-PCR experiments revealed that exposure of *S. aureus* to miconazole leads to an increase in the expression of the *katA* gene encoding catalase, a marker of oxidative stress, showing *katA* a 11.7±1.4-fold increase in wild-type strain but only a 7.8±0.4-fold increase in the *hmp* mutant, *i.e*, in the absence of flavohaemoglobin the expression of *katA* decreased ~ 30 %. These results are in agreement with a lower production of ROS in the *hmp* mutant.

Flavohaemoglobin decreases survival of miconazole-treated *S. aureus* in macrophages

The increased resistance of *S. aureus* Δ*hmp* to imidazoles when compared to the parent strain (Figure 4.2A-B) led us to examine the effect of flavohaemoglobin on the survival of miconazole-treated *S. aureus* cells phagocytised by macrophages. In the absence of the antibiotic, the Δ*hmp* strain was killed more efficiently by activated macrophages (Figure 4.5A) due to the lack of the NO detoxifying activity of flavohaemoglobin. For miconazole-treated cells we observed that while incubation in macrophages of antibiotic-treated wild-type cells resulted in a decrease of survival of approximately 50 %, the Δ*hmp* cell counts showed no statistical difference between cells unexposed and exposed to miconazole (Figure 4.5A). Similar data were obtained in assays performed in the presence of L-NMMA, the mammalian inhibitor of iNOS, which shows that in the presence or absence of NO, flavohaemoglobin contributes for the lower survival of azole-treated cells (Figure 4.5B). The decreased viability of antibiotic-treated *S. aureus* in macrophage cell lines can be rationalised taking into consideration that the simultaneous presence of flavohaemoglobin and miconazole leads to an increase in the level of deleterious reactive oxygen species, as previously demonstrated by fluorometric and EPR experiments.

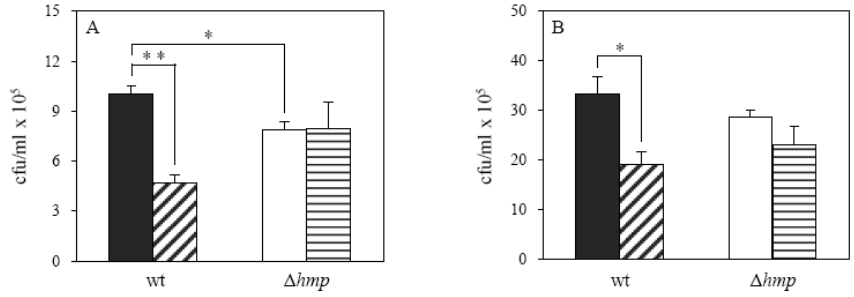

FIGURE 4.5 Intracellular survival in murine macrophages of miconazole-treated wild-type and Δ*hmp S. aureus* cells. Murine macrophages activated with IFN-γ/LPS (A) and treated with the mammalian iNOS inhibitor L-NMMA (B) were infected with *S. aureus* wild-type in the absence (black bar) and in the presence of miconazole (diagonal striped bar), or incubated with Δ*hmp* cells grown without antibiotic (white bar) and with miconazole (horizontal striped bar). After a period of infection of 30 min macrophages were lysed and bacterial counts determined. Two independent assays were performed in triplicate, exhibiting the indicated standard errors (**P<0.01; *P<0.05).

4.4 Discussion

In this study, we show that *S. aureus* is susceptible to several azole antibiotics with miconazole being, among those tested, the most effective one. The mechanism by which imidazole-treated bacteria undergo growth inhibition was assessed and the results provide the first evidence that imidazoles induce intracellular ROS production in *S. aureus* after miconazole and ketoconazole treatment. Remarkably, a strain of *S. aureus* lacking flavohaemoglobin resulted in increased cell viability, while over-expression of flavohaemoglobin restored the azole-susceptibility phenotype. These results indicate that flavohaemoglobin and imidazoles act together in the killing mechanism.

The binding of miconazole to *S. aureus* flavohaemoglobin was analysed by UV-Visible, EPR and Resonance Raman and revealed that miconazole acts as a strong field haem ligand, since upon miconazole binding the five coordinated high-spin configuration in both oxidation states is converted to a six-coordinated low-spin species. Interestingly, the binding of similar-size imidazoles led to similar MIC values, while the larger imidazole ketoconazole exhibits a much higher MIC value. Intriguingly, the two triazoles tested, itraconazole and fluconazole, had no activity against *S. aureus*. As seen in Figure 4.1, this lack of activity cannot solely be related to the size of these molecules since fluconazole is, in fact, the smallest system investigated. Both fluconazole and itraconazole are potent azole anti-fungals, but rather than having an imidazole side-chain, they possess 1,2,4-triazoles. These triazoles do bind well to their P450 targets; since in the P450 enzymes there is an axial cysteine, rather than an axial histidine (as in flavohaemoglobin), it seems likely there may simply be large differences between imidazoles and triazoles, binding to P450 or flavohaemoglobin, due to electronic effects.

Flavohaemoglobins have been reported to have several enzymatic activities, namely NO denitrosylase and alkyl hydroperoxide reductase, as well as to produce superoxide [21, 23]. We noticed that ligation of imidazoles to *S. aureus* flavohaemoglobin leads to impairment of denitrosylase activity (data not shown), which is in accordance with results described for fungal, yeast and *E. coli*

flavohaemoglobins [9]. However, both the susceptibility of wild-type *S. aureus* to imidazoles and the increase in the resistance of *S. aureus* to imidazoles upon deletion of the flavohaemoglobin gene occurs in the absence of any source of nitric oxide. Hence, we tested if binding of imidazoles could interfere with superoxide generation by flavohaemoglobin. In fact, EPR spin trap experiments showed that the binding of miconazole magnifies the superoxide production by *S. aureus* flavohaemoglobin. Since the haem is blocked with miconazole, we concluded that the superoxide was generated at the level of the FAD centre. In this mechanism, FAD receives electrons from NAD(P)H and reduces oxygen to superoxide. This hypothesis is supported by the ability of several flavin-containing proteins to generate superoxide upon one-electron oxidation by dioxygen (e.g., [24]). Also, it was previously shown that NAD(P)H is oxidised by flavohaemoglobin with the electrons transferred via FAD to external acceptors when the ferrous haem is blocked [22].

Therefore, upon ligation of azoles to flavohaemoglobin the *S. aureus* cells become exposed to higher amounts of deleterious ROS, which explains the lower survival of antibiotic-treated *S. aureus* wild-type cells and the higher resistance of the Δ*hmp* mutant strain.

Note that in the simultaneous presence of azoles and NO, the higher level of ROS and the inhibition of the NO scavenging activity of flavohaemoglobin both will contribute to the more efficient killing of wild-type *S. aureus* that was indeed detected in activated macrophages (Figure 4.5A). Interestingly, the present results may explain the previously observed synergistic antimicrobial action of imidazole antibiotics and NO releasers exerted on *Candida* species [25], which can be now rationalized considering that in the presence of the two compounds *Candida* flavohaemoglobin no longer can act as a fungal protective factor.

Finally, we consider future prospects and possible applications. The observation that some azole antifungals also have antibacterial activity is not new with Janssen *et al.* [26] reporting as early as 1969 that miconazole had potent (10 nM) activity against *S. hemolyticus* (*S. pyogenes*). It is also of interest to note that earlier works reported that miconazole killed *S. aureus* [16] and that cells grown in

the presence of miconazole had decreased levels of vitamin K_2 and increased levels on octaprenyl diphosphate [27]. This would be consistent with targeting MenA biosynthesis, which could also potentially contribute to an increase in ROS levels, and indeed we did observe that in the *hmp* mutant strain there is still ROS production (Figure 4.4A), i.e., even in the absence of flavohaemoglobin. So, it is possible that the imidazole antibiotics have more than one target in *S. aureus*, and potentially, in other bacteria as well.

In *S. aureus*, the buildup of ROS is of particular interest since *S. aureus* has a protective golden carotenoid "shield" called staphyloxanthin [28] that protects the bacterium from attack by neutrophil-generated ROS [29]. Inhibiting carotenoid biosynthesis [30] results in bacteria that are white (since they lack the carotenoid pigment) and are thus cleared by host defences. Combining azoles with staphyloxanthin biosynthesis inhibitors may enhance intracellular levels of ROS and NO killing by stripping bacteria of their defences.

In conclusion, this work revealed for the first time that the binding of azoles to *S. aureus* flavohaemoglobin leads to an increase of the intracellular level of reactive oxygen species, therefore enhancing the antimicrobial activity of these antibiotics.

4.5 References

[1] Klevens, R.M., Morrison, M.A., Nadle, J., Petit, S., Gershman, K., Ray, S., Harrison, L.H., Lynfield, R., Dumyati, G., Townes, J.M., Craig, A.S., Zell, E.R., Fosheim, G.E., McDougal, L.K., Carey, R.B. and Fridkin, S.K. (2007) Invasive methicillin-resistant *Staphylococcus aureus* infections in the United States. Jama 298, 1763-1771.

[2] Van Cutsem, J.M. and Thienpont, D. (1972) Miconazole, a broad-spectrum antimycotic agent with antibacterial activity. Chemotherapy 17, 392-404.

[3] Hornby, J.M. and Nickerson, K.W. (2004) Enhanced production of farnesol by *Candida albicans* treated with four azoles. Antimicrob. Agents Chemother. 48, 2305-2307.

[4] Kobayashi, D., Kondo, K., Uehara, N., Otokozawa, S., Tsuji, N., Yagihashi, A. and Watanabe, N. (2002) Endogenous reactive oxygen species is an important mediator of miconazole antifungal effect. Antimicrob. Agents Chemother. 46, 3113-3117.

[5] Thevissen, K., Ayscough, K.R., Aerts, A.M., Du, W., De Brucker, K., Meert, E.M., Ausma, J., Borgers, M., Cammue, B.P. and Francois, I.E. (2007) Miconazole induces changes in actin cytoskeleton prior to reactive oxygen species induction in yeast. J. Biol. Chem. 282, 21592-21597.

[6] De Nollin, S., Van Belle, H., Goossens, F., Thone, F. and Borgers, M. (1977) Cytochemical and biochemical studies of yeasts after in vitro exposure to miconazole. Antimicrob. Agents Chemother. 11, 500-513.

[7] François, I.E.J.A., Cammue, B.P., Borgers, M., Ausma, J., Dispersyn, G.D. and Thevissen, K. (2006) Azoles: mode of antifungal action and resistance development. Effect of miconazole on endogenous reactive oxygen species production in *Candida albicans*. Anti-Infective Agents in Medicinal Chemistry 5, 3-13.

[8] Trivedi, V., Chand, P., Srivastava, K., Puri, S.K., Maulik, P.R. and Bandyopadhyay, U. (2005) Clotrimazole inhibits hemoperoxidase of *Plasmodium falciparum* and induces oxidative stress. Proposed antimalarial mechanism of clotrimazole. J. Biol. Chem. 280, 41129-41136.

[9] Helmick, R.A., Fletcher, A.E., Gardner, A.M., Gessner, C.R., Hvitved, A.N., Gustin, M.C. and Gardner, P.R. (2005) Imidazole antibiotics inhibit the nitric oxide dioxygenase function of microbial flavohaemoglobin. Antimicrob. Agents Chemother. 49, 1837-1843.

[10] Goncalves, V.L., Nobre, L.S., Vicente, J.B., Teixeira, M. and Saraiva, L.M. (2006) Flavohaemoglobin requires microaerophilic conditions for nitrosative protection of *Staphylococcus aureus*. FEBS Lett. 580, 1817-1821.

[11] Nobre, L.S., Seixas, J.D., Romao, C.C. and Saraiva, L.M. (2007) Antimicrobial action of carbon monoxide-releasing compounds. Antimicrob. Agents Chemother. 51, 4303-4307.

[12] Sullivan, M.A., Yasbin, R.E. and Young, F.E. (1984) New shuttle vectors for *Bacillus subtilis* and *Escherichia coli* which allow rapid detection of inserted fragments. Gene 29, 21-26.

[13] Nobre, L.S., Goncalves, V.L. and Saraiva, L.M. (2008) Flavohaemoglobin of *Staphylococcus aureus*. Methods Enzymol. 436, 203-216.

[14] Zhao, H., Joseph, J., Zhang, H., Karoui, H. and 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.

[15] Miller, A. and Tanner, J. (2008) Essentials of chemical biology structure and dynamics of biological macromolecules, John Wiley & Sons.

[16] Sud, I.J. and Feingold, D.S. (1982) Action of antifungal imidazoles on *Staphylococcus aureus*. Antimicrob. Agents Chemother. 22, 470-474.

[17] Salerno, J.C. (1984) Cytochrome electron spin resonance line shapes, ligand fields, and components stoichiometry in ubiquinol-cytochrome *c* oxidoreductase. J. Biol. Chem. 259, 2331-2336.

[18] Hu, S., Smith, K.M. and Spiro, T.G. (1996) Assignment of Protoheme Resonance Raman Spectrum by Haem Labeling in Myoglobin. J. Am. Chem. Soc. 118, 12638–12646.

[19] Siebert, F. and Hildebrandt, P. (2008) Vibrational spectroscopy in life science, Wiley-VCH.

[20] Yeh, S.R., Couture, M., Ouellet, Y., Guertin, M. and Rousseau, D.L. (2000) A cooperative oxygen binding haemoglobin from *Mycobacterium tuberculosis*. Stabilization of heme ligands by a distal tyrosine residue. J. Biol. Chem. 275, 1679-1684.

[21] Membrillo-Hernandez, J., Ioannidis, N. and Poole, R.K. (1996) The flavohaemoglobin (HMP) of *Escherichia coli* generates superoxide in vitro and causes oxidative stress *in vivo*. FEBS Lett. 382, 141-144.

[22] Wu, G., Corker, H., Orii, Y. and Poole, R.K. (2004) Escherichia coli Hmp, an "oxygen-binding flavohaemoprotein", produces superoxide anion and self-destructs. Arch. Microbiol. 182, 193-203.

[23] Bonamore, A., Gentili, P., Ilari, A., Schinina, M.E. and Boffi, A. (2003) *Escherichia coli* flavohaemoglobin is an efficient alkylhydroperoxide reductase. J. Biol. Chem. 278, 22272-22277.

[24] Imlay, J.A. (2008) Cellular defenses against superoxide and hydrogen peroxide. Annu. Rev. Biochem. 77, 755-776.

[25] McElhaney-Feser, G.E., Raulli, R.E. and Cihlar, R.L. (1998) Synergy of nitric oxide and azoles against *Candida* species *in vitro*. Antimicrob. Agents Chemother. 42, 2342-2346.

[26] Godefroi, E.F., Heeres, J., Van Cutsem, J. and Janssen, P.A. (1969) The preparation and antimycotic properties of derivatives of 1-phenethylimidazole. J. Med. Chem. 12, 784-791.

[27] Bossche, H.V., Lauwers, W., Willemsens, G., Marichal, P., Cornelissen, F. and Cools, W. (2006) Molecular basis for the antimycotic and antibacterial activity of N-substituted imidazoles and triazoles: The inhibition of isoprenoid biosynthesis Pesticide Science 15, 188-198.

[28] Pelz, A., Wieland, K.P., Putzbach, K., Hentschel, P., Albert, K. and Gotz, F. (2005) Structure and biosynthesis of staphyloxanthin from *Staphylococcus aureus*. J. Biol. Chem. 280, 32493-32498.

[29] Liu, G.Y., Essex, A., Buchanan, J.T., Datta, V., Hoffman, H.M., Bastian, J.F., Fierer, J. and Nizet, V. (2005) *Staphylococcus aureus* golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. Journal of Experimental Medicine 202, 209-215.

[30] Liu, C.I., Liu, G.Y., Song, Y., Yin, F., Hensler, M.E., Jeng, W.Y., Nizet, V., Wang, A.H. and Oldfield, E. (2008) A cholesterol biosynthesis inhibitor blocks *Staphylococcus aureus* virulence. Science 319, 1391-1394.

CHAPTER 5

The antimicrobial action of carbon monoxide releasing compounds

Summary

Carbon monoxide is endogenously produced in the human body, mainly from the oxidation of haem catalysed by haem oxygenase enzymes. The induction of HO and the consequent increase in CO production play important physiological roles in vasorelaxation and neurotransmission and in the immune system. The exogenous administration of CO gas and CO-releasing molecules has been shown to induce vascular effects and to alleviate hypoxia-reoxygenation injury of mammalian cells. In particular, due to its anti-inflammatory, anti-apoptotic, and anti-proliferative properties, CO inhibits ischemic-reperfusion injury and provides potent cytoprotective effects during organ and cell transplantation. In spite of these findings regarding the physiology and biology of mammals, nothing is known about

the action of CO on bacteria. In the present work, we examined the effect of CO on bacterial cell proliferation. Cell growth experiments showed that CO caused the rapid death of the two pathogenic bacteria tested, *Escherichia coli* and *Staphylococcus aureus*, particularly when delivered through organometallic CO-RMs. Of importance is the observation that the effectiveness of the CO-RMs was greater in near-anaerobic environments, as many pathogens are anaerobic organisms and pathogen colonisation occurs in environments with low oxygen concentrations. Our results constitute the first evidence that CO can be utilised as an antimicrobial agent. We anticipate our results to be the starting point for the development of novel types of therapeutic drugs designed to combat antibiotic-resistant pathogens, which are widespread and presently a major public health concern.

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5.1 Introduction

Carbon monoxide is a colourless and odourless diatomic gas, chemically inert, that occurs in nature as a product of oxidation or combustion of organic matter. Owing to its lethal effect when present in high concentrations, CO was considered for many years to be only an environmental toxicant that results from air pollution by automobile exhaust. The knowledge that the human body is able to produce small quantities of CO and the evidence that CO derived from haem oxygenase activity contributes to important intracellular functions have modified our perception of CO as a pernicious toxin to include its beneficial effects [1-3]. In consequence, the application of CO gas or CO-releasing molecules has emerged as a new therapeutic strategy in medicine [4-6]. The evolution of CO from a toxicant to a molecule of mounting importance in mammals finds a parallel in another diatomic molecule, nitric oxide [7]. NO is produced in the body by the nitric oxide synthase and shares with CO many down-stream signalling pathways and regulatory functions, in particular, those associated with the activation of soluble guanylate cyclase [8-10]. In addition, there is an interplay between the two molecules, since it is proposed that CO is a modulator of nitric oxide synthase [3, 4] and NO up-regulates haem oxygenase [11, 12], which in turn catalyses the oxidative degradation of free haem into biliverdin, with the concomitant release of iron and CO. NO also constitutes one of the weapons that the mammalian immune system uses to fight pathogens [13, 14]. The bactericidal function of NO relies on the deleterious effects caused in the pathogen, e.g., the nitrosylation of iron centres. Although CO is a stable neutral molecule with a long half-life, it shares with NO the high affinity for iron of haem proteins, which is the basis of its toxicity. We therefore set out to explore the possible action of CO on bacterial growth rates. For this purpose, we tested the bioactivity of CO, applied either in the gaseous form or via treatment with CO-RMs, on *E. coli* and *S. aureus*. These bacteria are major human pathogens that are widespread in the community and are responsible for hospital-acquired infections, exhibiting a concerning degree of antibiotic resistance.

5.2 Material and Methods

Reagents

The different sources or references for CO were as follows: tricarbonyldichlororuthenium(II) dimer (CORM-2), Sigma; tricarbonylchloro(glycinate)ruthenium(II) (CORM-3), [15]; bromo(pentacarbonyl)manganese (ALF 021), [16]; and tetraethylammonium molybdenum pentacarbonyl bromide (ALF 062), [17]. All compounds were freshly prepared as 10 mM stock solutions by dissolution in dimethyl sulfoxide, pure distilled water, or methanol.

Bacterial strains and growth conditions

E. coli K-12 ATCC 23716 and *S. aureus* NCTC 8325 were grown in minimal salts (MS) medium (1.3 % [wt/vol] Na_2HPO_4, 0.3 % [wt/vol] KH_2PO_4, 0.05 % [wt/vol] NaCl, and 0.1 % [wt/vol] NH_4Cl supplemented with 20 mM glucose, 2 mM $MgSO_4$, 100 µM $CaCl_2$, and 0.25 % [wt/vol] Casamino Acids) and in LB medium (1 % [wt/vol] tryptone, 0.5 % [wt/vol] yeast extract, and 1 % [wt/vol] NaCl), respectively, under different oxygen supply conditions. Aerobic experiments were undertaken with flasks filled to one-fifth of their volume, microaerobic tests were conducted with closed flasks filled to one-half of their volume, and anaerobic conditions were produced in rubber-sealed flasks that, once filled with medium and closed, were extensively fluxed with nitrogen gas.

CO gas and CO-RM treatment

Overnight cultures of *E. coli* or *S. aureus* grown in LB or TSB, respectively, were used to inoculate fresh MS medium (*E. coli*) or LB medium (*S. aureus*), and the cultures on fresh medium were incubated at 37 °C under the required aeration conditions to an optical density at 600 nm of 0.3. At this point, cells were exposed to a flux of CO gas for 15 min or to CO-RMs. Untreated cells were bubbled with nitrogen gas or treated with dimethyl sulfoxide, water, or methanol, depending on the solvent used to dissolve the CO-RM. The inactive form of ALF 062 was prepared by mixing vigorously with 20 % methanol in a closed flask over 2 to 3 h.

The counter ion of ALF 062, tetraethyl ammonium bromide, and one of the products of ALF 062 decomposition, sodium molybdate, were used at the same concentration as ALF 062 (50 μM).

Viability assays

The number of viable cells was evaluated by measuring the CFU per milliliter upon plating serial dilutions of the various cultures onto agar plates. The percent survival was calculated as the number of colonies originated by treated cultures divided by the number of colonies formed upon the plating of untreated cultures. Sensitivity tests were conducted by plating 5 μl serial dilutions of cultures grown for 4 h and treated with CO-RMs, with or without the CO scavenger haemoglobin [bovine form used at 20 μM; Sigma]), onto agar. The experiments were performed with a minimum of three independent cultures, and the results are presented in the figures as averaged values with error bars representing one standard deviation.

The investigation of MICs and minimal bactericidal concentrations (MBCs) was carried out by the tube dilution test. Briefly, 2.5 ml of minimal medium was inoculated with an overnight culture of *E. coli* or *S. aureus* to give an optical density at 600 nm of 0.005 to 0.01. Different concentrations of CORM-2, between 150 μM and 2 mM, were added to the diluted suspensions in the wells of 24-well plates, and the plates were incubated for at least 18 h at 37°C and 90 rpm. The concentration of CORM-2 in the first well in the series with no sign of visible growth was reported as the MIC. All the cultures that exhibited a lack of cell growth were then subsequently plated onto agar devoid of any drug. After incubation at 37 °C for 24 h, the lowest concentration of CORM-2 in a culture with no growth was assumed to be the MBC.

CO release kinetics

CO-RMs were mixed with MS or LB medium in sealed vessels, and the vessels were incubated at room temperature under constant stirring and protected from light. Gas samples were collected after 30 min and 4 h and analysed using a gas

chromatograph (Thermo Finnigan Trace) equipped with a CTRI column (Alltech) and a thermal conductivity detector. The CO released was quantified using a calibration curve recorded prior to the reaction course.

Inductively coupled plasma mass spectrometry analysis

E. coli cells cultured in MS medium with or without 50 µM ALF 062 were collected after 1 h of growth, and the cellular metal content was analysed at Instituto de Investigacão das Pescas e do Mar, Lisbon, Portugal. The intracellular concentration of Mo in *E. coli* cultures was assayed on a quadropole inductively coupled plasma mass spectrometer (X series; Thermo Elemental) equipped with a Peltier impact bead spray chamber and a concentric Meinhard nebulizer. The experimental parameters were as follows: 790 W of forward power, peak jumping mode, and 150 sweeps per replicate (dwell time, 10 ms; dead time, 30 ns). A seven-point calibration within a range of 1 to 100 µg liter^{-1} was used to quantify metal concentrations. Coefficients of variation for determinations of metal content (n = 5) ranged between 0.5 and 2 %. The precision and accuracy of metal concentration measurements, as determined through the repeated analysis of reference materials (TORT-1, TORT-2, DORM-2, and DORM-3 from the National Research Council of Canada) by using indium as an internal standard, were within 1 to 2 %. Procedural blanks always accounted for less than 1 % of the total molybdenum concentrations in the samples.

5.3 Results and discussion

The effect of CO on the viability of bacteria was investigated first by the direct delivery of CO gas. The administration of CO gas, fluxed into the growing cultures, led to a significant growth impairment of *E. coli* and *S. aureus* (Figure 5.1). To evaluate the potential of CO-RMs, the compounds indicated in Figure 5.2 were selected.

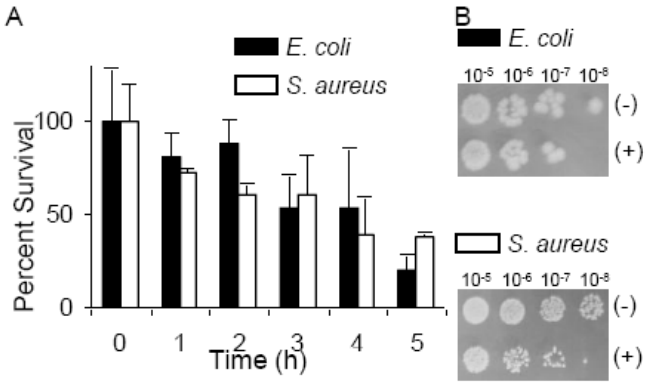

FIGURE 5.1 Effects of CO gas on *E. coli* and *S. aureus* viability. (A) *E. coli* and *S. aureus* cells were grown under microaerobic conditions in MS and LB media, respectively, and exposed to a flux of CO gas for 15 min. (B) Sensitivity tests were conducted by plating the indicated serial dilutions of the cultures collected after 4 h of exposure to CO gas (+) or to nitrogen gas (-).

CORM-2 and CORM-3 are active in a variety of CO-mediated biological processes, both *in vitro* and *in vivo* [18]. In the first series of experiments, the effect of CO released from CORM-2 on the growth of *E. coli* and *S. aureus* was studied with bacteria cultured under different levels of oxygen supply. Shortly after the exposure to CORM-2, the percentage of surviving cells significantly diminished (Figure 5.3). Experiments using water-soluble CORM-3 revealed that, albeit requiring higher concentrations than CORM-2 due to its chemical composition, the compound also strongly decreased the viability of *E. coli* and *S. aureus* cells (Figure 5.4). However, while the addition of CORM-3 resulted in a strong inhibition of *E. coli* cell growth, *S. aureus* was more resistant to CORM-3 (Figure 5.4A), particularly under aerobic conditions. In general, the action of the two compounds was rapid and extended over time, as

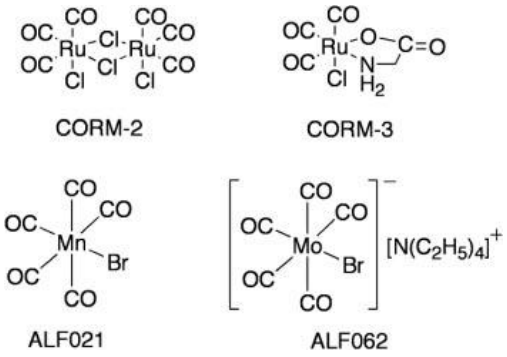

FIGURE 5.2 Chemical structures of CO-RMs used in this study.

cells did not resume growth over the subsequent 4 h (Figures 5.3 and 5.4) or after 8 h (data not shown).

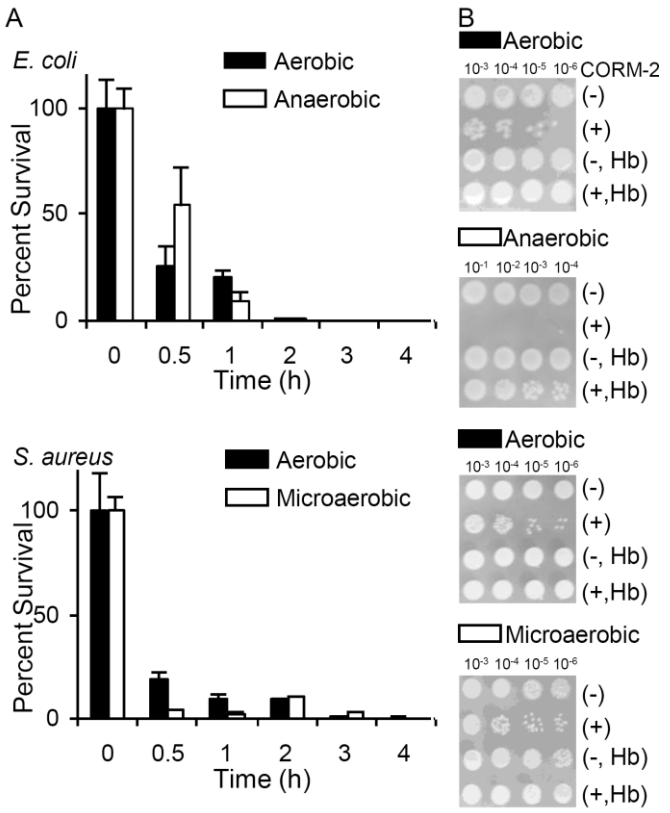

FIGURE 5.3 Effects of CORM-2 on *E. coli* and *S. aureus* cell viability. (A) *E. coli* cells were grown in MS under aerobic and anaerobic conditions and treated with 250 μM CORM-2. *S. aureus* cells were grown aerobically and microaerobically in LB medium and exposed to 250 μM CORM-2. (B) Results of tests of the sensitivity of cultures to CORM-2 (see Materials and Methods). The indicated dilutions of cultures were treated with CORM-2 (+; 250 μM) or left untreated (-) and assayed in the absence or in the presence of haemoglobin (Hb).

In order to examine whether the bactericidal effect of CO-RMs was due to CO, cell growth experiments with CO-RMs were also performed in the presence of Hb, a high-affinity CO scavenger. In all cases, the bactericidal effect on *E. coli* and *S. aureus* was completely lost (Figures 5.3B and 5.4B), thus demonstrating that the antimicrobial action of CO-RMs is dependent on their release of CO.

Bactericidal activity has been defined as a ratio of the MBC to the MIC of < 4 [19]. The determination of the CORM-2 MBC/MIC ratios for *E. coli* and *S. aureus* to be 1.5 and 1.0, respectively, revealed the bactericidal character of CORM-2. The two other CO-RMs used to investigate the bactericidal effect of CO, namely, manganese carbonyl ALF 021 and molybdenum carbonyl ALF 062, were also seen to be capable of strongly reducing the viability of *E. coli* and *S. aureus* (Figures 5.5

and 5.6). Again, the addition of Hb completely eliminated the harmful action of ALF compounds on the two bacteria (Figures 5.5 and 5.6). Furthermore, to ensure that the activity of ALF 062 was not related to its decomposition products, we tested the effects of tetraethyl ammonium bromide, sodium molybdate, and a solution of inactivated ALF 062, obtained after the cessation of CO release (see Materials and Methods), on bacterial growth. None of these compounds had bactericidal properties or altered growth kinetics (data not shown). Therefore, the bactericidal effects of ALF 062 are due to its capacity to release CO.

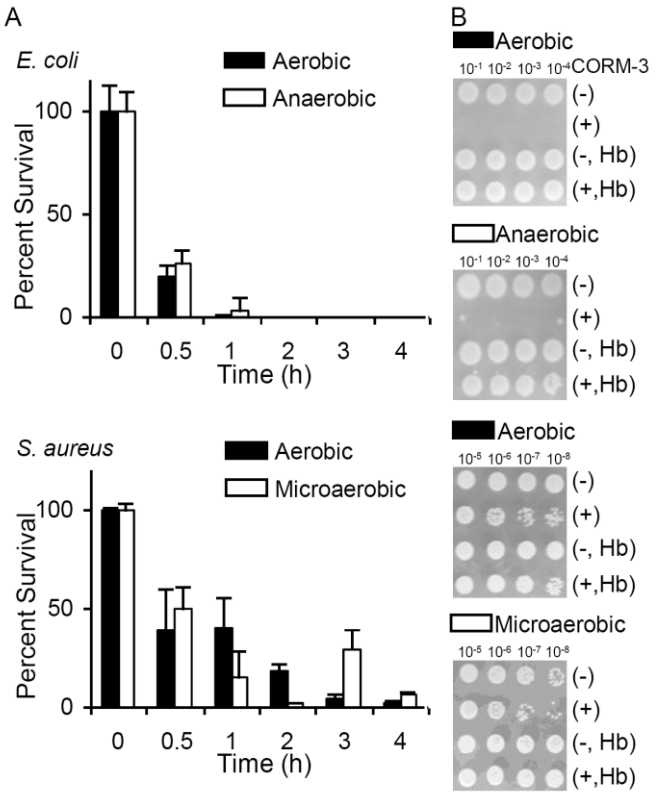

FIGURE 5.4 Effects of CORM-3 on *E. coli* and *S. aureus* cell viability. (A) *E. coli* cells were grown in MS medium either aerobically or anaerobically and treated with 400 μM CORM-3. *S. aureus* cells were grown aerobically or microaerobically in LB medium to which 500 or 400 μM CORM-3 was added, respectively. (B) Sensitivity tests were conducted by plating dilutions of cultures grown as described in Materials and Methods after exposure to CORM-3 (+) or no treatment (-) in the absence or in the presence of haemoglobin (Hb). The concentrations of CORM-3 used were the same as those indicated in the legend to panel A.

It should be mentioned that neither CORM-2 nor CORM-3 releases CO gas when dissolved in the media utilised, even at concentrations higher than those used in our experiments (Table 5.1). Furthermore, although ALF 021 and ALF 062 release CO gas upon dissolution in the medium, they do so in rather small amounts

within the time scale of the experiment (Table 5.1). However, inductively coupled plasma mass spectrometry analysis of *E. coli* cells incubated with ALF 062 revealed a very large increase in the content of Mo (155 μg/g) compared to that in control cells (2.5 μg/g), confirming that the Mo from ALF 062 accumulates inside the *E. coli* cells, where it releases CO to the cellular targets. Since the bactericidal effect of the CO-RMs does not require the release of CO gas to the extracellular medium (Table 5.1), we must conclude that CO has to be delivered to the cellular targets directly from the CO-RMs. Because Mo from bactericidally active (CO-loaded) ALF 062 is found to accumulate rapidly within cells, we infer that it transports CO and delivers it into the intracellular space, where it reaches the cellular targets and causes the decrease of bacterial cell viability. If Hb is present in the medium, the high affinity of Hb for CO results in a fast transfer (or abstraction) of the active CO from the CO-RMs (or from gas) to the protein haems and the effective scavenging of CO as COHb (see below). Under these conditions, no CO will be available for intracellular delivery and the cells remain alive.

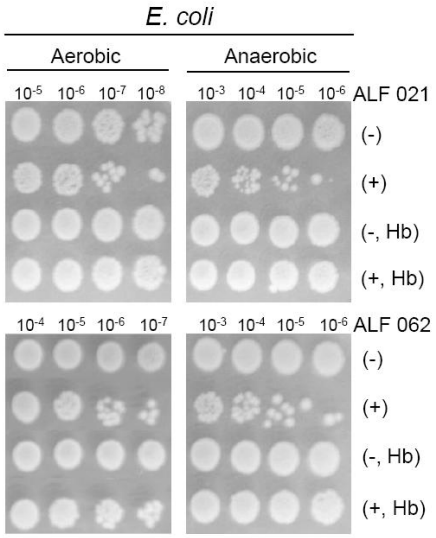

FIGURE 5.5 Sensitivity of *E. coli* to ALF 021 and ALF 062 compounds. *E. coli* cells grown under aerobic or anaerobic conditions were treated with 500 or 200 μM ALF 021, respectively, and with 50 μM ALF 062 (see Materials and Methods) in the absence or in the presence of Hb. The indicated dilutions of cultures exposed to CO-RMs (+) or not exposed (-) were subjected to sensitivity tests.

Albeit with some minor deviations, the general pattern of our results shows that CO-RM toxicity is enhanced when growth occurs under lower oxygen concentrations. For example, ALF 021 was more effective in reducing the viability

of *E. coli* cells grown anaerobically (200 μM ALF 021) than that of cells grown aerobically (500 μM ALF 021). The augmentation of the effect of CO at low oxygen concentrations may be explained by the preferential binding of CO to the ferrous form of haem proteins, which are predominant under reducing environments. More importantly, the bactericidal effect of CO-RMs under anaerobic conditions indicates that growth inhibition is not restricted to the impairment of the respiratory chain by the binding of CO to cytochrome oxidase, which is likely to contribute to the bactericidal activity of these compounds under aerobic conditions. This fact is quite important since pathogen colonisation occurs in near-anaerobic environments and since many pathogens are anaerobic organisms. On the other hand, the type of bacterial cell wall also seems not to interfere with the action of CO-RMs, as judged by the similar decreases in cell viability observed for the gram-positive (*S. aureus*) and gram-negative (*E. coli*) species upon treatment with the same CO-RM. Hence, CO-RMs have the potential for use as bactericides against a wide range of microorganisms independently of the type of bacterial cell wall and oxygen growth requirements.

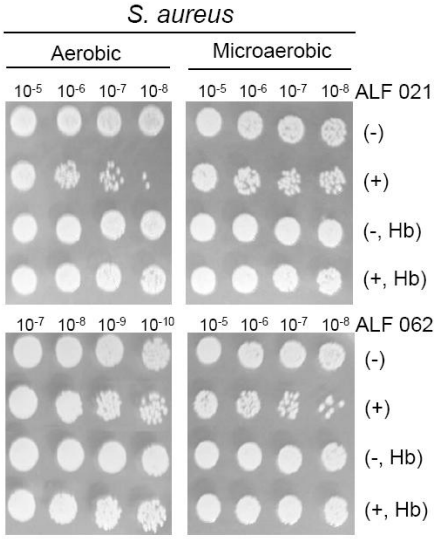

FIGURE 5.6 Sensitivity of *S. aureus* to ALF 021 and ALF 062 compounds. *S. aureus* cells grown under aerobic and microaerobic conditions were treated with 600 μM ALF 021 and 50 μM ALF 062. The indicated dilutions of cultures exposed to CO-RMs (+) or not exposed (-) were subjected to sensitivity tests in the absence or in the presence of Hb, as described in Materials and Methods.

The difference between the degrees of action of dissolved molecular CO gas and CO-RMs is striking. When administered as gas, CO had to be present in rather

high concentrations (ca. 1 mM) to become effective as a bactericide. The ability of CO-RMs to accumulate inside bacterial cells before they release CO makes these compounds highly effective CO donors to bacterial targets, thereby strongly enhancing the bactericidal efficacy of CO. In fact, the CO-RMs used in this study were able to transfer CO to Hb to form COHb, as judged by the shift of the Hb Soret band from 413 to 418 nm (data not shown) and by the results depicted in Figures 5.3B, 5.4B, 5.5, and 5.6. Hence, CO-RMs are capable of delivering CO to haem-containing molecules, as had been shown before for the rapid carbonylation of myoglobin by CORM-3 [20]. Likewise, the carbonylation of Hb by CORM-2 and CORM-3 occurs within the mixing time, while that by ALF 021 and ALF 062 takes place in less than 15 min. It is well known that the biological effect of CO on mammalian cells is due mainly to its interaction with iron-containing proteins, such as the above mentioned cytochrome oxidase. In addition to haem proteins and sensors, CO may bind to almost all transition metal-containing proteins, giving rise to structural modifications and alterations of their biological functions. Hence, in bacteria, there are a large number of likely intracellular targets that can account for the toxic effect of CO revealed in this study.

TABLE 5.1. CO released into medium by CO-RMs.*

Medium	CO Equivalents *			
	MS		LB	
Time (min)	30	240	30	240
CORM-2 (5 mM)	0	0	0	0.1
CORM-3 (12 mM)	0	0	0	0
ALF 021 (6 mM)	0	0.5	0	0.5
ALF 062 (6 mM)	1.4	3.8	0.2	1.6

*Amounts of CO are expressed as CO equivalents (number of CO groups released per CO-RM molecule

In spite of the increasing expectations for the use of CO in medicine [4-6], until now, the role of CO as a bactericidal compound had remained unexplored. Nevertheless, in the early 1970s it was reported that the addition of CO to an aerobic culture of *E. coli* caused a decrease in DNA replication [21]. However, as

the authors of the study did not observe any effect of CO on cells growing anaerobically on glucose, they concluded that the inhibition of DNA synthesis in cells grown under aerobic conditions was not due to a direct effect on the replication apparatus but resulted from indirect effects, such as ATP or deoxynucleoside triphosphate depletion [21]. In more recent years, in spite of several public concerns, CO has been used by the food industry to generate the bright red color of the dark muscle tissue of meat and fish, which results from the great affinity of CO for the Fe (II) binding site of myoglobin. Interestingly, a very recent study of the influence of different packing systems on meat preservation indicated that packages to which CO gas had been added exhibited less bacterial growth than other packages. These results suggest that CO may be one of the packaging gases responsible for the inhibition of the growth of microorganisms [22]. We now show that CO and, in particular, CO-RMs have the ability to kill bacteria under aerobic and anaerobic conditions. We submit that CO-RMs constitute a novel class of antibacterial molecules that may become drug candidates upon the development of safe and controllable methods of CO delivery to bacterial targets that avoid the *in vivo* scavenging of CO by the red blood cells [4]. In particular, non systemic bactericides may be a relatively easy application for CO-RMs. Although this is a first visualisation of a still very distant goal, bactericides based upon completely new concepts are urgently required, as the emergence and spread of drug-resistant bacterial pathogens reveal a concerning decrease in the effectiveness of currently available antibiotics.

5.4 References

[1] Piantadosi, C.A. (2002) Biological chemistry of carbon monoxide. Antioxid. Redox Signal 4, 259-270.

[2] Ryter, S.W., Alam, J. and Choi, A.M. (2006) Haem oxygenase-1/carbon monoxide: from basic science to therapeutic applications. Physiol. Rev. 86, 583-650.

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

[4] Motterlini, R., Mann, B.E. and Foresti, R. (2005) Therapeutic applications of carbon monoxide-releasing molecules. Expert Opin. Investig. Drugs 14, 1305-1318.

[5] Nakao, A., Choi, A.M. and Murase, N. (2006) Protective effect of carbon monoxide in transplantation. J. Cell Mol. Med. 10, 650-671.

[6] Ryter, S.W. and Otterbein, L.E. (2004) Carbon monoxide in biology and medicine. Bioessays 26, 270-280.

[7] Ryter, S.W., Morse, D. and Choi, A.M. (2004) Carbon monoxide: to boldly go where NO has gone before. Sci STKE 2004, RE6.

[8] Kharitonov, V.G., Sharma, V.S., Pilz, R.B., Magde, D. and Koesling, D. (1995) Basis of guanylate cyclase activation by carbon monoxide. Proc. Natl. Acad. Sci. U S A 92, 2568-2571.

[9] Moncada, S. and Higgs, E.A. (2006) The discovery of nitric oxide and its role in vascular biology. Br. J. Pharmacol. 147 Suppl 1, S193-201.

[10] Mungrue, I.N., Bredt, D.S., Stewart, D.J. and Husain, M. (2003) From molecules to mammals: what's NOS got to do with it? Acta Physiol. Scand. 179, 123-135.

[11] Ryter, S.W., Otterbein, L.E., Morse, D. and Choi, A.M. (2002) Haem oxygenase/carbon monoxide signaling pathways: regulation and functional significance. Mol. Cell Biochem. 234-235, 249-263.

[12] Srisook, K., Kim, C. and Cha, Y.N. (2005) Role of NO in enhancing the expression of HO-1 in LPS-stimulated macrophages. Methods Enzymol. 396, 368-377.

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

[14] Fang, F.C. (2004) Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. Nat. Rev. Microbiol. 2, 820-832.

[15] Johnson, T.R., Mann, B.E., Teasdale, I.P., Adams, H., Foresti, R., Green, C.J. and Motterlini, R. (2007) Metal carbonyls as pharmaceuticals? [Ru(CO)($_3$)Cl(glycinate)], a CO-releasing molecule with an extensive aqueous solution chemistry. Dalton Trans. 1500-1508.

[16] Herrmann-Brauer (1997) Synthetic Methods of Organometallic and Inorganic Chemistry, 36 . Stuttgart.

[17] Burgmayer, S.J.N. and Templeton, J.L. (1985) Synthesis and Structure of a 7-Coordinate Molybdenum Carbonyl Fluoride Derivative - Et_4M $Mo(CO)_2(S_2CNEt_2)2F$. Inorganic. Chemistry 24, 2224-2230.

[18] Motterlini, R., Clark, J.E., Foresti, R., Sarathchandra, P., Mann, B.E. and Green, C.J. (2002) Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. Circ. Res. 90, E17-24.

[19] Pankey, G.A. and Sabath, L.D. (2004) Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections. Clin. Infect. Dis. 38, 864-870.

[20] Motterlini, R., Sawle, P., Hammad, J., Bains, S., Alberto, R., Foresti, R. and Green, C.J. (2005) CORM-A1: a new pharmacologically active carbon monoxide-releasing molecule. Faseb. J. 19, 284-286.

[21] Weigel, P.H. and Englund, P.T. (1975) Inhibition of DNA replication in *Escherichia coli* by cyanide and carbon monoxide. J. Biol. Chem. 250, 8536-8542.

[22] Brashears, M.M. and Brooks, M.M. (2006) Effect of meat packaging technologies on the safety and spoilage-indicating characteristics of ground beef - phase 1: safety characteristics.

CHAPTER 6

Exploring the antimicrobial action of a carbon monoxide releasing compound through whole genome transcription profiling of *Escherichia coli*

Summary

We recently reported that carbon monoxide has bactericidal activity. To understand its mode of action we analysed the gene expression changes occurring when *Escherichia coli*, grown aerobically and anaerobically, is treated with the carbon monoxide releasing molecule, CORM-2. The *E. coli* microarray analysis shows that *E. coli* CORM-2 response is multifaceted with a high number of differentially regulated genes spread through several functional categories, namely genes involved in inorganic ion transport and metabolism, regulators, and genes implicated in posttranslational modification, such as chaperones. CORM-2 has higher impact in *E. coli* cells grown anaerobically, as judged by the existence of repressed genes belonging to eight functional classes which are absent in aerobically CORM-2 treated cells.

The biological relevance of the variations caused by CORM-2 was substantiated by studying the CORM-2 sensitivity of selected *E. coli* mutants. The results show that the deletion of redox-sensing regulators SoxS and OxyR increased the sensitivity to CORM-2 and suggest that while SoxS plays an important role in protection against CORM-2 under both oxygen growth conditions, OxyR seems to participate only in the aerobic CORM-2 response. Under anaerobic conditions, we found that the heat shock proteins IbpA and IbpB contribute to CORM-2 defence since the deletion of these genes increases the sensitivity of the strain. The induction of several *met* genes and the hypersensitivity to CORM-2 of the Δ*metR*, Δ*metI* and Δ*metN* mutant strains suggest that CO has effects on the methionine metabolism of *E. coli*. CORM-2 also affects the transcription of several *E. coli* biofilm-related genes and increases the biofilm formation in *E. coli*. In particular, the absence of *tqsA* or *bhsA* increases the *E. coli* resistance to CORM-2, and deletion of *tqsA* leads to a strain that loses its capacity to induce the biofilm formation upon treatment with CORM-2.

In spite of the relatively stable nature of the CO molecule, our results show that CO is able to trigger a significant alteration in the transcriptome of *E. coli* which necessarily has effects in several key metabolic pathways.

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6.1 Introduction

Carbon monoxide formed in nature results from the incomplete oxidation of organic matter and as the by-product of the activity of the haem oxygenase enzyme that oxidises haem to biliverdin [1]. In the latter process, which can occur in bacteria, plants and animals, the amount of CO endogenously produced by the various cell types is low. In humans, the CO formed has several physiological functions, namely in the neuronal and cardiovascular systems [2]. On the contrary, high concentrations of CO have a poisoning effect in mammals due to the binding of CO to haemoglobin, and the consequent impairment of oxygen storage and transport by haemoglobin causes hypoxia [2]. CO binds preferentially to transition metal-containing proteins, including haem-based proteins/sensors, giving rise to structural modifications and alteration of their biological function which may also account for the toxic effects of CO. However, it was shown that living cells can tolerate CO at the concentration of 0.01% (100 ppm) for several hours [3], and rodents could be exposed continuously for up to two years to 500 ppm CO without deleterious effects [3-5].

Following the discovery that carbon monoxide is physiologically important, molecules acting as CO-carriers, namely CO-releasing molecules, were developed. In order to mimic the role of CO derived from haem oxygenase [6], these molecules have to be able to liberate CO in a biological environment. This liberation is triggered by some physical, chemical, or enzymatic stimulus. For many CO-RMs that carry molecular CO as a ligand to a central transition metal, the lack of a pharmacological effect of a similar compound devoid or depleted of CO has been taken as a positive proof for the biological action of CO [7]. Following this principle, the beneficial therapeutic action of several CO-RMs in a wide range of vasoactive and anti-inflammatory situations and models has been attributed to CO [8].

Recently, we reported that four transition metal based CO-RMs at micromolar concentrations have antibiotic activity reducing the viability of bacteria, such as *E. coli* and *S. aureus*, both under aerobic and anoxic conditions [9]. In that work we also showed that the biological action of those CO-RMs as bactericides was only

effective when CO is taken up by the cells. In fact, we demonstrated that one of the CO-RMs led to extensive accumulation of the transition metal molybdenum inside bacterial cells. In all cases, the addition of haemoglobin to the medium, before addition of the CO-RM, prevented any bactericidal action. Separate experiments [10] (JD Seixas, personal communication) showed that these CO-RMs transfer CO to haemoglobin at a very fast rate. Taken together these results strongly suggest that the bactericidal action of a given CO-RM depends on its ability to sequentially enter bacterial cells and enable CO to reach specific targets.

We have now extended these studies to the analysis of the global transcriptome of *E. coli* treated with tricarbonyldichlororuthenium (II) dimer, also named CORM-2, which reflects the organism's immediate response to the environment. Additionally, we have examined the phenotype of twelve mutant strains to test the causative role of each deleted gene in *E. coli* CORM-2 response. Regulatory genes and genes involved in methionine metabolism and biofilm formation were found to play an important role when the microorganism is exposed to the bactericidal action of CORM-2.

6.2 Material and methods

Bacterial strains and growth conditions

Escherichia coli MG1655 was grown, at 37 ºC and 150 rpm, in minimal medium salts (MS) [9] in the presence and in the absence of oxygen. Aerobic experiments were undertaken in flasks filled with 1/5 of its volume and anaerobic conditions were performed in rubber sealed flasks that, once filled with media and closed, were extensively fluxed with nitrogen gas. Cultures were grown until an optical density at 600 nm of 0.3, and at this point cells were left untreated or treated with tricarbonyldichlororuthenium (II) dimer, CORM-2 (Sigma).

RNA isolation

The hot-phenol method was used to extract total RNA from two independent cultures of cells grown either aerobically or anaerobically and treated with 250 μM

CORM-2 during 15 min. The concentration of the drug added to the cultures is equivalent to 0.5 times the MIC (500 μM) which was previously determined [9]. After the isolation procedure, RNA was incubated with DNase (Ambion), and once the absence of any residual DNA was confirmed, concentration and purity were determined in a Nanodrop ND-1000 UV-visible spectrophotometer. RNA integrity was checked in an Agilent 2100 Bioanalyser coupled to a RNA Nano-Assay (Agilent Technologies, Palo Alto, CA).

Microarray analysis

Total RNA (10 μg) was processed for use in the Affymetrix GeneChip *E. coli* Genome 2.0 Arrays, according to the manufacturer's instructions. Arrays were scanned in an Affymetrix GeneChip scanner 2500 and analysed first with Affymetrix MAS 5.0 software to obtain Absent/Present calls and to assure that all quality parameters were within the recommended range. Hybridization, scanning and detection procedures were done at the Genomics Unit of Instituto Gulbenkian de Ciência (Portugal). The advantage of using the Affymetrix GeneChip is that it contains 20 probe pairs to detect each ORF, providing multiple independent measurements for each transcript. The subsequent analysis was performed with dChip software program [11]. The invariant set method [12] was used to normalise arrays at the probe cell level for comparison purposes, and the model-base (Perfect Match-only model) was used to probe selection and computing expression values. The expression profile of 4306 genes encoding proteins of *E. coli* K-12 MG155 were analysed (intergenic regions were not included) and genes were considered to have significant differential expression when showing a transcriptional fold change >2 (with a 90 % confidence interval) and a *P* value < 0.05 for a paired Student's t-test. To minimise occurrence of false positives the comparison criterion was carefully chosen to ensure that the empirical false discovery rate (FDR) was low (random permuting of the samples by 200 times led to a median of FDR of 0 % and 1.1 % for the arrays acquired for cells grown anaerobically and aerobically, respectively).

Gene Ontology analysis

To investigate the biological relevance of the differential gene expression profile, the microarray data was analysed with the FatiGO algorithm (Al-Shahrour *et al.*, 2004; Al-Shahrour *et al.*, 2006). Briefly, FatiGO considers two lists of genes (in this case the genes up or down regulated by CORM-2 anaerobically or aerobically were compared with the unchanged genes) and converts them into two lists of Gene Ontology (GO) terms. Subsequently, FatiGO determines separately the percentage of the relative frequency of genes in each group that is annotated with a particular GO term (biological process or molecular function) and finally, a Fisher's exact test is used to determine if the GO term is significantly over- or under- represented in one of the groups with respect to the other one. Hence, it retrieves information about the predominant biological process or molecular function for the genes that are differently expressed in a certain experimental condition. In this study, the GO terms were considered differently represented when exhibited a FDR-adjusted *P* value lower than 0.2. The FatiGO is implemented in a web tool at: http://www.fatigo.org and within the Babelomics environment (http://www.babelomics.org).

Quantitative real-time RT-PCR

DNA microarray data was validated by quantitative real-time RT-PCR. To this end, 2.5 µg of *E. coli* total RNA, derived from two independent samples grown aerobically or anaerobically and treated with 250 µM of CORM-2 or with 500 µM of $Ru(II)Cl_2(DMSO)_4$ was used to synthesise cDNA, using the first strand synthesis protocol of the Universal RiboClone cDNA Synthesis System (Promega). Each cDNA synthesis reaction was performed using 40 µg/ml of random primers, 40 U of RNasin Ribonuclease Inhibitor, 4 mM of sodium pyrophosphate and 30 U of AMV Reverse Transcriptase. After amplification, cDNA was purified by phenol extraction and ethanol precipitation. Real-time PCRs were performed in a LightCycler Instrument using LightCycler FastStart DNA Master SYBER Green I Kit according to the manufacturer's instructions (Roche Applied Science). For each target gene, specific oligonucleotides were designed to amplify nucleotide fragments of 200 to

580 base pairs (Table 6.A3). The amplification reactions were carried out with equal amounts of cDNA (120 ng), as initial template, and each reaction contained 0.5 μM of primers, 2 mM of $MgCl_2$, and hot-start PCR reaction mix (Roche Applied Science). The expression ratio of the target gene was determined relatively to a reference gene, the *E. coli* glyceraldehyde 3-phosphate dehydrogenase (*gapA*), which did not show variation in the transcription abundance under all the conditions tested by microarray analysis or real-time RT-PCR experiments.

Viability assays and complementation studies

In order to compare the sensitivity to CORM-2 of the *E. coli* parent strain versus the several mutant strains, cell viability was determined by evaluation of the number of CFU per millilitre, after 7 h of growth in the presence or in the absence of CORM-2. The percentage survival was calculated as the number of colonies obtained from CORM-2 treated cultures divided by the number of colonies formed upon platting untreated cultures. The experiments were performed in duplicate with a minimum of two independent cultures of the mutant strain and of its correspondent parental strain (Table 6.A4) and the results are presented as averaged values with error bars representing standard errors. For complementation purposes, *ibpAB* and *metR* genes were amplified from *E. coli* genomic DNA using the appropriated oligonucleotides (Table 6.A3), and cloned into pUC-19 to generate plasmids p*ibpAB* and p*metR* (Table 6.A5). These plasmids or the empty vector were transformed into the correspondent mutant strain and the phenotype was analysed by plating on agar successive dilution of the cultures treated with CORM-2 or left untreated. Concerning Δ*soxS*, Δ*oxyR*, Δ*tqsA* and Δ*bhsA* the complementation experiments were performed using the plasmids described in Table 6.A5.

Biofilm assays

Biofilm formation was quantified by the crystal violet method [22, 23]. Briefly, overnight LB-grown cultures of wild type *E. coli*, Δ*tqsA* and Δ*bhsA* mutant strains were used to inoculate fresh medium to an OD_{600nm} of 0.05 and then incubated for

6 h, at 37 ºC. At this stage, 250 μM of CORM-2 was added and the plates were incubated for another 18 h, at 37º C. The total biofilm formed was stained with a solution of 0.1 % crystal violet for 30 min and quantified by measuring the OD at 540 nm. The relative biofilm formation was calculated as the total biofilm formed by the cultures treated with CORM-2 divided by the total biofilm obtained in untreated cultures; each data point represents the average of six replicated wells from four independent cultures.

6.3 Results and discussion

Analysis of the *E. coli* transcriptome of cells treated with CORM-2

Microarray technology was utilised to analyse the genome-wide transcriptional pattern of *E. coli* cells, grown aerobically and anaerobically, in the presence of CORM-2. The complete list of genes that exhibited a twofold, or higher, increase or decrease (with $P < 0.05$) of the transcriptional expression is presented in Tables 6.A1 (anaerobic growth) and 6.A2 (aerobic growth) in annexe section. In order to independently confirm the microarrays results, quantitative real-time PCR was carried out on ten selected genes that were found to be up-regulated or down-regulated by the action of CORM-2. The values acquired in the real-time PCR experiments are in agreement with the fold variation obtained in the microarrays (Table 6.1-2).

The microarray data acquired for cells grown under anaerobic conditions and treated with CORM-2 showed that 396 genes had their transcription altered (~ 9 % of the total genome). Of these, the transcription of 228 genes was repressed (~ 5 % of the transcriptome) with genes dispersed through nearly all functional categories, particularly the genes related to amino acid transport and metabolism (Figure 6.1A). The FatiGO analysis showed that the genes down-regulated are over-represented in the cellular metabolic processes (which correspond to 84 % of all repressed-genes), namely in the catabolic processes, nucleotide metabolisms, and energy production by oxidation of organic compounds (Figure 6.2A), indicating that CORM-2 induces metabolic adaptation.

TABLE 6.1 Quantitative real-time RT-PCR analysis performed in cells exposed to CORM-2 and to the CO free $Ru(II)Cl_2(DMSO)_4$ under anaerobic conditions.

	Anaerobic		
		Real-Time PCR	
Gene	Microarray	CORM-2	$Ru(II)Cl_2(DMSO)_4$
ibpB	40	427 ± 33	-2 ± 0
soxS	10	14 ± 0	-3 ± 0
marA	14	14 ± 0	2 ± 1
frmA	10	57 ± 1	-1 ± 0
lrhA	16	14 ± 5	-2 ± 0
yncJ	42	85 ± 8	12 ± 2
bhsA	26	33 ± 2	3 ± 2
metR	-	-	-
gadX	-10	-21 ± 0	2 ± 1
sucA	-9	-6 ± 0	1 ± 0

TABLE 6.2 Quantitative real-time RT-PCR analysis performed in cells exposed to CORM-2 and to the CO free $Ru(II)Cl_2(DMSO)_4$ under aerobic conditions.

	Aerobic		
		Real-Time PCR	
Gene	Microarray	CORM-2	Ru(II)Cl2(DMSO)4
ibpB	79	2798 ± 27	12 ± 8
soxS	15	268 ± 63	-3 ± 0
marA	10	56 ± 3	-2 ± 0
frmA	16	133 ± 0	-2 ± 0
lrhA	5	23 ± 8	-2 ± 0
yncJ	62	162 ± 49	7 ± 2
bhsA	-	-	-
metR	21	432 ± 85	4 ± 0
gadX	-4	-4 ± 0	-2 ± 0
sucA	-5	-4 ± 0	-1 ± 0

The largest class of genes that were up-regulated belongs to the category of the hypothetical proteins (~ 39 %). Apart from these, the genes that showed increased transcription are divided essentially into three classes: inorganic ion transport, posttranslational modification and transcription (Figure 6.1A). Further analysis revealed that the genes up-regulated by CORM-2 were significantly over-represented within the class of transcriptional regulation and protein folding (Figure 6.2B). Among the latter, two heat shock proteins that are directly connected with protein stability, *ibpA* and *ibpB*, were strongly induced (19 and 40-fold, respectively). The data also showed that, under anaerobic conditions, the addition of CORM-2 had effects on iron metabolism since several genes related to this function were altered, e.g. ferritin (*ftn*), and bacterioferritin (*bfr*) (Table 6.A1).

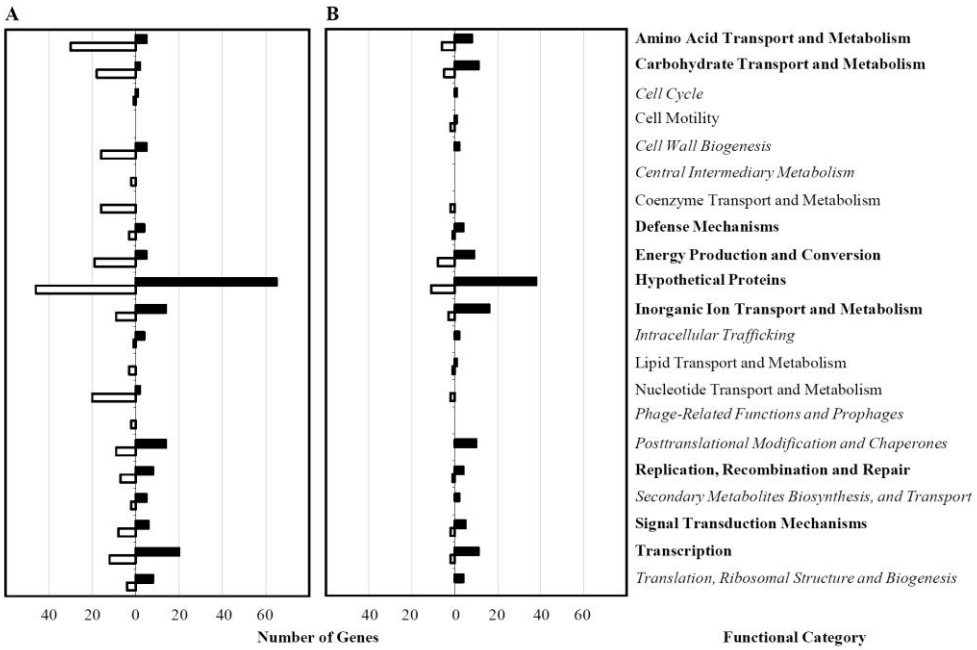

FIGURE 6.1 Effect of CORM-2 on *E. coli* transcriptome analysed according to the distribution of the genes with altered expression into functional categories. The differentially regulated genes in cells grown under anaerobic (A) and aerobic conditions (B) were divided into 21 functional categories according to NBCI and EcoCyc databases. The black and white bars represent the number of genes whose transcription was up- and down-regulated by CORM-2, respectively. In bold are highlighted the functional categories that include genes up- and down-regulated by CORM-2 in both oxygen growth conditions and in italics are represented the eight functional classes containing genes that were repressed by CORM-2 under anaerobic conditions and are absent in aerobically grown cells.

E. coli cells grown aerobically and exposed to CORM-2 displayed alteration in the transcription of 175 genes, ~ 4 % of the global gene expression profile, with 46 genes repressed and 129 genes induced. Apart from genes encoding hypothetical proteins, three functional categories exhibited a similar number of transcriptionally repressed genes, namely the classes of energy production, amino acid and carbohydrate transport and metabolism (Figure 6.1B). The FatiGO algorithm revealed over-representation of repressed genes in the coenzyme catabolic processes, tricarboxylic acid cycle (TCA) and aerobic respiration classes (Figure 6.2C). This trend would ultimately lead to the inhibition of the aerobic respiratory metabolism.

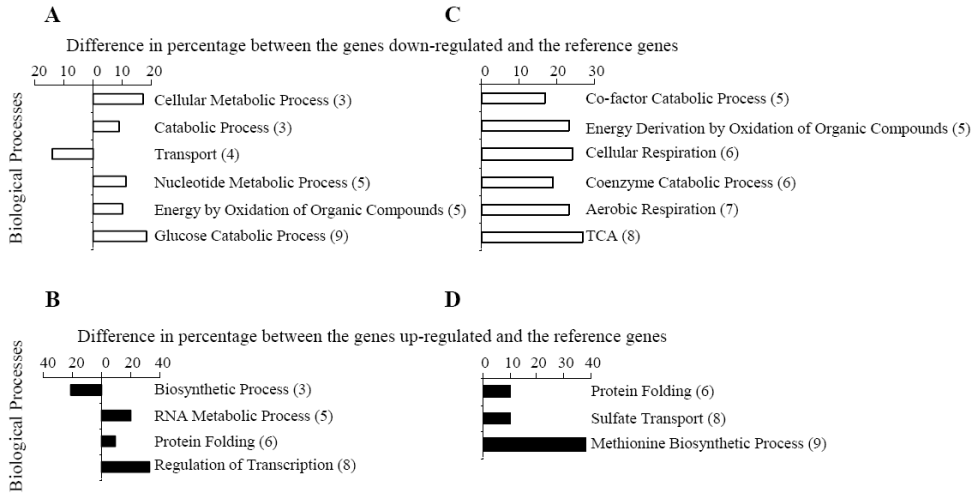

FIGURE 6.2 Biological processes modified by CORM-2. The genes down- and up-regulated by CORM-2 anaerobically (A and B) or aerobically (C and D) were compared with those unaltered by CORM-2 (reference genes), using the FatiGO algorithm (see Methods). The *y*-axis displays the biological processes that were considered differentially represented between the two groups compared in each case. The specificity of the biological process increases from the top to the bottom of the *y*-axis, as indicated in parenthesis. The *x*-axis represents the differences in percentages of the relative frequency of genes annotated for each biological process. The right and left part of the *x*-axis correspond to the biological processes over- and under-represented, in the down-regulated genes (A and C) and in up-regulated genes (B and D), respectively.

The genes whose expression was induced by CORM-2 were spread through several functional classes, containing the inorganic ion transport category the second highest number of genes transcriptionally modified (Figure 6.1B). Interestingly, the FatiGO analysis indicates that under aerobic conditions the addition of CORM-2 to *E. coli* cells led to a significant up-regulation of genes involved in sulphur metabolism, such as *tauABC*, *ssuAD*, *cysWA* and *sbp*, and in methionine metabolism, like the gene clusters *metNI* and *metBLF* (Figure 6.2D).

Transcriptional alterations caused by CORM-2 are dependent on CO release

Administration of any CO-RM produces some metabolite(s) along with the CO that is liberated. Therefore, we have determined whether the biological effect of a given CO-RM actually results from the liberated CO and not from any of its metabolites or the CO-RM's molecular skeleton itself. The commercially available tricarbonyldichlororuthenium (II) dimer, $[Ru(CO)_3Cl_2]_2$, also known as CORM-2 is, by far, the most tested CO-RM and we have reported its bactericidal activity [9]. The dimer complex is insoluble in aqueous media and its solubilisation, usually carried out in DMSO, entails a number of chemical transformations. According to Motterlini and co-workers [10], 23 min after dissolution three species are already formed, namely $RuCl_2(CO)_3(DMSO)$ and two isomers of $RuCl_2(CO)_2(DMSO)_2$. Of necessity, a certain amount of free CO is liberated into the DMSO solution and eventually lost to the atmosphere at an undetermined rate. More species appear upon standing or warming of the solution and further CO loss to give $RuCl_2(CO)(DMSO)_3$ and eventually $RuCl_2(DMSO)_4$ is to be expected since they are all synthetically interrelated in the presence of CO gas [27]. Nothing is presently known about the biological activity of any of these pure species in separate. However, the octahedral structure, the oxidation state as well as the ligands other than CO are retained along this series strongly supporting our choice of $Ru(II)Cl_2(DMSO)_4$ as a control for the bactericidal activity of the CO free $Ru(II)Cl_2(DMSO)_x$ fragment. It must also be mentioned that, regardless of the species present in the DMSO solution of $[Ru(CO)_3Cl_2]_2$, when an aliquot of this

solution is added to the cell culture medium no CO is released to the gas phase as previously reported [9]. We challenged *E. coli* with 500 μM of CO free $Ru(II)Cl_2(DMSO)_4$ and analysed the expression of several genes. Table 6.1-2 shows that no significant alteration in the transcription is observed when it is used a CO devoid compound, which allow to conclude that the main biological activity is due to the release of CO from CORM-2.

Genes transcriptionally regulated in response to CORM-2, independently of oxygen

The *E. coli* microarray data showed that upon exposure to CORM-2 the cells grown anaerobically exhibited a higher number of repressed genes than the cells grown aerobically. In particular, eight functional classes containing genes that were repressed by CORM-2 under anaerobic conditions were not detected in aerobically grown cells (Figure 6.1A-B). These results are in agreement with our previous observation that the decrease in cell viability caused by CO-RMs is higher for cells grown under anaerobic conditions [9]. However, a large set of genes were found in common between the two oxygen growth conditions and the fold of induction or repression generated by CORM-2 did not vary significantly between them (Table 6.3). There is also no example of a gene that was repressed under anoxic conditions and induced under aerobic conditions, and vice-versa. The major fold difference was observed for the heat shock protein *ibpB*, two hypothetical proteins (*yncJ* and *ymgG*), a flagellar repressor (*lrhA*) and an envelope stress induced periplasmic protein (*spy*). In all these cases, the genes showed a higher fold of CORM-2 dependent induction in aerobically grown cells than in cells cultured anaerobically (Table 6.3).

TABLE 6.3 Genes of *E. coli* differentially regulated by CORM-2 under both anaerobic and aerobic conditions.

Gene Name	Locus	Gene Product	Fold Change	
			Anaerobic	Aerobic
Inorganic ion transport and metabolism				
alx	b3088	Predicted inner membrane protein	12.3	3.3
yheL	b3343	Predicted intracellular sulfur oxidation protein	6.8	7.4
exuT	b3093	Hexuronate transporter	6.5	2.5
chaA*	b1216	Calcium/proton antiporter	6.0	8.4
yqjH	b3070	Predicted siderophore interacting protein	4.4	2.7
metN	b0199	D-methionine transport ATP-binding protein	4.3	9.9
metI	b0198	D-methionine transport system permease	4.0	6.5
pspE	b1308	Phage shock protein E precursor	2.4	7.0
yegH	b2063	Putative transport protein	-2.4	-2.5
apaG	b0050	Protein associated with Co^{2+}/Mg^{2+} efflux	-3.6	-2.4
Transcription				
lrhA	b2289	DNA-binding transcriptional repressor of flagellar	15.5	4.6
cpxP	b4484	Periplasmic protein combats stress	14.0	12.2
soxS	b4062	DNA-binding transcriptional dual regulator	10.0	14.5
frmR	b0357	Regulator protein that represses *frmRAB* operon	8.5	6.7
marA	b1531	Multiple antibiotic resistance protein	7.3	9.5
lexA	b4043	LexA repressor	6.8	3.8
zntR	b3292	Zinc-responsive transcriptional regulator	5.1	6.1
oxyR	b3961	Hydrogen peroxide-inducible genes activator	4.5	4.1
gadX	b3516	DNA-binding transcriptional dual regulator	-10.4	-4.3
Posttranslational modification, protein turnover, chaperones				
ibpB	b3686	16 kDa heat shock protein B	39.6	79.3
ibpA	b3687	16 kDa heat shock protein A	19.2	18.7
hspQ	b0966	DNA-binding protein, hemimethylated	7.7	4.8
yeeD	b2012	Predicted redox protein	7.4	3.3
yggG	b2936	Predicted peptidase	6.0	2.7
hslO	b3401	Hsp33-like chaperonin	4.6	5.4
dnaJ	b0015	Chaperone protein	3.7	4.1
clpB	b2592	Protein disaggregation chaperone	3.1	4.8
Signal transduction mechanisms				
ydeH	b1535	Hypothetical protein with DGC or GGDEF domain	17.5	23.5
pspB	b1305	Phage shock protein B	4.4	10.5

TABLE 6.3 Continuation.

Gene Name	Locus	Gene Product	Fold Change	
			Anaerobic	Aerobic
Signal transduction mechanisms (continuation)				
pspG	b4050	phage shock protein G	3.9	11.4
narP	b2193	Nitrate/nitrite response regulator protein	2.7	2.8
ydiV	b1707	C-di-GMP phosphodiesterase class I	-3.7	-5.9
Energy production and conversion				
frmA	b0356	Alcohol dehydrogenase class III	15.8	8.1
nemA	b1650	N-ethylmaleimide reductase	3.1	3.6
sucA	b0726	2-oxoglutarate decarboxylase	-9.1	-5.1
Translation, ribosomal structure and biogenesis				
rrmJ	b3179	Ribosomal RNA large subunit methyltransferase J	4.6	3.2
hslR	b3400	Ribosome-associated heat shock protein Hsp15	4.2	5.1
miaA	b4171	tRNA delta(2)-isopentenylpyrophosphate transferase	2.5	2.5
Amino acid transport and metabolism				
ybaT	b0486	Predicted transporter protein	-3.4	-3.7
gss	b2988	Fused glutathionylspermidine amidase	-3.5	-3.1
Carbohydrate transport and metabolism				
tktB	b2465	Transketolase	-2.5	-2.9
otsA	b1896	Alpha-trehalose-phosphate synthase	-4.9	-2.8
Replication, recombination and repair				
fimE	b4313	Tyrosine recombinase	5.6	3.9
recN	b2616	Recombination and repair protein	5.4	8.4
Defence mechanisms				
rdoA	b3859	Thr/Ser kinase implicated in Cpx stress response	2.8	3.5
aidB	b4187	Isovaleryl CoA dehydrogenase	-2.6	-2.7
Coenzyme transport and metabolism				
btuD	b1709	Vitamin B12-transporter ATPase	-3.1	-2.3

TABLE 6.3 Continuation.

Gene Name	Locus	Gene Product	Fold Change	
			Anaerobic	Aerobic
Cell wall/membrane/envelope biogenesis				
btuD	b1709	Vitamin B12-transporter ATPase	-3.1	-2.3
Secondary metabolites biosynthesis, transport and catabolism				
ybbA	b0495	Hypothetical ABC transporter	7.7	7.1
Intracellular trafficking, secretion, and vesicular transport				
spy	b1743	Envelope stress induced periplasmic protein	16.0	30.0
Cell cycle control, cell division, chromosome partitioning				
sulA	b0958	Cell division inhibitor	9.5	5.4
Hypothetical proteins				
yncJ	b1436	Hypothetical protein	42.2	62.0
yjfN	b4188	Hypothetical protein	19.8	22.9
ycfJ	b1110	Hypothetical protein	18.0	13.5
yebE	b1846	Hypothetical protein	16.3	22.9
ycfS	b1113	Hypothetical protein	12.0	16.9
yhdV	b3267	Hypothetical protein	9.0	9.0
yneM	b4599	Hypothetical protein	8.4	6.2
yqjA	b3095	Hypothetical protein	8.0	5.7
ypfG	b2466	Hypothetical protein	6.7	8.6
fxsA	b4140	FxsA protein	6.7	13.8
yaiY	b0379	Hypothetical protein	6.5	5.8
tqsA	b1601	Predicted permease	6.3	9.1
yeeE	b2013	Predicted inner membrane protein	6.3	2.7
ybeD	b0631	Hypothetical protein	6.1	6.0
yqaE	b2666	Hypothetical protein	5.9	4.5
ytfK	b4217	Hypothetical protein	5.3	5.3
ycjX	b1321	Hypothetical protein	5.0	7.7
ybfA	b0699	Hypothetical protein	4.5	4.5
bax	b3570	BAX protein	4.3	3.3
ymgG	b1172	Hypothetical protein	4.2	16.0
sraF	b4448	Unknown RNA	3.5	2.5
yrfG	b3399	Predicted hydrolase	3.4	4.3
ynfD	b1586	Hypothetical protein	3.4	4.5

TABLE 6.3 Continuation.

Gene Name	Locus	Gene Product	Fold Change	
			Anaerobic	Aerobic
Hypothetical proteins (continuation)				
yciS	b1279	Hypothetical protein	3.3	3.2
ycjF	b1322	Hypothetical protein	3.2	10.0
yhdN	b3293	Hypothetical protein	3.1	4.3
yciC	b1255	Hypothetical protein	2.6	2.3
slp	b3506	Outer membrane protein Slp precursor	-2.2	-4.1
yahK	b0325	Predicted oxidoreductase, Zn-dependent and NAD(P)-binding	-4.0	-3.6
yciX	b4523	Hypothetical protein	-4.6	-8.0

* strains mutated in the genes represented in bold were phenotipically analysed

Phenotypic analysis of *E. coli* regulators induced by CORM-2

To gain further insight into the function of the genes whose transcription was perturbed by treatment with CORM-2 we have analysed the phenotype of twelve *E. coli* mutant strains to test if the deleted gene has a role in the *E. coli* CORM-2 response. The genes were chosen on the basis of their highest fold of induction or repression and by exhibiting variation in transcript abundance both in cells grown under aerobic or anaerobic conditions (see Table 6.3). Hence, genes encoding regulators (*soxS*, *oxyR*, *zntR*, *metR*, and *gadX*), a putative combat stress protein (*cpxP*), heat shock proteins (*ibpA* and *ibpB*), proteins involved in biofilm formation (*ydgG* (*tqsA*), *ycfR* (*bhsA*)), an antiporter protein (*chaA*), and genes involved in the methionine metabolism (*metN*, *metI*) were studied. However, the study of the *E. coli* single mutant strains deleted in the genes *zntR*, *gadX* and *chaA* revealed that the strains did not display a CORM-2 growth induced delay when compared to the parent strain (data not shown).

Concerning *soxS*, whose transcription is controlled directly by SoxR, the gene showed a high fold of induction upon treatment with CORM-2, under aerobic and anaerobic conditions (Table 6.3). Together with SoxR, SoxS participates in the regulation of several genes involved in the response to oxidative stress [21]. However, with the exception of the *marRAB* operon, none of the other known

members of the SoxRS regulon was induced. A similar behaviour, which so far remains unexplained, was previously observed in other microarray experiments of *E. coli* grown in the presence of hydrogen peroxide and nitric oxide [28, 29]. The phenotypic study of the *E. coli* strain deficient in the *soxS* gene showed that the mutation leads to an increase of sensitivity to CORM-2 of the cells grown under aerobic (> 70 %) and anaerobic conditions (> 35 %) (Figure 6.3A-B).

The *oxyR* gene, whose encoded protein participates in the regulation of genes involved in the response to oxidative stress [28], was up-regulated by CORM-2 under aerobic and anaerobic conditions (≥ 4-fold) (Table 6.3). Under aerobic conditions and in the presence of CORM-2, the sensitivity of the *oxyR* mutant strain was found to be ~ 70 % higher than that of the wild type strain (Figure 6.3C), while no differences were observed for growth performed under anaerobic conditions.

The microarray data showed that in aerobically grown *E. coli* the transcription of *metR* was up-regulated by CORM-2. Analysis of the Δ*metR* strain revealed that this mutant display a sensitivity ~ 80 % higher than that of the parental strain for cells grown aerobically (Figure 6.3F), while no effect was detected in anoxic conditions (data not shown).

In all cases, the wild-type behaviour was restored upon expression of the genes from a plasmid containing SoxS, OxyR or MetR (Figure $6.3A_1$-C_1 and $6.3F_1$), showing that these transcription factors have an important role in the *E. coli* regulatory mechanisms triggered by exposure to CORM-2.

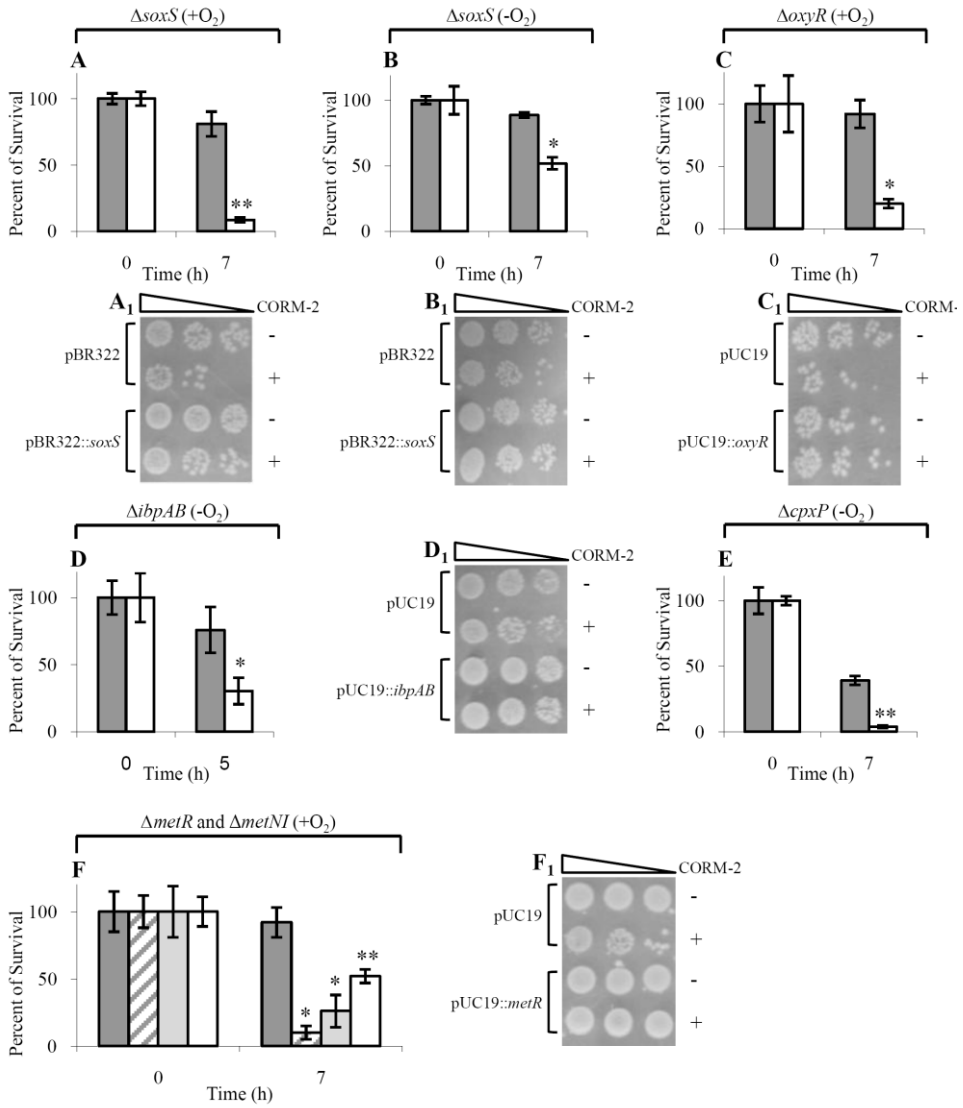

FIGURE 6.3 Analysis of the sensitivity of several *E. coli* mutant strains to CORM-2. Parental *E. coli* strain (gray bars), and mutant strains (white bars), were grown aerobically ($+O_2$; panels A, C and F) or anaerobically ($-O_2$; panels B, D and E) and exposed to 150 μM or 100 μM of CORM-2, respectively. The *E. coli ΔcpxP* mutant and its parental strain were treated with 125 μM of CORM-2 (panel E). In Panel F, the *ΔmetR* (striped bars), *ΔmetI* (light gray bars) and *ΔmetN* (white bars) mutant strains were grown aerobically and treated with 150 μM of CORM-2. The error bars represent the standard error of the average values obtained from at least two independent biological samples performed in duplicate (* P <0.01, ** P <0.05). Panels A_1-F_1: complementation analysis of Δ*soxS* (A_1-B_1), Δ*oxyR* (C_1), Δ*ibpAB* (D_1) and Δ*metR* (F_1) using the plasmids indicated in each panel.

Identification of other genes involved in the CORM-2 sensitivity of anaerobically grown *E. coli* cells

A significant variation of the expression of *ibpA* and *ibpB* genes was measured in aerobically and anaerobically grown cells of *E. coli* submitted to CORM-2 (Table 6.3). These genes encode two small heat shock proteins, IbpA and IbpB, that bind to protein aggregates and inclusion bodies formed during heterologous protein expression [30]. The two proteins are known to cooperate with ClpB and DnaK forming a functional triad of chaperones [17, 30]. Interestingly, the transcription of *clpB* and *dnaK* as well as that of genes coding for other chaperones like *dnaJ*, *grpE* and *htpG*, was also up-regulated anaerobically by CORM-2 (Table 6.A1). Actually, under anaerobic conditions, inactivation of the genes *ibpAB* led to an increase of the *E. coli* CORM-2 sensitivity of 45 % relative to the parental strain (Figure 6.3D), while no differences were observed under aerobic conditions (data not shown). As expected, complementation of *ibpAB* mutation induced the rescue of the wild type phenotype (Figure 6.3D_1).

The transcription of *E. coli cpxP*, which encodes a periplasmic protein putatively involved in combating extracytoplasmic protein-mediated toxicity [31], was up-regulated 12-14-fold in cells exposed to CORM-2 and cultured in both oxygen conditions. CpxP belongs to the *E. coli* Cpx system, which senses perturbations in the bacterial cell envelope and responds through the up-regulation of many gene products involved in protein folding and degradation [32]. In accordance, we verified that together with the induction of *cpxP* occurred a high number of transcriptionally modified genes involved in cell wall biogenesis and in protein folding, including heat shock proteins (*ibpAB*, *hslJ*, *htpX*), chaperones (*dnaJ*, *htpG*, *clpB*) and proteases (*ftsH*) (Table 6.A1), mainly under anaerobic conditions. These genes are under the control of *rpoH* factor (σ^{32}) [33], a gene that was also found to be up-regulated by CORM-2 under anaerobic conditions. The study of the growth behaviour of the Δ*cpxP* mutant revealed that this strain is approximately 35 % more sensitive to CORM-2 than the parental type but only under anaerobic conditions (Figure 6.3E), not under aerobic conditions (data not shown).

CORM-2 interferes with methionine biosynthesis

Addition of CORM-2 to aerobically grown cells of *E. coli* caused a marked increase in the transcription of several genes implicated in methionine biosynthesis and uptake, with *metR* being the gene that showed the highest induction (21-fold) (Table 6.A2). Apart from the genes presented in Table 6.A2, induction of the expression of genes *metA* (12.4-fold; *P*=0.0527) and *metE* (5.5-fold; *P*=0.0565) was also observed. To further clarify this issue, growth experiments were conducted aerobically in the presence of CORM-2 for the *E. coli* Δ*metR*, Δ*metI* and Δ*metN* mutant strains. All mutant strains are hypersensitive to CORM-2 (80 %, 66 %, and 40 %, higher, respectively) (Figure 6.3F). Furthermore, the complementation assay showed that *E. coli* Δ*metR* regains resistance to CO upon expression of the regulator MetR (Figure 6.3F_1). These findings suggest that, under aerobic conditions, CORM-2 affects the *E. coli* metabolism of methionine.

CORM-2 influences biofilm formation

Previous microarray studies revealed that apart from the *E. coli* genes that are directly implicated in the process of biofilm formation such as *tqsA*, *mqsR*, *bhsA*, *yceP* (*bssS*) and *yliH* (*bssR*), genes involved in the response to stress conditions are also induced during biofilm formation, namely *ibpAB*, *soxS*, *cpxP* and *spy* [34, 35]. Many of these genes were also found to be transcriptionally modified when *E. coli* is exposed to CORM-2 (Tables 6.3 and 6.A6). In particular, comparison of the microarray data showed that approximately 40 % of the genes that are found to be transcriptionally modified during the formation of the *E. coli* biofilm [34] are in common with those altered by CORM-2 under anaerobic conditions, and 12 % of the common genes are observed when growth is performed aerobically (Table 6.A6).

To evaluate the effect of CORM-2 in the process of biofilm formation, quantification of the total biofilm formed during aerobic growth in LB and in the presence of the compound was performed. The results showed that CORM-2 increased by 1.6-fold the total biofilm of the *E. coli* wild type strain (Figure 6.4A). This indicates that *E. coli* produces higher biofilm content as a defensive response

against CORM-2, as previously observed for other stress conditions [22]. The *E. coli* gene related with biofilm formation, *bhsA* [22], was significantly induced under anaerobic conditions (26-fold). Although the Δ*bhsA* mutant showed an elevated resistance to CORM-2 anaerobically (Figure 6.4B), and the complementation experiment performed with a multiple-copy clone containing the *bhsA* regulatory region rescued the wild type behaviour (Figure 6.4B_2), the values of total biofilm measured for the mutant strain exposed to CORM-2 matched those of the parental strain (Figure 6.4A).

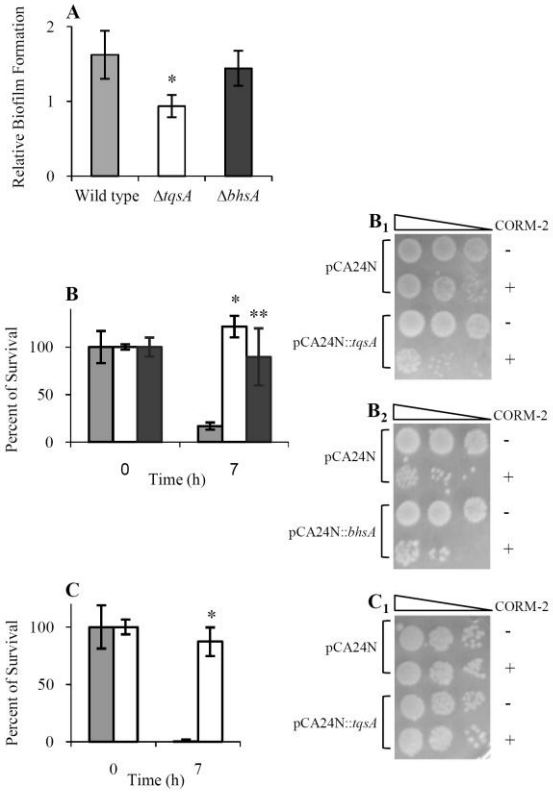

FIGURE 6.4 Effect of CORM-2 in biofilm formation and cell viability of *E. coli ΔtqsA* and *ΔbhsA* mutant strains. Biofilm was assayed in *E. coli* wild type and in *ΔtqsA* and *ΔbhsA* mutant strains, in the absence or in the presence of 250 μM of CORM-2. Cell viability of *E. coli* parental strain (gray bars), Δ*tqsA* (white bars) and Δ*bhsA* (black bars) mutants strains grown anaerobically (B) or aerobically (C) in the presence of 100 μM or 180 μM of CORM-2, respectively. The number of colonies (cfu) was determined for at least two biological samples and in duplicate. Error bars represent the standard error of average values (*P <0.01, ** P <0.05). Complementation analysis of *ΔtqsA* and *ΔbhsA* mutant strains (B_1 and B_2, anaerobic conditions), and *ΔtqsA* (C_1, aerobic conditions) using the plasmids indicated in each panel.

In *E. coli*, the *tqsA* gene is proposed to encode a putative transport protein involved in biofilm formation [19]. The gene was up-regulated in aerobically and anaerobically grown CORM-2 treated cells with a 9- and 6-fold increase, respectively. Analysis of the *E. coli* Δ*tqsA* mutant revealed that deletion of *tqsA* yielded a strain with higher resistance to CORM-2 than the parental strain, either

under oxic or anoxic growth conditions (Figure 6.4B-C), and the phenotype of the *tqsA* mutant strain could be fully complemented by a clone containing the *tqsA* gene (Figure $6.4B_1$-C_1). This result is in agreement with those obtained by Herzberg and co-workers, which show that inactivation of *tqsA* increases the resistance to several antibiotics [19]. Additionally, deletion of *tqsA* abolished the increase in biofilm formation observed in the wild type strain upon exposure to CORM-2 (Figure 6.4A).

6.4 Conclusion

In this study, we provide the first microarray analysis of a microorganism treated with the new bactericide CORM-2. The choice of *E. coli* was based on the fact that, besides being a model bacterium, this Gram-negative microorganism is able to live in aerobic environments and also adapts to anaerobic niches as part of its normal colonisation-transmission cycle within a host. Hence, it allowed us to compare the transcriptional response of a bacterium when treated with CORM-2 in both anaerobic and aerobic environments. The broad effect of the CORM-2 on gene expression levels is evident from the distribution of induced and repressed genes over the spectrum of all functional categories. In particular, the higher number of genes affected in anaerobically CORM-2-exposed cells illustrates fundamental differences between the way in which microbes control external stress under the two oxygen conditions, and highlights the importance of specific investigations to understand the different adaptation strategies.

Although it has been assumed that CO targets are haem containing enzymes or proteins, they were not yet identified and little is known about the trigger event or mechanism that mediates the transfer of CO from the CO-RM to the haem target. The present data shows that the action of CO extends further beyond the action on haem proteins since a wide range of transcriptional modifications is observed in cells grown under fermentative conditions and exposed, aerobically or anaerobically, to CORM-2.

The changes in the expression level of key transcription regulators together with the phenotypic analysis of the mutant strains reveals that CORM-2 triggers a

complex network of responses. Like all other microarray studies performed in *E. coli* cells submitted to toxic chemicals such as hydrogen peroxide and NO, exposure to CORM-2 increases the transcription of *soxS* regulator but it does not induce any of the genes of the regulon, a result that requires further studies to be clarified. A particular observation, common to all transcriptional studies, is the large representation of "unknown" genes, which leaves a wide field to be still explored, until the bacterial physiology is fully understood and indeed ready for the application of a systems biology approach. Nevertheless, the data provided by this study will certainly be valuable for guiding future research on the pharmacological application of CO-RMs.

6.5 References

[1] Li, C., Hossieny, P., Wu, B.J., Qawasmeh, A., Beck, K. and Stocker, R. (2007) Pharmacologic induction of haem oxygenase-1. Antioxid. Redox Signal 9, 2227-2239.

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

[3] Otterbein, L.E. and Choi, A.M. (2000) Haem oxygenase: colors of defense against cellular stress. Am. J. Physiol. Lung Cell Mol. Physiol. 279, L1029-1037.

[4] Stupfel, M. and Bouley, G. (1970) Physiological and biochemical effects on rats and mice exposed to small concentrations of carbon monoxide for long periods. Ann. N. Y. Acad. Sci. 174, 342-368.

[5] Verma, A., Hirsch, D.J., Glatt, C.E., Ronnett, G.V. and Snyder, S.H. (1993) Carbon monoxide: a putative neural messenger. Science 259, 381-384.

[6] Ryter, S.W., Alam, J. and Choi, A.M. (2006) Haem oxygenase-1/carbon monoxide: from basic science to therapeutic applications. Physiol. Rev. 86, 583-650.

[7] Dulak, J., Jozkowicz, A., Foresti, R., Kasza, A., Frick, M., Huk, I., Green, C.J., Pachinger, O., Weidinger, F. and Motterlini, R. (2002) Haem oxygenase activity modulates vascular endothelial growth factor synthesis in vascular smooth muscle cells. Antioxid. Redox. Signal 4, 229-240.

[8] Motterlini, R., Mann, B.E. and Foresti, R. (2005) Therapeutic applications of carbon monoxide-releasing molecules. Expert. Opin. Investig. Drugs 14, 1305-1318.

[9] Nobre, L.S., Seixas, J.D., Romao, C.C. and Saraiva, L.M. (2007) Antimicrobial action of carbon monoxide-releasing compounds. Antimicrob. Agents Chemother. 51, 4303-4307.

[10] Motterlini, R., Clark, J.E., Foresti, R., Sarathchandra, P., Mann, B.E. and Green, C.J. (2002) Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. Circ. Res. 90, E17-24.

[11] Li, C. and Wong, W.H. (2001) Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection. Proc. Natl. Acad. Sci. U S A 98, 31-36.

[12] Li, C. and Hung Wong, W. (2001) Model-based analysis of oligonucleotide arrays: model validation, design issues and standard error application. Genome Biol. 2, RESEARCH0032.

[13] Flatley, J., Barrett, J., Pullan, S.T., Hughes, M.N., Green, J. and Poole, R.K. (2005) Transcriptional responses of *Escherichia coli* to S-nitrosoglutathione under defined chemostat conditions reveal major changes in methionine biosynthesis. J. Biol. Chem. 280, 10065-10072.

[14] Weinstein-Fischer, D., Elgrably-Weiss, M. and Altuvia, S. (2000) *Escherichia coli* response to hydrogen peroxide: a role for DNA supercoiling, topoisomerase I and Fis. Mol. Microbiol. 35, 1413-1420.

[15] Carlioz, A. and Touati, D. (1986) Isolation of superoxide dismutase mutants in *Escherichia coli*: is superoxide dismutase necessary for aerobic life? Embo J. 5, 623-630.

[16] Casadaban, M.J. (1976) Transposition and fusion of the lac genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. J. Mol. Biol. 104, 541-555.

[17] Thomas, J.G. and Baneyx, F. (1998) Roles of the *Escherichia coli* small heat shock proteins IbpA and IbpB in thermal stress management: comparison with ClpA, ClpB, and HtpG *in vivo*. J. Bacteriol. 180, 5165-5172.

[18] DiGiuseppe, P.A. and Silhavy, T.J. (2003) Signal detection and target gene induction by the CpxRA two-component system. J. Bacteriol. 185, 2432-2440.

[19] Herzberg, M., Kaye, I.K., Peti, W. and Wood, T.K. (2006) YdgG (TqsA) controls biofilm formation in *Escherichia coli* K-12 through autoinducer 2 transport. J. Bacteriol. 188, 587-598.

[20] Christman, M.F., Storz, G. and Ames, B.N. (1989) OxyR, a positive regulator of hydrogen peroxide-inducible genes in *Escherichia coli* and *Salmonella typhimurium*, is homologous to a family of bacterial regulatory proteins. Proc. Natl. Acad. Sci. U S A 86, 3484-3488.

[21] Wu, J. and Weiss, B. (1992) Two-stage induction of the *soxRS* (superoxide response) regulon of *Escherichia coli*. J. Bacteriol. 174, 3915-3920.

[22] Zhang, X.S., Garcia-Contreras, R. and Wood, T.K. (2007) YcfR (BhsA) influences *Escherichia coli* biofilm formation through stress response and surface hydrophobicity. J. Bacteriol. 189, 3051-3062.

[23] Ren, D., Zuo, R., Gonzalez Barrios, A.F., Bedzyk, L.A., Eldridge, G.R., Pasmore, M.E. and Wood, T.K. (2005) Differential gene expression for investigation of *Escherichia coli* biofilm inhibition by plant extract ursolic acid. Appl. Environ. Microbiol. 71, 4022-4034.

[24] Al-Shahrour, F., Diaz-Uriarte, R. and Dopazo, J. (2004) FatiGO: a web tool for finding significant associations of Gene Ontology terms with groups of genes. Bioinformatics 20, 578-580.

[25] Al-Shahrour, F., Minguez, P., Tarraga, J., Montaner, D., Alloza, E., Vaquerizas, J.M., Conde, L., Blaschke, C., Vera, J. and Dopazo, J. (2006) BABELOMICS: a systems biology perspective in the functional annotation of genome-scale experiments. Nucleic Acids Res. 34, W472-476.

[26] Al-Shahrour, F., Minguez, P., Tarraga, J., Medina, I., Alloza, E., Montaner, D. and Dopazo, J. (2007) FatiGO +: a functional profiling tool for genomic data. Integration of functional annotation, regulatory motifs and interaction data with microarray experiments. Nucleic Acids Res. 35, W91-96.

[27] Alessio, E., Milani, B., Bolle, M., Mestroni, G., Faleschini P., Todone, F., Geremia S. and Calligaris, M. (1995) Carbonyl Derivatives of Chloride-Dimethyl Sulfoxide-Ruthenium(II) Complexes: Synthesis,

Structural Characterization, and Reactivity of Ru(CO)x(DMSO)$_4$-xCl2 Complexes (x = 1-3). Inorganic Chemistry 34 (19), 4722-4734.

[28] Zheng, M., Wang, X., Templeton, L.J., Smulski, D.R., LaRossa, R.A. and Storz, G. (2001) DNA microarray-mediated transcriptional profiling of the *Escherichia coli* response to hydrogen peroxide. J. Bacteriol. 183, 4562-4570.

[29] Justino, M.C., Vicente, J.B., Teixeira, M. and Saraiva, L.M. (2005) New genes implicated in the protection of anaerobically grown *Escherichia coli* against nitric oxide. J. Biol. Chem. 280, 2636-2643.

[30] Lethanh, H., Neubauer, P. and Hoffmann, F. (2005) The small heat-shock proteins IbpA and IbpB reduce the stress load of recombinant *Escherichia coli* and delay degradation of inclusion bodies. Microb. Cell Fact 4, 6.

[31] Danese, P.N. and Silhavy, T.J. (1998) CpxP, a stress-combative member of the Cpx regulon. J. Bacteriol. 180, 831-839.

[32] Fleischer, R., Heermann, R., Jung, K. and Hunke, S. (2007) Purification, reconstitution, and characterization of the CpxRAP envelope stress system of *Escherichia coli*. J. Biol. Chem. 282, 8583-8593.

[33] Zhao, K., Liu, M. and Burgess, R.R. (2005) The global transcriptional response of *Escherichia coli* to induced sigma 32 protein involves sigma 32 regulon activation followed by inactivation and degradation of sigma 32 in vivo. J. Biol. Chem. 280, 17758-17768.

[34] Ren, D., Bedzyk, L.A., Thomas, S.M., Ye, R.W. and Wood, T.K. (2004) Gene expression in *Escherichia coli* biofilms. Appl. Microbiol. Biotechnol. 64, 515-524.

[35] Beloin, C., Valle, J., Latour-Lambert, P., Faure, P., Kzreminski, M., Balestrino, D., Haagensen, J.A., Molin, S., Prensier, G., Arbeille, B. and Ghigo, J.M. (2004) Global impact of mature biofilm lifestyle on *Escherichia coli* K-12 gene expression. Mol. Microbiol. 51, 659-674.

Annexes

TABLE 6.A1 *E. coli* genes differentially expressed following anaerobic exposure to CORM-2.

Gene Name	Locus	Fold Change	*P* value	Gene Product
Amino acid transport and metabolism				
glnQ	b0809	8.0	0.0350	Glutamine ABC transporter ATP-binding protein
glnP	b0810	6.4	0.0179	Glutamine ABC transporter permease protein
cycA	b4208	6.4	0.0024	D-alanine/D-serine/glycine transporter
glnH	b0811	3.6	0.0172	Glutamine ABC transporter periplasmic-binding protein
aspA	b4139	3.5	0.0167	Aspartate ammonia-lyase
pepD	b0237	-2.4	0.0029	Aminoacyl-histidine dipeptidase (peptidase D)
dcp	b1538	-2.4	0.0029	Dipeptidyl carboxypeptidase II
hisB	b2022	-2.4	0.0101	Imidazole glycerol-phosphate dehydratase
potC	b1124	-2.5	0.0462	Polyamine transporter subunit
hisH	b2023	-2.5	0.0034	Imidazole glycerol phosphate synthase subunit HisH
hisF	b2025	-2.6	0.0407	Imidazole glycerol phosphate synthase subunit HisF
aroB	b3389	-2.6	0.0030	3-dehydroquinate synthase
gadC	b1492	-2.6	0.0088	Predicted glutamate:gamma-aminobutyric acid antiporter
artI	b0863	-2.6	0.0055	Arginine-binding periplasmic protein 1 precursor
gcvP	b2903	-2.8	0.0025	Glycine dehydrogenase; glycine decarboxylase
aspC	b0928	-2.9	0.0036	Aspartate aminotransferase PLP-dependent
proC	b0386	-2.9	0.0368	Pyrroline-5-carboxylate reductase, NAD(P)-binding
leuB	b0073	-3.0	0.0352	3-isopropylmalate dehydrogenase
speD	b0120	-3.0	0.0046	S-adenosylmethionine decarboxylase proenzyme
solA	b1059	-3.0	0.0147	N-methyltryptophan oxidase, FAD-binding
gadB	b1493	-3.3	0.0113	Glutamate decarboxylase B, PLP-dependent
artJ	b0860	-3.3	0.0240	Arginine transporter subunit
ybaT	b0486	-3.4	0.0323	Predicted transporter
gss	b2988	-3.5	0.0035	Fused glutathionylspermidine amidase/ synthetase
gcvH	b2904	-3.5	0.0032	Glycine cleavage system protein
ansA	b1767	-3.5	0.0113	Cytoplasmic asparaginase I
artP	b0864	-3.6	0.0141	Arginine transport ATP-binding protein
dapB	b0031	-3.8	0.0039	Dihydrodipicolinate reductase
ybaS	b0485	-3.8	0.0015	Glutaminase
gcvT	b2905	-4.1	0.0037	Glycine cleavage system aminomethyltransferase
tppB	b1634	-4.4	0.0009	Dipeptide/tripeptide permease
pepN	b0932	-4.6	0.0016	Aminopeptidase N
aroG	b0754	-4.6	0.0043	3-deoxy-D-arabino-heptulosonate-7-phosphate synthase
gadA	b3517	-8.0	0.0027	Glutamate decarboxylase A, PLP-dependent

TABLE 6.A1 Continuation.

Gene Name	Locus	Fold Change	*P* value	Gene Product
Amino acid transport and metabolism (continuation)				
dadA	b1189	-9.3	0.0483	D-amino acid dehydrogenase small subunit
Transcription				
ygaV	b2667	30.4	0.0041	Predicted DNA-binding transcriptional regulator
lrhA	b2289	15.5	0.0057	DNA-binding transcriptional repressor of flagellar, motility and chemotaxis genes
cpxP	b4484	14.0	0.0088	Periplasmic protein combats stress
marR	b1530	13.8	0.0003	Multiple antibiotic resistance protein
bssS	b1060	13.0	0.0002	Biofilm formation regulatory protein
soxS	b4062	10.0	0.0036	Regulatory protein SoxS
yqjI	b3071	9.4	0.0052	Predicted transcriptional regulator
frmR	b0357	8.5	0.0020	Regulator protein that represses *frmRAB* operon
marA	b1531	7.3	0.0005	Multiple antibiotic resistance protein
ydhM	b1649	6.9	0.0044	Predicted DNA-binding transcriptional regulator
lexA	b4043	6.8	0.0076	LexA repressor
ycfQ	b1111	6.5	0.0019	Predicted DNA-binding transcriptional regulator
rpoH	b3461	6.1	0.0482	RNA polymerase sigma factor
zntR	b3292	5.1	0.0044	DNA-binding transcriptional activator in response to Zn(II)
oxyR	b3961	4.5	0.0013	Hydrogen peroxide-inducible genes activator
exuR	b3094	4.0	0.0194	DNA-binding transcriptional repressor
greA	b3181	3.4	0.0017	Transcription elongation factor
yhaJ	b3105	3.1	0.0024	Predicted DNA-binding transcriptional regulator
cspA	b3556	2.8	0.0127	Major cold shock protein
hdfR	b4480	2.3	0.0289	DNA-binding transcriptional regulator
appY	b0564	-2.4	0.0040	DLP12 prophage
chaB	b1217	-2.7	0.0427	Cation transport regulator
yheO	b3346	-2.8	0.0043	Predicted DNA-binding transcriptional regulator
cspC	b1823	-2.8	0.0057	Cold shock-like protein
gadE	b3512	-3.2	0.0169	DNA-binding transcriptional activator
yiiD	b3888	-3.4	0.0108	Predicted acetyltransferase
rnb	b1286	-3.4	0.0018	Ribonuclease II
yhgF	b3407	-4.2	0.0434	Predicted transcriptional accessory protein
csgD	b1040	-4.4	0.0299	DNA-binding transcriptional activator
putA	b1014	-5.0	0.0057	Fused DNA-binding transcriptional regulator
gadW	b3515	-6.1	0.0358	DNA-binding transcriptional activator
gadX	b3516	-10.4	0.0075	DNA-binding transcriptional dual regulator

TABLE 6.A1 Continuation.

Gene Name	Locus	Fold Change	*P* value	Gene Product
Energy production and conversion				
frmA	b0356	15.8	0.0215	Alcohol dehydrogenase class III
ybdH	b0599	3.7	0.0358	Hypothetical oxidoreductase
yeaX	b1803	3.1	0.0362	Predicted oxidoreductase
nemA	b1650	3.1	0.0262	N-ethylmaleimide reductase, FMN-linked
rsxB	b1628	2.9	0.0238	Predicted iron-sulfur protein
sdhB	b0724	-2.3	0.0049	Succinate dehydrogenase
pntA	b1603	-2.4	0.0076	NAD(P) transhydrogenase, subunit alpha
nuoC	b2286	-2.4	0.0143	NADH:ubiquinone oxidoreductase
ppa	b4226	-2.4	0.0087	Inorganic pyrophosphatase
aceE	b0114	-2.5	0.0177	Pyruvate dehydrogenase subunit E1
gpsA	b3608	-2.6	0.0024	NAD(P)H-dependent glycerol-3-phosphate dehydrogenase
ydfG	b1539	-2.6	0.0124	L-allo-threonine dehydrogenase, NAD(P)-binding
aceA	b4015	-2.7	0.0133	Isocitrate lyase
ackA	b2296	-2.7	0.0193	Acetate kinase
aceB	b4014	-2.7	0.0132	Malate synthase
nuoA	b2288	-2.9	0.0024	NADH dehydrogenase subunit A
hyaB	b0973	-3.0	0.0040	Hydrogenase 1, large subunit
nuoB	b2287	-3.1	0.0040	NADH dehydrogenase subunit B
ydiJ	b1687	-3.6	0.0017	Predicted FAD-linked oxidoreductase
fpr	b3924	-3.6	0.0182	Ferredoxin-NADP reductase
ppc	b3956	-3.7	0.0366	Phosphoenolpyruvate carboxylase
cybB	b1418	-3.8	0.0009	Cytochrome b_{561}
hyaA	b0972	-5.0	0.0030	Hydrogenase 1
sucA	b0726	-9.1	0.0049	2-oxoglutarate decarboxylase
Posttranslational modification, protein turnover, chaperones				
ibpB	b3686	39.6	0.0018	16 kDa heat shock protein B
ibpA	b3687	19.2	0.0009	16 kDa heat shock protein A
htpX	b1829	8.6	0.0018	Heat shock protein HtpX
hspQ	b0966	7.7	0.0176	DNA-binding protein, hemimethylated
yeeD	b2012	7.4	0.0179	Predicted redox protein
hslJ	b1379	6.1	0.0033	Heat shock protein
yggG	b2936	6.0	0.0015	Predicted peptidase
hslO	b3401	4.6	0.0029	Hsp33-like chaperonin
ftsH	b3178	4.0	0.0063	Protease, ATP-dependent zinc-metallo
ybbK	b0489	3.8	0.0231	Predicted protease

TABLE 6.A1 Continuation.

Gene Name	Locus	Fold Change	*P* value	Gene Product
Posttranslational modification, protein turnover, chaperones (continuation)				
dnaJ	b0015	3.7	0.0038	Chaperone Hsp40, co-chaperone with DnaK.
clpB	b2592	3.1	0.0019	Protein disaggregation chaperone
htpG	b0473	2.5	0.0359	Heat shock protein 90
hflC	b4175	2.4	0.0164	Modulator for HflB protease specific for phage λ cII repressor
glnE	b3053	-2.3	0.0064	Glutamate-ammonia-ligase adenylyltransferase
sppA	b1766	-2.5	0.0015	Protease IV (signal peptide peptidase)
hchA	b1967	-2.6	0.0177	Hsp31 molecular chaperone
tpx	b1324	-3.3	0.0108	Lipid hydroperoxide peroxidase
grxB	b1064	-3.4	0.0228	Glutaredoxin 2
trxB	b0888	-4.1	0.0453	Thioredoxin reductase, FAD/NAD(P)-binding
cbpA	b1000	-4.3	0.0496	Curved DNA-binding protein, DnaJ homologue
cbpM	b0999	-4.3	0.0103	Modulator of CbpA co-chaperone
msrB	b1778	-4.7	0.0057	Methionine sulfoxide reductase B
Inorganic ion transport and metabolism				
mgtA	b4242	57.0	0.0112	Magnesium transporter
ygaP	b2668	17.7	0.0017	Predicted inner membrane protein
alx	b3088	12.3	0.0214	Predicted inner membrane protein, part of terminus
yheL	b3343	6.8	0.0010	Predicted intracellular sulfur oxidation protein
exuT	b3093	6.5	0.0433	Hexuronate transporter
chaA	b1216	6.0	0.0031	Calcium/sodium:proton antiporter
yqjH	b3070	4.4	0.0420	Predicted siderophore interacting protein
metN	b0199	4.3	0.0426	D-methionine transport ATP-binding protein
ftnB	b1902	4.3	0.0038	Ferritin-like protein
metI	b0198	4.0	0.0071	D-methionine transport system permease protein
hcaE	b2538	3.8	0.0261	3-phenylpropionate dioxygenase, large (alpha) subunit
feoA	b3408	2.8	0.0361	Ferrous iron transport protein A
pspE	b1308	2.4	0.0097	Phage shock protein E
corA	b3816	2.4	0.0041	Magnesium/nickel/cobalt transporter
yegH	b2063	-2.4	0.0301	Putative transport protein
yheN	b3345	-2.5	0.0130	Predicted intracellular sulfur oxidation protein
ppx	b2502	-2.8	0.0483	Exopolyphosphatase
mdoG	b1048	-2.9	0.0054	Glucan biosynthesis protein
bcp	b2480	-3.4	0.0092	bacterioferritin comigratory protein
ftnA	b1905	-3.5	0.0042	Ferritin iron storage protein
bfr	b3336	-3.5	0.0023	Bacterioferritin

TABLE 6.A1 Continuation.

Gene Name	Locus	Fold Change	*P* value	Gene Product
Inorganic ion transport and metabolism (continuation)				
apaG	b0050	-3.6	0.0103	Uncharacterized protein associated with Co^{2+}/Mg^{2+} efflux
can	b0126	-5.3	0.0100	Carbonic anhydrase.
Nucleotide transport and metabolism				
gmk	b3648	2.9	0.0087	Guanylate kinase
cmk	b0910	2.6	0.0043	Cytidylate kinase
apt	b0469	-2.3	0.0041	Adenine phosphoribosyltransferase
thyA	b2827	-2.5	0.0065	Thymidylate synthase
pyrG	b2780	-2.6	0.0035	CTP synthetase
deoC	b4381	-2.7	0.0129	2-deoxyribose-5-phosphate aldolase
purE	b0523	-2.8	0.0435	Phosphoribosylaminoimidazole carboxylase
purD	b4005	-2.8	0.0097	Phosphoribosylglycinamide synthetase
purH	b4006	-2.9	0.0428	Cyclohydrolase
purK	b0522	-3.1	0.0368	Phosphoribosylaminoimidazole carboxylase
guaB	b2508	-3.2	0.0019	Inositol-5-monophosphate dehydrogenase
purB	b1131	-3.3	0.0142	Adenylosuccinate lyase
amn	b1982	-3.8	0.0413	AMP nucleosidase
carB	b0033	-4.3	0.0105	Carbamoyl-phosphate synthase large subunit
purU	b1232	-4.4	0.0357	Formyltetrahydrofolate deformylase
ndk	b2518	-4.6	0.0038	Nucleoside diphosphate kinase
pyrC	b1062	-4.6	0.0249	Dihydroorotase
prsA	b1207	-4.9	0.0235	Ribose-phosphate pyrophosphokinase
hinT	b1103	-5.0	0.0181	Purine nucleoside phosphoramidase
carA	b0032	-8.3	0.0088	Carbamoyl-phosphate synthase small subunit
codA	b0337	-8.3	0.0265	Cytosine deaminase
codB	b0336	-9.7	0.0310	Cytosine transporter
Cell wall/membrane/envelope biogenesis				
mltD	b0211	3.7	0.0034	Membrane-bound lytic murein transglycosylase D precursor
yiaD	b3552	3.0	0.0249	Predicted outer membrane lipoprotien
lpxC	b0096	2.5	0.0040	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase
amiC	b2817	2.4	0.0082	N-acetylmuramoyl-L-alanine amidase
tolA	b0739	2.3	0.0258	Membrane anchored protein in TolA-TolQ-TolR complex

TABLE 6.A1 Continuation.

Gene Name	Locus	Fold Change	*P* value	Gene Product
Cell wall/membrane/envelope biogenesis (continuation)				
blc	b4149	-2.3	0.0036	Outer membrane lipoprotein
yncA	b1448	-2.3	0.0038	Predicted acyltransferase with acyl-CoA N-acyltransferase domain
mdoH	b1049	-2.6	0.0031	Glucan biosynthesis: glycosyl transferase.
osmF	b2131	-2.8	0.0181	Predicted transporter subunit
rfaL	b3622	-3.0	0.0311	O-antigen ligase
rfaF	b3620	-3.2	0.0216	ADP-heptose--LPS heptosyltransferase II
galE	b0759	-3.2	0.0072	UDP-galactose-4-epimerase
lolC	b1116	-3.3	0.0110	Outer membrane-specific lipoprotein transporter
imp	b0054	-3.3	0.0105	Exported protein required for envelope biosynthesis and integrity
lolB	b1209	-3.9	0.0308	Chaperone for lipoproteins
murF	b0086	-4.1	0.0033	UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate--D-alanyl-D-alanyl ligase
yfdI	b2352	-4.8	0.0056	CPS-53 (KpLE1) prophage; predicted inner membrane protein
murE	b0085	-5.0	0.0013	UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-diaminopimelate ligase
mdtE	b3513	-5.4	0.0161	Multidrug resistance efflux transporter
yfdH	b2351	-6.2	0.0025	CPS-53 (KpLE1) prophage
cfa	b1661	-8.3	0.0295	Cyclopropane fatty acyl phospholipid synthase
Carbohydrate transport and metabolism				
yhbE	b3184	6.8	0.0062	Hypothetical transport protein
yicI	b3656	3.4	0.0350	Predicted alpha-glucosidase
nadK	b2615	-2.3	0.0063	Inorganic polyphosphate/ATP-NAD kinase
ybjS	b0868	-2.4	0.0211	Predicted NAD(P)H-binding oxidoreductase
gnd	b2029	-2.5	0.0222	6-phosphogluconate dehydrogenase
pgk	b2926	-2.5	0.0036	Phosphoglycerate kinase
tktB	b2465	-2.5	0.0102	Transketolase 2, thiamin-binding
rpe	b3386	-2.5	0.0133	Ribulose-phosphate 3-epimerase
pgi	b4025	-2.6	0.0046	Glucose-6-phosphate isomerase
treA	b1197	-2.7	0.0052	Periplasmic trehalase
ptsN	b3204	-2.7	0.0083	Sugar-specific enzyme IIA component of PTS
tpiA	b3919	-2.8	0.0188	Triosephosphate isomerase
pykF	b1676	-2.9	0.0384	Pyruvate kinase I
nagB	b0678	-3.2	0.0300	Glucosamine-6-phosphate deaminase

TABLE 6.A1 Continuation.

Gene Name	Locus	Fold Change	*P* value	Gene Product
Carbohydrate transport and metabolism (continuation)				
amyA	b1927	-3.2	0.0018	Cytoplasmic alpha-amylase
pykA	b1854	-3.5	0.0428	Pyruvate kinase
talA	b2464	-3.7	0.0007	Transaldolase A
ybhC	b0772	-4.0	0.0165	Predicted pectinesterase
pgl	b0767	-4.3	0.0151	6-phosphogluconolactonase
otsA	b1896	-4.9	0.0479	Trehalose-6-phosphate synthase
Coenzyme transport and metabolism				
moaA	b0781	-2.3	0.0042	Molybdenum cofactor biosynthesis protein A.
sufB	b1683	-2.3	0.0034	SufB protein; component of SufBCD complex
pdxA	b0052	-2.4	0.0251	4-hydroxythreonine-4-phosphate dehydrogenase
sufC	b1682	-2.5	0.0157	cysteine desulfurase ATPase component
pta	b2297	-2.7	0.0162	Phosphate acetyltransferase
folD	b0529	-2.7	0.0079	5,10-methylene-tetrahydrofolate dehydrogenase
sufD	b1681	-2.8	0.0096	Cysteine desulfurase activator
pdxY	b1636	-3.0	0.0386	Pyridoxine kinase
btuE	b1710	-3.1	0.0014	Vitamin B12 transport periplasmic protein
btuD	b1709	-3.1	0.0034	Vitamin B12-transporter ATPase
sufA	b1684	-3.5	0.0226	Iron-sulfur (Fe-S) cluster assembly scaffold protein
poxB	b0871	-3.7	0.0084	Pyruvate dehydrogenase
lipA	b0628	-3.9	0.0444	Lipoyl synthase
pdxH	b1638	-4.3	0.0020	Pyridoxamine 5'-phosphate oxidase
bioB	b0775	-4.4	0.0100	Biotin synthase
serC	b0907	-6.5	0.0264	Phosphoserine aminotransferase
Replication, recombination and repair				
fimE	b4313	5.6	0.0467	Tyrosine recombinase
recN	b2616	5.4	0.0137	Recombination and repair protein
dinJ	b0226	4.8	0.0007	Predicted antitoxin of YafQ-DinJ
insL	b0016	3.7	0.0203	Transposase
dnaG	b3066	3.0	0.0200	DNA primase
priC	b0467	2.6	0.0212	Primosomal replication protein N
dnaA	b3702	2.6	0.0171	Chromosomal replication initiator protein
nudE	b3397	2.6	0.0072	ADP-ribose diphosphatase
nudJ	b1134	-2.3	0.0228	Thiamin pyrophosphate hydrolase
nei	b0714	-2.4	0.0106	Endonuclease VIII

TABLE 6.A1 Continuation.

Gene Name	Locus	Fold Change	*P* value	Gene Product
Replication, recombination and repair (continuation)				
nudC	b3996	-2.5	0.0476	NADH pyrophosphatase
nfi	b3998	-2.5	0.0078	Endonuclease V
xthA	b1749	-3.1	0.0129	Exonuclease III
holA	b0640	-3.1	0.0051	DNA polymerase III subunit delta
uvrC	b1913	-3.3	0.0242	Excinuclease ABC subunit C
Signal transduction mechanisms				
ydeH	b1535	17.5	0.0002	Hypothetical protein with Diguanylate-cyclase (DGC) or GGDEF domain
pspC	b1306	4.6	0.0055	Phage shock protein C
pspB	b1305	4.4	0.0147	Phage shock protein B
pspG	b4050	3.9	0.0290	Phage shock protein G
uspG	b0607	3.3	0.0065	Universal stress protein UP12
narP	b2193	2.7	0.0056	Nitrate/nitrite response regulator protein
ybeZ	b0660	-2.5	0.0224	Predicted protein with nucleoside triphosphate hydrolase domain
rstA	b1608	-2.5	0.0054	DNA-binding response regulator in two-component regulatory system with RstB
ptsP	b2829	-2.9	0.0121	Phosphoenolpyruvate-protein phosphotransferase; fused PTS enzyme
yebR	b1832	-2.9	0.0080	Hypothetical protein; GAF domain
yeaP	b1794	-3.0	0.0192	Predicted diguanylate cyclase
spoT	b3650	-3.2	0.0093	Guanosine-3',5'-bis pyrophosphate 3'-pyrophosphohydrolase
rseB	b2571	-3.5	0.0100	Periplasmic negative regulator of sigmaE
ydiV	b1707	-3.7	0.0245	C-di-GMP phosphodiesterase class I
Translation, ribosomal structure and biogenesis				
rttR	b4425	15.9	0.0002	Regulatory sRNA
rrmJ	b3179	4.6	0.0061	23S rRNA methyltransferase
deaD	b3162	4.3	0.0012	ATP-dependent RNA helicase
hslR	b3400	4.2	0.0292	Ribosome-associated heat shock protein Hsp15.
rprA	b4431	4.1	0.0271	Regulatory sRNA
miaA	b4171	2.5	0.0330	tRNA delta(2)-isopentenylpyrophosphate transferase
trmJ	b2532	2.5	0.0151	Hypothetical tRNA/rRNA methyltransferase
truA	b2318	2.3	0.0138	tRNA pseudouridine synthase A

TABLE 6.A1 Continuation.

Gene Name	Locus	Fold Change	*P* value	Gene Product
Translation, ribosomal structure and biogenesis (continuation)				
glyQ	b3560	-2.2	0.0025	Glycyl-tRNA synthetase alpha subunit
yeiP	b2171	-2.4	0.0182	Elongation factor P
rluE	b1135	-2.5	0.0086	23S rRNA pseudouridine synthase
rimN	b3282	-2.5	0.0310	Predicted ribosome maturation factor
Defence mechanisms				
mdtA	b2074	13.6	0.0449	Multidrug efflux system, subunit A
acrD	b2470	4.5	0.0227	Aminoglycoside/multidrug efflux system
rdoA	b3859	2.8	0.0054	Thr/Ser kinase implicated in Cpx stress response
yefM	b2017	2.4	0.0460	Antitoxin of the YoeB-YefM toxin-antitoxin system
lolD	b1117	-2.5	0.0391	Outer membrane-specific lipoprotein transporter
aidB	b4187	-2.6	0.0045	Isovaleryl CoA dehydrogenase
katE	b1732	-4.0	0.0015	Catalase
Secondary metabolites biosynthesis, transport and catabolism				
macB	b0879	9.6	0.0012	Fused macrolide transporter subunits of ABC superfamily
ybbA	b0495	7.7	0.0047	Hypothetical ABC transporter
yrbE	b3194	4.2	0.0137	Predicted toluene transporter subunit
yrbF	b3195	3.8	0.0349	Predicted toluene transporter subunit
yrbD	b3193	2.4	0.0082	Predicted ABC-type organic solvent transporter
ycaC	b0897	-3.9	0.0421	Predicted hydrolase
pptA	b1461	-4.2	0.0220	4-oxalocrotonate tautomerase homolog
Intracellular trafficking, secretion, and vesicular transport				
spy	b1743	16.0	0.0002	Envelope stress induced periplasmic protein
zraP	b4002	10.4	0.0335	Zn-binding periplasmic protein
secG	b3175	2.5	0.0224	Preprotein translocase membrane subunit
secF	b0409	2.4	0.0021	SecYEG protein translocase auxillary subunit
fimC	b4316	-3.9	0.0493	Chaperone, periplasmic
Lipid transport and metabolism				
fabI	b1288	-2.3	0.0153	Enoyl-[acyl-carrier-protein] reductase
ispD	b2747	-2.8	0.0297	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase
yegS	b2086	-3.2	0.0220	Phosphatidylglycerol kinase

TABLE 6.A1 Continuation.

Gene Name	Locus	Fold Change	*P* value	Gene Product
Central intermediary metabolism				
appA	b0980	-2.5	0.0375	Phosphoanhydride phosphorylase
psiF	b0384	-2.7	0.0127	Phosphate starvation-inducible protein.
Cell cycle control, cell division, chromosome partitioning				
sulA	b0958	9.5	0.0097	SOS cell division inhibitor
yhdE	b3248	-2.4	0.0056	Maf-like protein
Phage-related functions and prophages				
lit	b1139	-3.1	0.0218	Cell death peptidase (e14 prophage)
ymfI	b1143	-4.1	0.0023	e14 prophage
Hypothetical proteins				
yncJ	b1436	42.2	0.0086	Hypothetical protein
yaaX	b0005	42.2	0.0098	Hypothetical protein
bhsA	b1112	25.7	0.0115	Hypothetical protein
yjfN	b4188	19.8	0.0029	Hypothetical protein
ycfJ	b1110	18.0	0.0177	Hypothetical protein
yebE	b1846	16.3	0.0029	Hypothetical protein
ycfS	b1113	12.0	0.0165	Hypothetical protein
yedX	b1970	10.3	0.0069	Transthyretin-like protein precursor
yhdV	b3267	9.0	0.0191	Hypothetical protein
ypfM	b4606	8.8	0.0018	Hypothetical protein
yneM	b4599	8.4	0.0082	Hypothetical protein
yqjA	b3095	8.0	0.0023	Hypothetical protein
yiiX	b3937	6.9	0.0218	Predicted peptidoglycan peptidase
ypfG	b2466	6.7	0.0310	Hypothetical protein
fxsA	b4140	6.7	0.0032	Hypothetical membrane protein
yqjB	b3096	6.6	0.0426	Hypothetical protein
yaiY	b0379	6.5	0.0187	Hypothetical protein
tqsA	b1601	6.3	0.0331	Predicted permease
yeeE	b2013	6.3	0.0469	Predicted inner membrane protein
ybeD	b0631	6.1	0.0029	Hypothetical protein
yqaE	b2666	5.9	0.0015	Hypothetical protein
mgrB	b1826	5.8	0.0340	Hypothetical protein
yhcN	b3238	5.8	0.0014	Hypothetical protein
ymgD	b1171	5.5	0.0216	Hypothetical proteins

TABLE 6.A1 Continuation.

Gene Name	Locus	Fold Change	*P* value	Gene Product
Hypothetical proteins (continuation)				
yebG	b1848	5.4	0.0017	Conserved protein regulated by LexA
bssR	b0836	5.4	0.0192	Hypothetical protein
ytfK	b4217	5.3	0.0027	Hypothetical protein
obgE	b3183	5.2	0.0091	Hypothetical GTP-binding protein
ves	b1742	5.0	0.0278	Hypothetical protein
ycjX	b1321	5.0	0.0015	Hypothetical protein
ybfA	b0699	4.5	0.0182	Hypothetical protein
rmuC	b3832	4.4	0.0199	Predicted recombination limiting protein
bax	b3570	4.3	0.0342	Hypothetical protein; ATP-binding protein
ymgG	b1172	4.2	0.0230	Hypothetical protein
ygdR	b2833	4.2	0.0203	Hypothetical protein
ycbB	b0925	3.7	0.0092	Predicted carboxypeptidase
yfcO	b2332	3.6	0.0159	Hypothetical protein
ymgI	b4593	3.6	0.0177	Hypothetical protein
ydcH	b1426	3.5	0.0470	Hypothetical protein
yohN	b2107	3.5	0.0033	Hypothetical protein
sraF	b4448	3.5	0.0140	Unknown RNA
yrfG	b3399	3.4	0.0253	Predicted hydrolase
ynfD	b1586	3.4	0.0284	Hypothetical protein
yciS	b1279	3.3	0.0274	Hypothetical protein
rygB	b4445	3.3	0.0028	Unknown RNA
yebO	b1825	3.2	0.0038	Hypothetical protein
ycjF	b1322	3.2	0.0093	Hypothetical protein
rygA	b4444	3.1	0.0347	Unknown RNA
yhdN	b3293	3.1	0.0076	Hypothetical protein
ryeE	b4438	2.9	0.0099	Unknown RNA
nlpI	b3163	2.8	0.0053	Lipoprotein, contains TPR repeats
ybbJ	b0488	2.7	0.0226	Hypothetical protein
yifN	b3776	2.7	0.0210	Hypothetical protein
ytfP	b4222	2.7	0.0195	Hypothetical protein
sroF	b4441	2.6	0.0119	Unknown RNA
ybaJ	b0461	2.6	0.0271	Hypothetical protein
ydcY	b1446	2.6	0.0052	Hypothetical protein
yciC	b1255	2.6	0.0044	hypothetical protein
pgaD	b1021	2.5	0.0111	Hypothetical protein
yhhA	b3448	2.5	0.0441	Hypothetical protein
yjgQ	b4262	2.4	0.0221	Conserved membrane protein
lolA	b0891	2.4	0.0084	Chaperone for lipoproteins

TABLE 6.A1 Continuation.

Gene Name	Locus	Fold Change	*P* value	Gene Product
Hypothetical proteins (continuation)				
yrbB	b3191	2.4	0.0030	Hypothetical protein. Predicted NTP binding protein (contains STAS domain)
b3022	b3022	2.3	0.0042	Predicted cyanide hydratase
yjgP	b4261	2.3	0.0023	Conserved membrane protein. Predicted permease
yccJ	b1003	-2.2	0.0034	Hypothetical protein
yeaH	b1784	-2.2	0.0352	Hypothetical protein
slp	b3506	-2.2	0.0068	Outer membrane lipoprotein.
yfbT	b2293	-2.3	0.0058	Predicted hydrolase or phosphatase
wrbA	b1004	-2.3	0.0166	Predicted flavoprotein in Trp regulation
yciH	b1282	-2.4	0.0066	Hypothetical protein
ybiB	b0800	-2.5	0.0304	Predicted transferase/phosphorylase
ydhZ	b1675	-2.5	0.0163	Hypothetical protein
yabQ	b0057	-2.5	0.0266	Hypothetical protein
msyB	b1051	-2.6	0.0052	Hypothetical protein
ybeY	b0659	-2.6	0.0126	Hypothetical protein
yihI	b3866	-2.6	0.0171	Hypothetical protein
yehE	b2112	-2.6	0.0247	Hypothetical protein
yceK	b1050	-2.6	0.0484	Predicted lipoprotein
yoaC	b1810	-2.6	0.0394	Hypothetical protein
yigL	b3826	-2.7	0.0033	Predicted hydrolase, phosphatase-like domain
yobF	b1824	-2.7	0.0310	Hypothetical protein
yidA	b3697	-2.7	0.0249	Predicted hydrolase
ydbJ	b4529	-2.7	0.0162	Hypothetical protein
yjdJ	b4127	-2.8	0.0206	Predicted acyltransferase
ytjA	b4568	-2.8	0.0411	Hypothetical protein
yeaC	b1777	-2.9	0.0086	Hypothetical protein
yecN	b1869	-2.9	0.0358	Hypothetical protein
elaB	b2266	-3.0	0.0076	Hypothetical protein
bcsE	b3536	-3.0	0.0223	Hypothetical protein
yiiQ	b3920	-3.0	0.0398	Hypothetical protein
ycfM	b1105	-3.0	0.0029	Predicted outer membrane lipoprotein
envC	b3613	-3.1	0.0024	Protease with a role in cell division
yceF	b1087	-3.4	0.0039	Hypothetical protein
bcsF	b3537	-3.5	0.0051	Hypothetical protein
ybjT	b0869	-3.5	0.0022	Conserved protein with NAD(P)-binding Rossmann-fold domain
ydiA	b1703	-3.6	0.0076	Hypothetical protein

TABLE 6.A1 Continuation.

Gene Name	Locus	Fold Change	*P* value	Gene Product
Hypothetical proteins (continuation)				
fklB	b4207	-3.6	0.0491	FKBP-type peptidyl-prolyl cis-trans isomerase (rotamase)
ycfL	b1104	-3.7	0.0171	Predicted lipoprotein
rcsF	b0196	-3.8	0.0423	Predicted outer membrane protein
yahK	b0325	-4.0	0.0036	Predicted oxidoreductase
yjdI	b4126	-4.4	0.0183	Hypothetical protein
ydiZ	b1724	-4.4	0.0031	Hypothetical protein
ychJ	b1233	-4.5	0.0008	Hypothetical protein
gph	b3385	-4.5	0.0020	Phosphoglycolate phosphatase
yciX	b4523	-4.6	0.0044	Hypothetical protein
yhiD	b3508	-5.1	0.0026	Predicted Mg^{2+} transport ATPase
yiiS	b3922	-5.3	0.0433	Hypothetical protein
yhiM	b3491	-6.9	0.0128	Hypothetical protein.
yeeN	b1983	-7.2	0.0238	Hypothetical protein
ybgA	b0707	-8.8	0.0334	Hypothetical protein

TABLE 6.A2 *E. coli* genes differentially expressed following aerobic exposure to CORM-2.

Gene Name	Locus	Fold Change	*P* value	Gene Product
Inorganic ion transport and metabolism				
tauA	b0365	19.4	0.0107	Taurine transporter subunit
tauB	b0366	17.7	0.0362	Taurine transport ATP-binding protein
sbp	b3917	10.7	0.0048	Sulfate transporter subunit
metN	b0199	9.9	0.0009	D-methionine transport ATP-binding protein
tauC	b0367	9.0	0.0019	Taurine transport system permease protein
chaA	b1216	8.4	0.0483	Calcium/sodium:proton antiporter
yheL	b3343	7.4	0.0016	Predicted intracellular sulfur oxidation protein
pspE	b1308	7.0	0.0438	Phage shock protein E. Thiosulfate:cyanide sulfurtransferase (rhodanese)
metI	b0198	6.5	0.0005	D-methionine transport system permease protein
cysA	b2422	5.2	0.0373	Sulfate/thiosulfate transporter subunit
ssuA	b0936	4.7	0.0181	Alkanesulfonate transporter subunit
cysW	b2423	3.4	0.0368	Sulfate/thiosulfate transporter subunit
alx	b3088	3.3	0.0196	Predicted inner membrane protein, part of terminus
yqjH	b3070	2.7	0.0022	Predicted siderophore interacting protein
exuT	b3093	2.5	0.0418	Hexuronate transporter
yqcE	b2775	2.3	0.0124	Predicted transporter
apaG	b0050	-2.4	0.0229	Uncharacterized protein associated with Co^{2+}/Mg^{2+} efflux
yegH	b2063	-2.5	0.0146	Hypothetical protein. Putative transport protein
cyaY	b3807	-2.8	0.0289	Frataxin, iron-binding and oxidizing protein
Energy production and conversion				
frmA	b0356	8.1	0.0042	Alcohol dehydrogenase class III
ldhA	b1380	4.8	0.0150	Fermentative D-lactate dehydrogenase
glpK	b3926	3.7	0.0334	Glycerol kinase
nemA	b1650	3.6	0.0428	N-ethylmaleimide reductase, FMN-linked
yqhD	b3011	3.6	0.0319	Alcohol dehydrogenase, NAD(P)-dependent
yfiD	b2579	3.2	0.0015	Pyruvate formate lyase subunit
fucO	b2799	3.0	0.0231	L-1,2-propanediol oxidoreductase
ssuD	b0935	3.0	0.0054	Alkanesulfonate monooxygenase
xdhD	b2881	2.9	0.0087	hypoxanthine oxidase
fdoH	b3893	-2.6	0.0127	Formate dehydrogenase-O, Fe-S subunit
sucB	b0727	-2.7	0.0019	Dihydrolipoamide acetyltransferase
lldD	b3605	-3.2	0.0162	L-lactate dehydrogenase, FMN-linked
gabD	b2661	-3.6	0.0341	Succinate-semialdehyde dehydrogenase I
fumA	b1612	-3.9	0.0146	Fumarate hydratase (fumarase A)

TABLE 6.A2 Continuation.

Gene Name	Locus	Fold Change	*P* value	Gene Product
Energy production and conversion (continuation)				
sucA	b0726	-5.1	0.0318	2-oxoglutarate decarboxylase, thiamin-requiring
sdhC	b0721	-7.8	0.0133	Succinate dehydrogenase
sdhD	b0722	-8.7	0.0305	Succinate dehydrogenase
Carbohydrate transport and metabolism				
uxuB	b4323	6.1	0.0043	D-mannonate oxidoreductase, NAD-binding
uxuA	b4322	5.9	0.0043	Mannonate dehydratase
yciM	b1280	3.7	0.0427	Hypothetical protein
uxaC	b3092	3.2	0.0188	Uronate isomerase
gntX	b3413	3.1	0.0323	Gluconate periplasmic binding protein
maa	b0459	3.0	0.0283	Maltose O-acetyltransferase
uhpT	b3666	2.8	0.0367	Hexose phosphate transport protein
srlE	b2703	2.7	0.0236	Glucitol/sorbitol-specific enzyme
rbsB	b3751	2.7	0.0487	D-ribose transporter subunit
uxaA	b3091	2.5	0.0128	Altronate hydrolase
fucI	b2802	2.4	0.0065	L-fucose isomerase
otsA	b1896	-2.8	0.0059	Trehalose-6-phosphate synthase
tktB	b2465	-2.9	0.0413	Transketolase 2, thiamin-binding
proP	b4111	-3.1	0.0431	Proline/glycine betaine transporter
ugpA	b3452	-3.9	0.0067	Glycerol-3-phosphate transporter subunit
tsgA	b3364	-4.2	0.0176	Predicted transporter
Amino acid transport and metabolism				
metF	b3941	17.9	0.0058	5,10-methylenetetrahydrofolate reductase
metB	b3939	4.5	0.0062	Cystathionine gamma-synthase, PLP-dependent
prlC	b3498	3.7	0.0441	Oligopeptidase A
metL	b3940	3.3	0.0246	Homoserine dehydrogenase II
sdaA	b1814	2.9	0.0327	L-serine deaminase I
ilvG	b4488	2.9	0.0427	Acetolactate synthase II, large subunit
glpB	b2242	2.7	0.0081	Glycerol-3-phosphate dehydrogenase
ilvM	b3769	2.6	0.0301	Acetolactate synthase II, small subunit
gss	b2988	-3.1	0.0248	Fused glutathionylspermidine amidase
lysP	b2156	-3.2	0.0482	Lysine transporter
yeaS	b1798	-3.3	0.0176	Neutral amino-acid efflux system
ybaT	b0486	-3.7	0.0074	Predicted transporter
ygjG	b3073	-5.9	0.0279	Putrescine: 2-oxoglutaric acid aminotransferase
aroH	b1704	-7.0	0.0421	2-dehydro-3-deoxyphosphoheptonate aldolase

TABLE 6.A2 Continuation.

Gene Name	Locus	Fold Change	*P* value	Gene Product
Transcription				
metR	b3828	20.5	0.0053	DNA-binding transcriptional activator
soxS	b4062	14.5	0.0251	Regulatory protein SoxS
cpxP	b4484	12.2	0.0455	Periplasmic protein combats stress
marA	b1531	9.5	0.0096	Multiple antibiotic resistance protein
frmR	b0357	6.7	0.0442	Regulator protein that represses *frmRAB* operon
zntR	b3292	6.1	0.0045	DNA-binding transcriptional activator in response to Zn(II)
araC	b0064	5.0	0.0083	Arabinose operon regulatory protein
lrhA	b2289	4.6	0.0180	DNA-binding transcriptional repressor of flagellar, motility and chemotaxis genes
oxyR	b3961	4.1	0.0059	Hydrogen peroxide-inducible genes activator
lexA	b4043	3.8	0.0160	LexA repressor
lsrR	b1512	3.0	0.0186	lsr operon transcriptional repressor
cspG	b0990	-3.2	0.0231	Cold shock-like protein
gadX	b3516	-4.3	0.0143	DNA-binding transcriptional dual regulator
Posttranslational modification, protein turnover, chaperones				
ibpB	b3686	79.3	0.0266	16 kDa heat shock protein B
ibpA	b3687	18.7	0.0022	16 kDa heat shock protein A
hslO	b3401	5.4	0.0096	Hsp33-like chaperonin
hspQ	b0966	4.8	0.0026	DNA-binding protein, hemimethylated
clpB	b2592	4.8	0.0378	Protein disaggregation chaperone
dnaJ	b0015	4.1	0.0014	Chaperone Hsp40, co-chaperone with DnaK.
yeeD	b2012	3.3	0.0023	Predicted redox protein
ybbN	b0492	2.9	0.0373	Predicted thioredoxin domain-containing protein
yggG	b2936	2.7	0.0444	Predicted peptidase
dnaK	b0014	2.6	0.0105	Chaperone Hsp70, co-chaperone with DnaJ
Signal transduction mechanisms				
ydeH	b1535	23.5	0.0122	Hypothetical protein with diguanylate-cyclase
pspG	b4050	11.4	0.0352	Phage shock protein G
pspB	b1305	10.5	0.0045	Phage shock protein B
uspF	b1376	3.8	0.0207	Stress-induced protein, ATP-binding protein
narP	b2193	2.8	0.0487	Nitrate/nitrite response regulator protein
apaH	b0049	-2.8	0.0394	diadenosinetetraphosphatase
ydiV	b1707	-5.9	0.0360	C-di-GMP phosphodiesterase class I

TABLE 6.A2 Continuation.

Gene Name	Locus	Fold Change	*P* value	Gene Product
Replication, recombination and repair				
phr	b0708	-2.6	0.0316	Deoxyribodipyrimidine photolyase, FAD-binding
ruvA	b1861	2.5	0.0438	Component of RuvABC resolvasome
fimE	b4313	3.9	0.0156	Tyrosine recombinase
mutM	b3635	6.0	0.0282	Formamidopyrimidine-DNA glycosylase
recN	b2616	8.4	0.0302	Recombination and repair protein
Defence mechanisms				
mdtB	b2075	19.0	0.0312	Multidrug efflux system, subunit B
marB	b1532	6.9	0.0049	Multiple antibiotic resistance protein
rdoA	b3859	3.5	0.0184	Thr/Ser kinase implicated in Cpx stress response
pmrD	b2259	2.8	0.0325	Polymyxin B resistance protein
aidB	b4187	-2.7	0.0373	Isovaleryl CoA dehydrogenase
Translation, ribosomal structure and biogenesis				
hslR	b3400	5.1	0.0010	Ribosome-associated heat shock protein Hsp15
micF	b4439	4.1	0.0291	Regulatory antisense sRNA affecting ompF expression
rrmJ	b3179	3.2	0.0166	23S rRNA methyltransferase
miaA	b4171	2.5	0.0151	tRNA delta(2)-isopentenylpyrophosphate transferase
Cell motility				
ydeS	b1504	3.2	0.0218	Predicted fimbrial-like adhesin protein
flgA	b1072	-2.6	0.0153	Assembly protein for flagellar basal-body periplasmic P ring
cheA	b1888	-2.9	0.0453	Sensory histidine kinase/signal sensing protein
Intracellular trafficking, secretion, and vesicular transport				
spy	b1743	30.0	0.0043	Envelope stress induced periplasmic protein
secA	b0098	3.4	0.0065	Preprotein translocase subunit, ATPase
Lipid transport and metabolism				
sbmA	b0377	3.2	0.0260	Predicted transporter
yihG	b3862	-2.7	0.0270	Predicted endonuclease
Nucleotide transport and metabolism				
guaC	b0104	-2.2	0.0167	GMP reductase
sthA	b3962	-5.0	0.0069	Soluble pyridine nucleotide transhydrogenase

TABLE 6.A2 Continuation.

Gene Name	Locus	Fold Change	*P* value	Gene Product
Secondary metabolites biosynthesis, transport and catabolism				
ybbA	b0495	7.1	0.0084	Hypothetical ABC transporter
ybbP	b0496	6.5	0.0154	Predicted inner membrane protein
Cell wall/membrane/envelope biogenesis				
osmB	b1283	3.1	0.0394	Osmotically inducible lipoprotein B
amiC	b2817	2.4	0.0154	N-acetylmuramoyl-L-alanine amidase
Coenzyme transport and metabolism				
thiC	b3994	-2.3	0.0080	Thiamin (pyrimidine moiety) biosynthesis protein
btuD	b1709	-2.3	0.0111	Vitamin B12-transporter ATPase
Cell cycle control, cell division, chromosome partitioning				
sulA	b0958	5.4	0.0232	SOS cell division inhibitor
Hypothetical proteins				
yncJ	b1436	62.0	0.0191	Hypothetical protein
yjfN	b4188	22.9	0.0030	Hypothetical protein
yebE	b1846	22.9	0.0056	Hypothetical protein
ycfS	b1113	16.9	0.0064	Hypothetical protein
ymgG	b1172	16.0	0.0312	Hypothetical protein
fxsA	b4140	13.8	0.0135	Hypothetical membrane protein
ycfJ	b1110	13.5	0.0390	Hypothetical protein
ycjF	b1322	10.0	0.0151	Hypothetical protein
tqsA	b1601	9.1	0.0461	Predicted permease
yhdV	b3267	9.0	0.0063	Hypothetical protein
ypfG	b2466	8.6	0.0319	Hypothetical protein
ycjX	b1321	7.7	0.0223	Hypothetical protein
yodA	b1973	6.6	0.0117	Conserved metal-binding protein
yneM	b4599	6.2	0.0438	Hypothetical protein
ybeD	b0631	6.0	0.0020	Hypothetical protein
yaiY	b0379	5.8	0.0109	Hypothetical protein
ydeT	b1505	5.7	0.0017	Hypothetical protein
yqjA	b3095	5.7	0.0100	Hypothetical protein
ryeD	b4437	5.6	0.0125	Unknown RNA
ytfK	b4217	5.3	0.0023	Hypothetical protein
ycgK	b1178	4.9	0.0338	Hypothetical protein

TABLE 6.A2 Continuation.

Gene Name	Locus	Fold Change	*P* value	Gene Product
Hypothetical proteins (continuation)				
yqaE	b2666	4.5	0.0145	Hypothetical protein
ybfA	b0699	4.5	0.0023	Hypothetical protein
ynfD	b1586	4.5	0.0385	Hypothetical protein
ydjM	b1728	4.4	0.0043	Predicted inner membrane protein
yedY	b1971	4.3	0.0035	Predicted reductase
yrfG	b3399	4.3	0.0104	Predicted hydrolase
yhdN	b3293	4.3	0.0341	Hypothetical protein
eptB	b3546	4.0	0.0315	Predicted metal dependent hydrolase
yobB	b1843	4.0	0.0060	Predicted amidohydrolase
bax	b3570	3.3	0.0284	ATP-binding protein
yciS	b1279	3.2	0.0134	Hypothetical protein
ldrB	b4421	3.1	0.0481	Toxic polypeptide, small
yeeE	b2013	2.7	0.0183	Predicted inner membrane protein
ybjK	b0846	2.6	0.0076	Predicted DNA-binding transcriptional regulator
sraF	b4448	2.5	0.0109	Unknown RNA
yciC	b1255	2.3	0.0298	Hypothetical protein
ucpA	b2426	2.3	0.0056	Predicted oxidoredutase
yeiB	b2152	-2.2	0.0138	Conserved inner membrane protein
yqcC	b2792	-2.3	0.0102	Hypothetical protein
yaeB	b0195	-2.7	0.0059	Hypothetical protein
yrdB	b3280	-2.8	0.0138	Hypothetical protein
ygfJ	b2877	-2.9	0.0288	Hypothetical protein
yafK	b0224	-3.0	0.0098	Hypothetical protein
yahK	b0325	-3.6	0.0290	Predicted oxidoreductase
ybaK	b0481	-3.9	0.0269	Hypothetical protein
slp	b3506	-4.1	0.0200	Outer membrane lipoprotein
yciX_2	b4523	-8.0	0.0152	Hypothetical protein
yagU	b0287	-10.4	0.0226	Conserved inner membrane protein

TABLE 6.A3 Oligonucleotides used in this study.

Oligonucleotides	Sequence (5′ – 3′)
RT_yncJ_up	GGACAACTCATGGCAGGGC
RT_yncJ_low	GCTCGCGCAAACCTTCC
RT_marA_up	GGACTGGATCGAGGACAACC
RT_marA_low	CGCCCTGCATATTGGTCAT
RT_soxS_up	GACCAGCCGCTTAACATTGA
RT_soxS_low	CGATAATCGCTGGGAGTGC
RT_lrhA_up	CGCGTGAGTTCGGTTTATCC
RT_lrhA_low	CTGCGCAGTACCAGTGTGTTG
RT_ibpB_up	CGCCGTACAACATTGAGAAA
RT_ibpB_low	CGGGACGTTCGCTGATAG
RT_frmA_up	CCGGCGTTGTGGTTGAAGT
RT_frmA_low	TCGCGCCAAGACCAAACAC
RT_gadX_up	CGCTTTCATTATTTCTCCCGA
RT_gadX_low	CACGGTTCGACAATCTCTGC
RT_metR_up	TCTGCGCATTGCCATTGAG
RT_metR_low	GGCGTACAGTCGGCTCCAC
RT_bhsA_up	GTAAAAACCCTCATCGCTGC
RT_bhsA_low	TGCTGTTCCATGGAGGGTAT
RT_sucA_up	GGCGTTTAACCCGTCTCACC
RT_sucA_low	GCGTCGCCACTTTTTCCTG
Comp_ibpAB_HindIII	ATCGTGGGTGTAAGCTTGATGAGTT
Comp_ibpAB_XbaI	CAGTCGAAATCTAGAGCATTGTTGAG
Comp_metR_HindIII	TCGTACCAAGCTTAATCGCCCACC
Comp_metR_ XbaI	GCTGGTAAACCTGTCTAGATGGCAC

TABLE 6.A4 Bacterial strains used in this study.

E. coli Strains	Description	Reference
MG1655	Wild type	Laboratory stock
Δ*metR*	MG1655 *metR*::Kan^r	CGSC*, [13]
Δ*metI*	MG1655 *metI*::Kan^r	CGSC, [13]
Δ*metN*	MG1655 *metN*::Kan^r	CGSC, [13]
SA29	MC4100 Δ*oxyR*::Kan^r	[14]
Δ*oxyR*	P1 transduction of SA29 to MG1655	This study
GC4468	Parental strain	[15]
BW831	GC4468 soxS3::Tn10	[15]
MC4100	Parental strain	[16]
JGT10	MC4100 Δ*ibpAB*::Kan^r	[17]
PAD282	Parental strain	[18]
PAD488	PAD282 *cpxP*::Kan^r	[18]
BW25113	Parental strain	[19]
Δ*tqsA*	BW25113 *tqsA*::Kan^r	[19]
Δ*bhsA*	BW25113 *bhsA*::Kan^r	[22]

*CGSC - Coli Genetic Stock Center

TABLE 6.A5 *E. coli* plasmids used in this study.

Plasmid	Description	Reference
pUC-19	Cloning vector (Amp^r)	Laboratory stock
p*ibpAB*	pUC-19 containing *ibpAB*	This study
p*metR*	pUC-19 containing *metR*	This study
pAQ17	pUC-19 containing *oxyR*	[20]
pBR322	Cloning vector (Amp^r)	[21]
pWB31	pBR322 containing *soxS*	[21]
pCA24N	Cloning vector (Cm^r)	[19]
pCA24N*ydgG*$^+$	pCA24N containing *ydgG*	[19]
pCA24N*ycfR*$^+$	pCA24N containing *ycfR*	[22]

TABLE 6.A6 Comparison of CORM-2- and biofilm-regulated genes.

Gene Name	Locus	Fold Change		
		Anaerobic CORM-2	Aerobic CORM-2	Biofilm*
ibpB	b3686	40	79	32
bhsA	b1112	26	-	12
ibpA	b3687	19	19	4
bssS	b1060	13	-	5
soxS	b4062	10	15	49
tqsA	b1601	6	9	6
bssR	b0836	5	-	3
ybaJ	b0461	3	-	36
yefM	b2017	2	-	3
b3022	b3022	2	-	8
yceK	b1050	-3	-	-8
artI	b0863	-3	-	-6
yobF	b1824	-3	-	-7
artP	b0864	-4	-	-6
gabD	b2661	-	-4	-8
hyaA	b0972	-5	-	-6
yhiD	b3508	-5	-	-5
yhiM	b3491	-7	-	-6

* Adapted from [34]

Discussion

CHAPTER 7

General Discussion

7.1 New insight into the role of *S. aureus* flavohaemoglobin

S. aureus is an opportunist pathogen that due to its high virulence and antibiotic resistance has high impact on human health and is responsible for several serious infections such as toxic shock syndrome and septicemia [1].

Macrophages and neutrophils are key components of the human innate immune system that provide defence against invading microorganisms. Nitric oxide is one of the most powerful antimicrobial products generated within phagocytic cells and has been shown to afford protection against *S. aureus* since the inhibition of nitric oxide synthase or its gene deletion leads to an increase of mice susceptibility to *S. aureus* infections [2, 3].

Microbes have developed several strategies to detoxify NO (Chapter 1) but the mechanisms by which *S. aureus* resist to nitrogen reactive species remain essentially unknown. Among the known NO-detoxifiers (see 1.2.2), *S. aureus* genome only encodes a flavohaemoglobin-like gene.

Flavohaemoglobins are important enzymes regarding the scavenging of NO [4-6]. In this thesis, the role of flavohaemoglobin in *S. aureus* protection against nitrosative stress was addressed. *S. aureus* Hmp exhibits 30 % amino acid sequence identity with *E. coli* Hmp, and is constituted by a high spin haem *b* and a flavin domain. Its biochemical characterisation revealed that it has all the typical features of the canonical flavohaemoglobins. Modeling studies, using the three-dimensional structure of the *E. coli* Hmp, predicted that *S. aureus* Hmp has a overall folding similar to that of the *E. coli* enzyme. In particular, the His85 residue (considering the residue numbering of the *S. aureus* amino acid sequence), that links the haem to the polypeptide chain, and the residues that control the interaction with ligands (Tyr29 and Gln53) are conserved (Figure 7.1) [7]. In addition, the residues that interact with phopholipids are conserved (Figure 7.1), which suggest that *S. aureus* Hmp may also bind lipids and detoxify toxic hydroperoxides [8, 9].

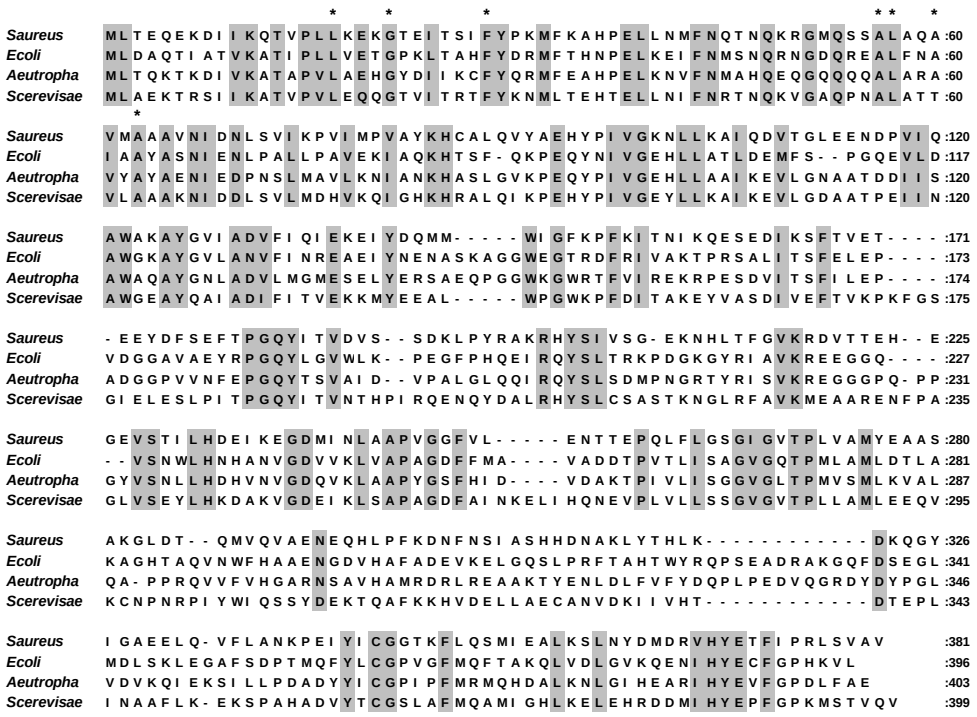

FIGURE 7.1 Amino acid sequence alignment of flavohaemoglobins. *S. aureus*, *E. coli*, *A. eutrophus* and *S. cerevisiae* flavohaemoglobins. Asterisks refer to amino acid residues involved in ligation to the phospholipids in the *A. eutrophus* enzyme.

We showed that *S. aureus* Hmp metabolises NO, under aerobic and anaerobic conditions, with activity values that are within the range of values measured to homologous proteins [10-12]. However, as the anaerobic activity of *E. coli* flavohaemoglobin is much lower than the aerobic one, it has been considered that flavohaemoglobins have a major role under aerobic conditions [13]. Yet, Hmp was reported to afford NO-protection to *S. typhimurium* under low levels of dioxygen, since the *hmp* deficient mutant showed high susceptible to GSNO under anaerobic conditions [14]. Similar results were describded for *S. cerevisiae*, underlining the potential protective role of flavohaemoglobins under anaerobic conditions [15]. Our results also demonstrated the anaerobic ability of *S. aureus* Hmp to detoxify nitric oxide since the over-expression of *S. aureus* Hmp in the *E. coli* flavorubredoxin-

mutant, a strain that display high susceptibility to nitric oxide anaerobically, is able to protect *E. coli* cells against nitrosative stress (Figure 3.3F).

Richardson and co-workers, have also analysed the role of flavohaemoglobin in *S. aureus* COL strain [16]. However, in this work, only the aerobic protection afforded by Hmp was explored exhibiting the *S. aureus hmp* null mutant higher susceptibility to nitrosative stress when compared to the wild type. Furthermore, the growth phenotypes were analysed after a long period of incubation (~ 12 h) and using a high S-nitroso-N-acetylpenicillamine concentration (5 mM), which is well above the estimated intracellular NO levels (μM range) [16, 17].

On contrary, all our results reinforce the proposal that *S. aureus* Hmp has a major role under low levels of dioxygen. In fact, the highest induction of *S. aureus hmp* expression is triggered by the limitation of dioxygen in bacterial growth conditions (Figure 3.2), and the deletion of the *hmp* gene generates a *S. aureus* strain sensitive to nitrosative stress but only under microaerobic conditions (Figure 3.3C-D). Therefore, *S. aureus* Hmp functions as a NO denitrosylase. Although this mechanism has already been proposed by Hausladen *et al.* as the one operative for flavohaemoglobin under physiological conditions (discussed 1.5.2), this was the first time that supportive experimental data was obtained.

Hmp is required for full virulence in mice as a *S. aureus hmp*-deleted strain was not able to induce mortality of infected mice, which highlights the importance of Hmp in *S. aureus* defence against nitrosative stress [16]. Nevertheless, since the *S. aureus* NO consuming activity is not completely abolished in the *hmp* mutant, the contribution of other enzymes to the *S. aureus* NO metabolism needs to be considered [16]. Recently, we have identified a bifunctional nitroreductase that, in addition to its nitroreductase activity, displays GSNO reductase activity. The enzyme affords protection to GSNO-treated *S. aureus* as the deletion of the nitroreductase gene increases the susceptibility of *S. aureus* to GSNO [18].

The importance of NO detoxifying systems is increased in *S. aureus*, since NO is also produced endogenously via the activity of NO synthase, which was reported to generate nitric oxide in response to methanol [19]. Although several bacteria contain NO synthase homologues, their biological function remains to be

elucidated. Recent studies suggest that bacterial NO synthase may increase the resistance to a broad spectrum of antibiotics as *B. subtilis* and *S. aureus* strains deleted in the NO synthase gene display higher susceptibility to antibiotics like acriflavine and pyocyanin [20]. The mechanisms by which NO-mediated antibiotic resistance include chemical modification of the antibiotic, and induction of the expression of genes involved in oxidative stress detoxification [20].

Interestingly we found that, on contrary, flavohaemoglobin confers antibiotic susceptibility to *S. aureus*. During the investigation of the ability of *S. aureus* Hmp to coordinate azoles antibiotics, that inhibit *S. aureus* Hmp NO denitrosylase activity, we observed that, the deletion of *hmp* gene increases the resistance of *S. aureus* to azole antibiotics (see Chapter 4), both in the absence and presence of NO.

Azoles are synthetic compounds that were initially used to treat fungal infections and its main target is the lanosterol 14α-demethylase, an essential enzyme in the biosynthesis of ergosterol [21]. Nowadays, it is well established that azoles are also active against bacteria, particularly Gram-positive species, but its cellular targets remain unknown [22, 23].

This work showed that *S. aureus* is susceptible to imidazoles like miconazole, sulconazole, clotrimazole, and ketozonazole, but not to triazoles such as fluconazole and itraconazole. The lack of *S. aureus* sensitivity to triazoles seems to result from the inability of these compounds to coordinate to Hmp.

To assed the mechanism by which flavohaemoglobin potentiates the antimicrobial action of imidazoles, it was first analysed the ability of the imidazole to bind the purified *S. aureus* Hmp. By UV-Visible, EPR and resonance Raman it was shown that miconazole coordinates *S. aureus* Hmp haem as a sixth ligand in both ferric and ferrous state given rise to low spin species (Figure 4.3 and Table 4.1). The miconazole binds tightly to the Hmp ferric haem with an association constant of 1.7×10^6 M^{-1}. More importantly, when provided with an electron donor (NADH), an increase of superoxide production by Hmp was observed (Figure 4.4B). In accordance, a high level of reactive oxygen species is detected in *S. aureus* cells expressing *hmp* and the deletion of *hmp* increases the resistance of *S. aureus* to

imidazoles by ~ 60 % for miconazole, 56 % for sulconazole and clotrimazole and ~ 40 % for ketoconazole.

Several studies have already reported that Hmp has the ability of reducing dioxygen and the production of superoxide results from the binding of dioxygen to the protein haem [24-27]. However, in the presence of imidazoles, the haem site is occupied by the antibiotic molecule and the superoxide reduction can only occur at the flavin domain. Hence, we proposed that Hmp transfers electrons directly from flavin to dioxygen, which would be in agreement with other's results showing that when haem is blocked (e.g. CO) Hmp transfers electrons to several external electron acceptors [28].

Concerning the generation of reactive oxygen species related to the azole action, previous studies in *C. albicans* and *S. cerevisiae* reported a similar occurrence. However, a completely different mechanism is operating as ROS production results from inhibition of ROS-detoxification enzymes like catalase and peroxidase [21, 29]. Hence, the presented data revealed a novel mode of action of the imidazoles antibiotics. Nevertheless, the existence of other cellular targets that contribute to the imidazole-ROS generation in *S. aureus* cannot be excluded. In fact, antibiotic treated *hmp* deleted strains still contain a non-contemptible amount of reactive oxygen species.

Regarding other potential imidazole targets that may contribute to the generation of ROS, an early study reported that *S. aureus* exposed to miconazole has decreased levels of vitamin K_2 and increased levels of octaprenyl diphosphate, which was proposed to be relating with the inhibition of MenA, the sixth enzyme in menaquinone (vitamin K_2) biosynthesis. Since menaquinones are involved in the electron transport in the respiratory chain, the inhibition of its synthesis might potentiate the increase of intracellular reactive oxygen species [30].

We also observed that the increase of *S. aureus* susceptibility to imidazole, and consequent increase of reactive oxygen species, was not dependent on the presence of NO as the survival of miconazole-treated *S. aureus* strains is strongly reduced in LPS- and INF-γ-activated macrophages (producing NO) as well as in macrophages in which the generation of NO was inhibited by L-NMMA.

Therefore, the simultaneous presence of NO and imidazoles will both contribute to the killing of *S. aureus* by: i) increasing the ROS production; and ii) decreasing the *S. aureus* ability to detoxify NO due to the impairment of NO binding to the haem when in the presence of the antibiotic.

It is interesting to note that in *S. cerevisiae* the simultaneous treatment with miconazole and NO increases the susceptibility of yeast to an extension similar to that occurring when *S. cerevisiae hmp*-mutant in exposed only to NO, thus suggesting that the increase of miconazole-sensitivity is related with the inhibition of Hmp NO denitrosylase activity [31].

In candida species, a synergistic effect between NO and imidazoles was reported, although the mechanism whereby the simultaneous treatment with NO and imidazoles enhances candida killing was not investigated. Our results, together with the observation that candida species (e.g. *C. albicans*, *C. tropicalis* and *C. dubliniensis*) containing a flavohaemoglobin show higher sensitivity to azole treatment and lower susceptibility to NO than candida strains lacking a *hmp* homologue gene (e.g. *C. krusei* and *C. glabrata*), we can speculate that the antifungal activity of azole also depends on flavohaemoglobin. Hence, in addition to the lanosterol 14α-demethylase, Hmp may constitute an important target of azole derivatives on fungal species [32].

The knowledge of microbial defence mechanisms to resist inside phagocytes is very important to design new strategies to combat pathogens. The work present in Chapter 3 provides evidences that *S. aureus* utilises Hmp to defend itself against nitrosative stress, and therefore inhibitors of its activity are good candidates for antibiotic development. The work described in Chapter 4 allowed the identification of a mechanism by which imidazoles are able to inhibit the growth of *S. aureus* that also involves Hmp. It was demonstrated that by ligating to flavohaemoglobin, imidazoles increase the *S. aureus* intracellular ROS production, which leads to the decrease of *S. aureus* survival. Hence, imidazoles both inhibit the NO denitrosylase activity of Hmp and potentiate the ROS formation constituting a good alternative treatment for *S. aureus* infections.

7.2 Carbon monoxide as antimicrobial agent

7.2.1 The bactericidal action of CO-releasing molecules

Carbon monoxide is produced in the human body by the activity of haem oxygenase enzymes, which also generates ferrous iron and biliverdin. However the relevance of the endogenous production of CO remains, so far, to be fully understood. In addition, the potential of utilising the "poisoning ability" of CO was until now never addressed. In this thesis, it was analysed, for the first time, the effect of CO on bacteria viability using two sources of CO: CO gas and CO-RMs. It was found that CO and, in particular, CO-RMs are indeed able to kill bacteria like *E. coli* and *S. aureus*, under oxic and anoxic conditions.

Although similar studies have never been performed, an early investigation reported the influence of different packing systems on meat preservation. Curiously, the authors observed that packages to which CO had been added exhibited less bacterial growth [33]. These results can now be explained based on the data presented in Chapter 5, which show that the treatment of cells with CO causes bacterial growth inhibition.

The CO released by CO-RMs elicits a more pronounced decrease of bacteria viability and the reason behind this difference is not yet clear. Nevertheless, we can speculate that the low solubility of CO gas in aqueous solutions may contribute to its reduced effectiveness and that the ability of CO-RMs to accumulate inside bacterial cells, as demonstrated by the increase of the metal content in *E. coli* cells after being treated with the CO-RMs (Table 7.1), may be responsible for its higher efficacy.

TABLE 7.1 *E. coli* metal content after the treatment with CO-RMs.

	[Metal] (µmol/g cell)					
	CORM-2		ALF 021		ALF 062	
	-	+	-	+	-	+
[Ru]	0.001	26				
[Mn]			0.2	20		
[Mo]					0.04	2

- *E. coli* cells untreated; + *E. coli* cells treated with CO-RM

Our results revealing that the antimicrobial action of CO-RMs is higher under low levels of dioxygen may be explained considering the preferential binding of CO to the ferrous form of haem proteins, which are present predominantly under anoxic conditions. This observation is not negligible since pathogen colonisation occurs in nearly-anaerobic environments. On the other hand, the type of bacterial cell wall seems not to interfere with the function of CO-RMs, as a similar decrease of cell viability was observed for *E. coli* and *S. aureus*, which are consider as prototypes of Gram-negative and Gram-positive bacteria, respectively.

Importantly, the addition of haemoglobin to the bacterial cultures abolishes the antimicrobial action of CO-RMs, suggesting that the antibacterial effect is dependent on the CO-released. Neither iCORM-2 nor an inactivated solution of ALF 062 shows any antimicrobial effect. Furthermore, the decomposition products of ALF 062, (tetraethyl ammonium bromide and sodium molybdate) are also innocuous toward bacterial growth. Hence, the bactericidal activity of CO-RMs is not due to the molecular skeleton of the compound but related to its ability of releasing CO.

An early study reported that the exogenous treatment of macrophages with CO gas enhances the phagocytic clearance of *E. coli* [34]. Since the authors measured the macrophages phagocytosis ability and did not evaluate the bacterial viability, the CO activity was only associated with the increase of surface expression of Toll-like receptor 4 of macrophages via the p38 MAPK pathway [34]. However, the results presented in this thesis suggest that the increase of bacteria clearance in macrophages also relates to the ability of CO gas to kill *E. coli*.

After our work, Chung and co-workers analysed the importance of HO-1 on microbial sepsis [35]. It was shown that HO-1 deficient mice display enhanced susceptibility to polymicrobial infections, while the over-expression of HO-1 has remarkable beneficial effects, enhancing bacterial clearance by increasing phagocytosis, which leads to the reduction of the number of viable bacteria in mice blood and organ homogenates. The beneficial effects of HO-1 on microbial sepsis are attributed to CO since the administration of CORM-2 to mice decreases the number of bacterial counts in blood, lungs, liver and spleen, and rescues the HO-1-

deficient mice from sepsis-induced lethality [35]. However, the data acquired with HO-1-deficient mice only demonstrate the antibacterial effect of the exogenous administration of CO-RMs and not of the endogenous production of CO since HO-1 produces, in addition to CO, bilirubin and ferrous iron, therefore not allowing to exclude the contribution of these other products of the HO-1 activity.

More recently, Desmard and co-workers showed that CO gas and CORM-3 are also able to decrease *P. aeruginosa* growth, and the antimicrobial effect of CORM-3 was correlated with the inhibition of the respiratory chain as the CORM-3 treatment impairs dioxygen consumption of *P. aeruginosa* cultures. Interestingly, CORM-3 exhibits bactericidal effect on *P. aeruginosa* cultures at concentrations lower than those required to kill *E. coli* and *S. aureus*. However, these results are most probably due to the use of a much lower number of *P. aeruginosa* cells when compared to those utilised in the *E. coli* and *S. aureus* experiments. In addition, while CORM-3 was added to the beginning of the *E. coli* exponential cell phase, the addition of CORM-3 to *P. aeruginosa* occurred at the lag phase [36, 37].

Importantly, Desmard *et al*. also reported that the prior administration of CORM-3 to mice followed *P. aeruginosa* infection prolongs its survival, being observed a decrease of the bacterial counts in the spleen of immunocompetent and immunosuppressed mice [36]. This indicates that carbon monoxide contributes, together with the stimulation of bacterial phagocytosis by the host, to the bacterial clearance, as previously proposed by Otterbein *et al.* and Chung and co-workers [34-36].

The present work and the subsequent studies reported above strongly suggest that the exogenous administration, whether in the form of CO gas or CO-RMs, has the potential to treat several bacterial infections. In particular, our results indicate that CO-RMs can be considered as a promising pharmaceutical tool for eradication of both Gram-positive and Gram–negative microorganisms. It is also important to refer that the bactericidal effect of CO-RMs occurs at concentrations not cytotoxic to eukaryotic cells, as the treatment of macrophages or smooth muscle cells with CO-RMs at concentrations up to 500 μM was reported to promote a non-significant cytotoxicity [36, 38, 39]. Furthermore, the administration of CO-

RMs to rats (5-20 μmol/Kg) does not increase carboxy-haemoglobin to dangerous levels (COHb levels < 10 %) [38, 40, 41]. In particular, the intravenous administration of 100 μM CORM-3 to mice only raised the COHb level to ~ 0.5 % [36]. Since the first symptoms of CO poisoning in humans appear in a range of 15 – 20 % COHb, it is not predictive significant side effects of CO-RMs therapy [36, 42].

7.2.2 On the search of bacterial targets of CO-releasing molecules

In humans, the biological activity of CO has been considered mainly due to its interaction with iron haem proteins. However, CO may bind to almost all transition metals-containing proteins, altering their structure and activity. Therefore, there are a large number of intracellular targets that may be responsible for the toxic effects of CO on bacteria.

To identify the potential bacterial CO targets, the transcriptional response of *E. coli* to CORM-2 was analysed by DNA microarray experiments (Chapter 6). Microarray studies allow the simultaneous examination of the expression levels of thousands of genes being consider an essential tool since it provides a global picture of the metabolic pathways that are affected by an external stimulus. The choice of *E. coli* was based on the fact that, besides being a model bacterium with the genome fully sequenced and annotated, this Gram-negative bacterium is a facultative anaerobic that grows in oxygenated as well as in anoxic environments during its normal colonisation-transmission cycle within the host. Therefore, it allows to compare the transcriptional response to CORM-2 under both anaerobic and aerobic conditions.

To confirm that the expression profile of *E. coli* cells exposed to CORM-2 was indeed due to the CO liberated by the molecule, quantitative Real-Time RT-PCR experiments were conducted in *E. coli* cells grown with the iCORM-2, a molecule depleted of all CO groups. In fact, it was observed that this CO-free molecule does not cause significant alterations in the transcription of the genes that are regulated by CORM-2.

Based on the results acquired, phenotypic studies were designed and performed for more than ten *E. coli* mutant strains that are deleted in genes whose expression was found to be significantly changed by CORM-2. In this way, insights into the biological function of CORM-2-regulated genes could be obtained.

In general, the microarray results demonstrated that for *E. coli* cells grown under aerobic conditions the main target of CORM-2 is the aerobic respiration. This was an expected result, since CO is known to be an inhibitor of cytochrome oxidase, the terminal electron acceptor of the aerobic respiratory chain. Although the transcription of cytochrome oxidase was not altered by CORM-2, the transcription of genes encoding enzymes of the citric acid cycle (e.g. *sdhCD, fumA, and sucB*) were found to be repressed. Interesting, all the above mentioned genes encode proteins that contain and/or utilise metals for their biological activity. SdhC and SdhD are the large and the small subunits, respectively, of the succinate dehydrogenase, a cytochrome b_{556}-containing protein. Fumarase (FumA) contains a [4Fe-4S] cluster, and SucB a subunit of 2-oxoglutarate dehydrogenase uses Mg^{2+} as co-factor. Therefore, the inhibition of citric acid cycle enzymes may result either from the binding of CO to the cytochrome oxidase which impairs the re-oxidation of NADH or from the direct binding of CO to these enzymes.

Results acquired for *E. coli* grown aerobically revealed that CORM-2 also strongly affects the transcription of genes involved in the methionine biosynthesis process, up-regulating the expression of *metNI* and *metBLF* operons. The deletion of *metR*, a regulator of several genes involved in methionine biosynthesis, and of *metN* and *metI*, encoding two methionine transporters, were found to increase the susceptibility of *E. coli* to CORM-2. Altogether, the data suggest that methionine protects *E. coli* cells against CORM-2 through a mechanism that needs to be elucidated.

Interestingly, *E. coli* showed to be more affected by the CORM-2 treatment when grown anaerobically, as judged by the higher number of repressed genes in comparison to those altered under aerobic conditions. In fact, these results are in agreement with the higher susceptibility of anaerobic *E. coli* cells exposed to CO-

RMs thus showing that the CO-RM action goes far beyond the impairment of aerobic respiratory chain.

Exposure of *E. coli* cells grown under anaerobic conditions to CORM-2 has a high impact on the glucose catabolic process. In particular, CORM-2 inhibits the transcription of several genes encoding glycolic enzymes, such as pyruvate kinase and phosphoglycerate kinase, both participate in the generation of ATP at the level of glycolysis. Interestingly, the two enzymes utilise Mg^{2+} as co-factor. Since in cells grown anaerobically the major fraction of ATP is generated glycolytically, the inhibition of those enzymes conduct to the depletion of ATP which probably contributes to the higher susceptibility that *E. coli* cells display upon treatment with CORM-2 under anaerobic conditions.

Another work reported that the exposure of aerobically grown of *E. coli* cells to CO decreased the thymine uptake and reduced the ATP levels. The authors could not observed alteration in thymine incorporation and ATP concentration in cells grown under anaerobic conditions in the presence of CO [43, 44]. However, the present results do suggest that ATP level is indeed altered in *E. coli* cells grown anaerobically since among the four genes encoding glycolytic enzymes that were repressed by CORM-2, two of them are involved in ATP formation (*pgk* and *pykF* encoding phosphoglycerate kinase and pyruvate kinase, respectively).

In addition, a significant number of genes found to be regulated by CORM-2 in *E. coli* cells grown anaerobically encode proteins involved in folding, namely, heat-shock proteins, chaperones and proteases. The analysis of the deletion of *cpxP* and *ibpAB* genes, which have been induced under both conditions of growth in the presence of CORM-2, give rise to strains that display elevated sensitivity to CORM-2. CpxP is a periplasmic combat stress protein that belongs to the Cpx system which is involved in the sensing of perturbations occurring at the bacterial cell envelope and responds through the up-regulation of several gene products involved in protein folding and degradation [45]. IbpA and IbpB are two heat-shock proteins that bind to protein aggregates and inclusion bodies formed during heterologous protein expression and contributes to the reduction of protein

disaggregation and degradation [46]. Hence, under anaerobic conditions CORM-2 affects the protein homeostasis.

The comparison of the microarray results obtained for *E. coli* cells grown aerobically with those acquired anaerobically reveals that a larger number of genes are regulated by CORM-2 independently of the presence of dioxygen (Table 6.6). The transcription of several genes encoding regulators was up-regulated by CORM-2 aerobically and anaerobically, namely *soxS*, *frmR*, *zntR*, *oxyR*, and *narP* (see Table 6.6 for the function of these regulators) whereas, the expression of *gadX* was repressed. The phenotypic analysis of *E. coli* strains lacking *OxyR* and *soxS*, two of the genes that participate in the oxidative stress response, revealed that the *soxS* null mutant exhibits high sensitivity to CORM-2 both under aerobic and anaerobic conditions, while *oxyR* mutant only displays elevated susceptibility to CORM-2 under aerobic conditions. These results suggest that CORM-2 increases the reactive oxygen species, which, under aerobic conditions, may be link to the inhibition of the respiration. Nevertheless, the increase of *soxS* transcription as well as the enhanced sensitivity displayed by the *soxS* mutant anaerobically needs to be rationalised.

CORM-2 also affects the expression of genes involved in biofilm formation, as jugged by the high number of CORM-2-regulated genes that were found in common with those whose expression was altered during the *E. coli* biofilm formation (see Table 6.A6). Hence, the total biofilm was measured in *E. coli* cells after being exposed to CORM-2. CORM-2 increases the biofilm production in *E. coli* as it also happens in the presence of several other stress agents like hydrogen peroxide, acid, low-temperature, and heavy metal stresses [47]. Given that bacterial biofilms display high resistance to antimicrobial agents by restricting penetration, slowing the bacterial growth owing to nutrient limitation and inducing the expression of genes involved in the general stress response, it is possible that *E. coli* cells exposed to CORM-2 trigger the production of biofilm as a defensive response [48].

Regarding the role of specific genes, phenotypic analysis of one of the biofilm-related genes, *bshA*, a gene that was highly induced in *E. coli* cells grown

anaerobically and treated with CORM-2, revealed that its deletion increases the resistance of *E. coli* to CORM-2, suggesting that BshA contribute to the susceptibility of *E. coli* to CORM-2 when grown under anaerobic conditions. BshA is reported to be a multiple stress resistance protein due to its relation with the increase of *E. coli* resistance to hydrogen peroxide, heat, acid, and cadmium. BshA is also proposed to be involved in the biofilm formation elicited by these stress agents [47]. However, we did not detect differences in the biofilm formation in Δ*bshA* cells exposed to CORM-2, when compared to the wild type, and hence concluded that BshA does not mediate the formation of biofilm elicited by CORM-2.

The expression of *tsqA* was induced by CORM-2 treatment and the study of the mutant strain revealed that the deletion of this gene increases the resistance of *E. coli* to CORM-2 and abolishes the increase of biofilm-induced by CORM-2. TsqA is proposed to export the quorum-sensing signal autoinducer-2 and is involved in the bacterial susceptibility to several antibiotics, namely streptomycin [49]. Our results demonstrate that TqsA not only increases the *E. coli* sensitivity to CORM-2, but also participates in the biofilm formation trigged by the presence of CORM-2 and suggest that TsqA may potentiate the entry of CORM-2 in *E. coli* cells.

The effect of CORM-3 in *E. coli* transcriptome was analysed by Davidge *et al*. [42]. In that study, *E. coli* cells grown aerobically in defined media with glycerol, and anaerobically in LB media supplemented with casamino acids using fumarate as terminal electron acceptor were treated with 30 μM and 100 μM CORM-3, respectively. As expected, the authors reported that the addition of CORM-3 to *E. coli* cells grown aerobically, down-regulates genes encoding proteins that form the aerobic respiratory complexes, namely the operon *cyoABCDE* encoding the cytochrome *bo´* haem-copper terminal oxidase. On the other hand, CORM-3 up-regulates many genes involved in zinc homeostasis, such as *yodA* and *znuA*, two genes that encode Zn-binding proteins. These genes were also induced in the microarray of the *E. coli* cells grown anaerobically together with *zraP*, a gene that also codes for a zinc-binding protein.

Comparing the transcriptional response of *E. coli* to CORM-2 and CORM-3, some genes were found to be regulated in common by the two CO-RMs. For

example, under aerobic conditions most of the genes involved in citric acid cycle were repressed by CORM-2 and CORM-3, while *metF*, involved in methionine metabolism and *mdtB*, a multidrug efflux system, were found to be induced. Under anaerobic conditions, *ftnA*, an iron storage protein was repressed whereas *zraP*, a zinc-binding periplasmic protein and *mdtA*, a multidrug efflux system, were found to be induced by the two CO-RMs. Interestingly, the genes *spy*, a envelope stress induced periplasmic protein and *cpxP*, whose function was mentioned above, were induced in the two microarray experiments and under both growth conditions, suggesting that CO-RMs affect the integrity of cell envelope proteins.

The most striking difference between the two microarray studies was that only CORM-3 seems to interfere with zinc homeostasis. However, Davidge and co-workers did not found any alteration in the total amount of intracellular zinc content after exposing *E. coli* cells to CORM-3 [42].

The differences that are detected between the two microarray experiments are most probable due to the different experimental conditions used regarding the growth media, the type and the concentration of CO-RM, and the compound used in control samples. Nevertheless, the two microarray studies revealed several potential targets for CO-RMs action that required further investigation.

Although CO is usually considered as a relatively inert molecule, its reactivity is significant with transition metals. Indeed, the data presented in this thesis indicate that CO is able to interfere with several genes revealing that targets other then haem iron need to be considered to account for the CO action.

Taking into consideration that CO also displays anti-inflammatory activity (Chapter 2) by inhibiting the expression of pro-inflammatory cytokines [50-52], and the pro-inflammatory action, by limiting sepsis [34], the function of CO may appear as paradoxal. In fact, as it happens with NO, the dosage of CO seems to control the balance between the pro- and anti-inflammatory action during infection. In this work, we started by showing that the exogenous administration of CO gas and CO-RMs efficiently kill bacteria and the role of the endogenous production of CO on bacteria needs now to be explored. As mentioned before, the results obtained, by Chung *et al.,* in mice deleted in HO-1 and in mice over-

expressing the enzyme, suggest that the products of HO activity are important in the control of the microbial sepsis. However, they do not prove the direct involvement of endogenous CO production in the killing of the pathogens since HO activity generates apart from CO, ferrous iron and bilirubin [35].

The emergence and spread of drug resistance bacteria, which reveals the ineffectiveness of currently available antibiotics, makes the search for new molecules to fight pathogens of great importance. The work developed during this thesis reveals novel strategies to improve bacterial clearance. In particular, it is described a novel class of antibiotics, the CO-releasing molecules and the potential mechanisms by which they mediate their bactericidal action, namely by causing alteration at the level of aerobic respiration, methionine metabolism, regulation of the transcription, protein homeostasis and biofilm formation.

Future work needs to be developed to understand how CO-RMs deliver CO to bacteria and why CO-RMs appear to be more effective than CO gas, as antimicrobial agent. The data acquired so far suggest that CO-RMs can form the basis for developing a novel therapeutic strategy against several pathogens. Nevertheless, novel CO-releasing molecules are needed, with different chemical characteristics to improve the bactericidal effectiveness of CO-RMs at lower concentrations, and to facilitate the delivery of CO to humans in a safe and more controlled way.

7.3 References

[1] Madigan, M.T., Martinko, J.M. and Parker, J. (2000) Biology of Microorganisms. Prentice-Hall, Inc, New Jersey.

[2] McInnes, I.B., Leung, B., Wei, X.Q., Gemmell, C.C. and Liew, F.Y. (1998) Septic arthritis following *Staphylococcus aureus* infection in mice lacking inducible nitric oxide synthase. J. Immunol. 160, 308-315.

[3] Sasaki, S., Miura, T., Nishikawa, S., Yamada, K., Hirasue, M. and Nakane, A. (1998) Protective role of nitric oxide in *Staphylococcus aureus* infection in mice. Infect. Immun. 66, 1017-1022.

[4] Gardner, P.R., Gardner, A.M., Martin, L.A. and Salzman, A.L. (1998) Nitric oxide dioxygenase: an enzymic function for flavohemoglobin. Proc. Natl. Acad. Sci. U S A 95, 10378-10383.

[5] Membrillo-Hernandez, J., Coopamah, M.D., Anjum, M.F., Stevanin, T.M., Kelly, A., Hughes, M.N. and Poole, R.K. (1999) The flavohemoglobin of *Escherichia coli* confers resistance to a nitrosating agent, a

"Nitric oxide Releaser," and paraquat and is essential for transcriptional responses to oxidative stress. J. Biol. Chem. 274, 748-754.

[6] Svensson, L., Marklund, B.I., Poljakovic, M. and Persson, K. (2006) Uropathogenic *Escherichia coli* and tolerance to nitric oxide: the role of flavohemoglobin. J. Urol. 175, 749-753.

[7] Ilari, A., Bonamore, A., Farina, A., Johnson, K.A. and Boffi, A. (2002) The X-ray structure of ferric *Escherichia coli* flavohemoglobin reveals an unexpected geometry of the distal heme pocket. J. Biol. Chem. 277, 23725-23732.

[8] Nobre, L.S., Goncalves, V.L. and Saraiva, L.M. (2008) Flavohemoglobin of *Staphylococcus aureus* . Methods Enzymol. 436, 203-216.

[9] Ermler, U., Siddiqui, R.A., Cramm, R. and Friedrich, B. (1995) Crystal structure of the flavohemoglobin from *Alcaligenes eutrophus* at 1.75 A resolution. Embo J. 14, 6067-6077.

[10] Gardner, P.R. (2005) Nitric oxide dioxygenase function and mechanism of flavohemoglobin, hemoglobin, myoglobin and their associated reductases. J. Inorg. Biochem. 99, 247-266.

[11] Kim, S.O., Orii, Y., Lloyd, D., Hughes, M.N. and Poole, R.K. (1999) Anoxic function for the *Escherichia coli* flavohaemoglobin (Hmp): reversible binding of nitric oxide and reduction to nitrous oxide. FEBS Lett. 445, 389-394.

[12] Poole, R.K. and Hughes, M.N. (2000) New functions for the ancient globin family: bacterial responses to nitric oxide and nitrosative stress. Mol. Microbiol. 36, 775-783.

[13] Gardner, A.M. and Gardner, P.R. (2002) Flavohemoglobin detoxifies nitric oxide in aerobic, but not anaerobic, *Escherichia coli*. Evidence for a novel inducible anaerobic nitric oxide-scavenging activity. J. Biol. Chem. 277, 8166-8171.

[14] Crawford, M.J. and Goldberg, D.E. (1998) Role for the Salmonella flavohemoglobin in protection from nitric oxide. J. Biol. Chem. 273, 12543-12547.

[15] Liu, L., Zeng, M., Hausladen, A., Heitman, J. and Stamler, J.S. (2000) Protection from nitrosative stress by yeast flavohemoglobin. Proc. Natl. Acad. Sci. U S A 97, 4672-4676.

[16] Richardson, A.R., Dunman, P.M. and Fang, F.C. (2006) The nitrosative stress response of *Staphylococcus aureus* is required for resistance to innate immunity. Mol. Microbiol. 61, 927-939.

[17] MacMicking, J., Xie, Q.W. and Nathan, C. (1997) Nitric oxide and macrophage function. Annu. Rev. Immunol. 15, 323-350.

[18] Tavares, A.F., Nobre, L.S., Melo, A.M. and Saraiva, L.M. (2009) A novel nitroreductase of *Staphylococcus aureus* with S-nitrosoglutathione reductase activity. J. Bacteriol. 191, 3403-3406.

[19] Hong, I.S., Kim, Y.K., Choi, W.S., Seo, D.W., Yoon, J.W., Han, J.W., Lee, H.Y. and Lee, H.W. (2003) Purification and characterization of nitric oxide synthase from *Staphylococcus aureus* . FEMS Microbiol. Lett. 222, 177-182.

[20] Gusarov, I., Shatalin, K., Starodubtseva, M. and Nudler, E. (2009) Endogenous nitric oxide protects bacteria against a wide spectrum of antibiotics. Science 325, 1380-1384.

[21] François, I.E.J.A., Cammue, B.P., Borgers, M., Ausma, J., Dispersyn, G.D. and Thevissen, K. (2006) Azoles: Mode of Antifungal Action and Resistance Development. Effect of Miconazole on Endogenous

Reactive Oxygen Species Production in *Candida albicans*. Anti-Infective Agents in Medicinal Chemistry 5, 3-13.

[22] Van Cutsem, J.M. and Thienpont, D. (1972) Miconazole, a broad-spectrum antimycotic agent with antibacterial activity. Chemotherapy 17, 392-404.

[23] Rautelin, H., Vaara, M., Renkonen, O.V., Kosunen, T.U. and Seppala, K. (1992) *In vitro* activity of antifungal azoles against *Helicobacter pylori*. Eur. J. Clin. Microbiol. Infect. Dis. 11, 273-274.

[24] Wu, G., Corker, H., Orii, Y. and Poole, R.K. (2004) *Escherichia coli* Hmp, an "oxygen-binding flavohaemoprotein", produces superoxide anion and self-destructs. Arch. Microbiol. 182, 193-203.

[25] Orii, Y., Ioannidis, N. and Poole, R.K. (1992) The oxygenated flavohaemoglobin from *Escherichia coli*: evidence from photodissociation and rapid-scan studies for two kinetic and spectral forms. Biochem. Biophys. Res. Commun. 187, 94-100.

[26] Poole, R.K., Rogers, N.J., D'Mello R, A., Hughes, M.N. and Orii, Y. (1997) *Escherichia coli* flavohaemoglobin (Hmp) reduces cytochrome *c* and Fe(III)-hydroxamate K by electron transfer from NADH via FAD: sensitivity of oxidoreductase activity to haem-bound dioxygen. Microbiology 143 (Pt 5), 1557-1565.

[27] Membrillo-Hernandez, J., Ioannidis, N. and Poole, R.K. (1996) The flavohaemoglobin (HMP) of *Escherichia coli* generates superoxide in vitro and causes oxidative stress *in vivo*. FEBS Lett. 382, 141-144.

[28] Wu, G., Wainwright, L.M. and Poole, R.K. (2003) Microbial globins. Adv. Microb. Physiol. 47, 255-310.

[29] De Nollin, S., Van Belle, H., Goossens, F., Thone, F. and Borgers, M. (1977) Cytochemical and biochemical studies of yeasts after *in vitro* exposure to miconazole. Antimicrob. Agents Chemother. 11, 500-513.

[30] Bossche, H.V., Lauwers, W., Willemsens, G., Marichal, P., Cornelissen, F. and Cools, W. (2006) Molecular basis for the antimycotic and antibacterial activity of N-substituted imidazoles and triazoles: The inhibition of isoprenoid biosynthesis Pesticide Science 15, 188-198.

[31] Helmick, R.A., Fletcher, A.E., Gardner, A.M., Gessner, C.R., Hvitved, A.N., Gustin, M.C. and Gardner, P.R. (2005) Imidazole antibiotics inhibit the nitric oxide dioxygenase function of microbial flavohemoglobin. Antimicrob. Agents Chemother. 49, 1837-1843.

[32] McElhaney-Feser, G.E., Raulli, R.E. and Cihlar, R.L. (1998) Synergy of nitric oxide and azoles against candida species *in vitro*. Antimicrob. Agents Chemother. 42, 2342-2346.

[33] Brashears, M.M. and Brooks, M.M. (2006) Effect of meat packaging technologies on the safety and spoilage-indicating characteristics of ground beef - phase 1: safety characteristics.

[34] Otterbein, L.E., May, A. and Chin, B.Y. (2005) Carbon monoxide increases macrophage bacterial clearance through Toll-like receptor (TLR)4 expression. Cell Mol. Biol. (Noisy-le-grand) 51, 433-440.

[35] Chung, S.W., Liu, X., Macias, A.A., Baron, R.M. and Perrella, M.A. (2008) Heme oxygenase-1-derived carbon monoxide enhances the host defense response to microbial sepsis in mice. J. Clin. Invest. 118, 239-247.

[36] Desmard, M., Davidge, K.S., Bouvet, O., Morin, D., Roux, D., Foresti, R., Ricard, J.D., Denamur, E., Poole, R.K., Montravers, P., Motterlini, R. and 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.

[37] Nobre, L.S., Seixas, J.D., Romao, C.C. and Saraiva, L.M. (2007) Antimicrobial action of carbon monoxide-releasing compounds. Antimicrob. Agents Chemother. 51, 4303-4307.

[38] Motterlini, R., Clark, J.E., Foresti, R., Sarathchandra, P., Mann, B.E. and Green, C.J. (2002) Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. Circ. Res. 90, E17-24.

[39] Motterlini, R., Mann, B.E. and Foresti, R. (2005) Therapeutic applications of carbon monoxide-releasing molecules. Expert. Opin. Investig. Drugs 14, 1305-1318.

[40] Foresti, R., Bani-Hani, M.G. and Motterlini, R. (2008) Use of carbon monoxide as a therapeutic agent: promises and challenges. Intensive Care Med. 34, 649-658.

[41] Vera, T., Henegar, J.R., Drummond, H.A., Rimoldi, J.M. and Stec, D.E. (2005) Protective effect of carbon monoxide-releasing compounds in ischemia-induced acute renal failure. J. Am. Soc. Nephrol. 16, 950-958.

[42] Davidge, K.S., Sanguinetti, G., Yee, C.H., Cox, A.G., McLeod, C.W., Monk, C.E., Mann, B.E., Motterlini, R. and Poole, R.K. (2009) Carbon monoxide-releasing antibacterial molecules target respiration and global transcriptional regulators. J. Biol. Chem. 284, 4516-4524.

[43] Weigel, P.H. and Englund, P.T. (1975) Inhibition of DNA replication in *Escherichia coli* by cyanide and carbon monoxide. J. Biol. Chem. 250, 8536-8542.

[44] Cairns, J. and Denhardt, D.T. (1968) Effect of cyanide and carbon monoxide on the replication of bacterial DNA *in vivo*. J. Mol. Biol. 36, 335-342.

[45] Danese, P.N. and Silhavy, T.J. (1998) CpxP, a stress-combative member of the Cpx regulon. J. Bacteriol. 180, 831-839.

[46] Lethanh, H., Neubauer, P. and Hoffmann, F. (2005) The small heat-shock proteins IbpA and IbpB reduce the stress load of recombinant *Escherichia coli* and delay degradation of inclusion bodies. Microb. Cell Fact. 4, 6.

[47] Zhang, X.S., Garcia-Contreras, R. and Wood, T.K. (2007) YcfR (BhsA) influences *Escherichia coli* biofilm formation through stress response and surface hydrophobicity. J. Bacteriol. 189, 3051-3062.

[48] Ito, A., Taniuchi, A., May, T., Kawata, K. and Okabe, S. (2009) Increased antibiotic resistance of *Escherichia coli* in mature biofilms. Appl. Environ. Microbiol. 75, 4093-4100.

[49] Herzberg, M., Kaye, I.K., Peti, W. and Wood, T.K. (2006) YdgG (TqsA) controls biofilm formation in *Escherichia coli* K-12 through autoinducer 2 transport. J. Bacteriol. 188, 587-598.

[50] Kim, H.P., Ryter, S.W. and Choi, A.M. (2006) CO as a cellular signaling molecule. Annu. Rev. Pharmacol. Toxicol. 46, 411-449.

[51] Otterbein, L.E., Bach, F.H., Alam, J., Soares, M., Tao Lu, H., Wysk, M., Davis, R.J., Flavell, R.A. and Choi, A.M. (2000) Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat. Med. 6, 422-428.

[52] Morse, D., Pischke, S.E., Zhou, Z., Davis, R.J., Flavell, R.A., Loop, T., Otterbein, S.L., Otterbein, L.E. and Choi, A.M. (2003) Suppression of inflammatory cytokine production by carbon monoxide involves the JNK pathway and AP-1. J. Biol. Chem. 278, 36993-36998.