The globins of *Campylobacter jejuni:*

A functional study in a heterologous host.

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Nada te turbe, nada te espante, todo se pasa, Dios no se muda. La paciencia todo lo alcanza. Quien a Dios tiene nada le falta: Sólo Dios basta.

Santa Teresa de Jesús

I hereby declare that no part of this thesis has previously been submitted for any degree or qualification at this, or any other University or Institute of learning.

Abstract

Campylobacter jejuni, a human pathogen, is exposed to NO and reactive nitrogen species (RNS) derived from the host during colonisation of the gut. As a response, *C. jejuni* expresses a single-domain globin (Cgb) and a truncated globin (Ctb) under control of the NssR regulator. Function of Cgb as an NO and RNS detoxification system and the involvement of Ctb in O_2 chemistry have been deduced from gene mutagenesis *in vivo* and spectroscopic and kinetic characterisation *in vitro*. However, confirmation of the Cgb activity and further exploration of the Ctb function(s) are restricted in *Campylobacter* by difficulties in complementation by transformation of plasmids, and the lack of the reductase domain that, in the flavohaemoglobins, reconstitutes the haem ferrous state required for ligand-binding activity. This limits additional insight into the molecular mechanisms of these globins.

In the present work, a functional study in the heterologous host *E. coli* was performed by cloning the *cgb and ctb* genes under control of arabinose-inducible promoters and expressing the globins in NO-sensitive strains. In this way, it was found that Cgb, but not Ctb, complements the *E. coli* NO and RNS resistance phenotype of an *E. coli hmp* mutant aerobically, confirming the function of Cgb as a NO and RNS resistance system. Interestingly, both Cgb- and Ctb-expressing cells consumed NO in an O_2-independent manner. However, Cgb failed to protect *E. coli* anaerobically. Spectroscopic changes of the Cgb and Ctb haems in cellular milieus were evaluated, showing that the haems are reduced in *E. coli* and *C. jejuni* soluble extracts even after oxidation by NO. Nevertheless, exploration of candidates for the Cgb electron donor revealed only a minor role for the *E. coli* flavorubredoxin reductase (NorW), and Cgb reduction was independent of the respiratory chain of *E. coli* and the lactate dehydrogenase (Cj1585) from *C. jejuni*, arguing in favour of a non-specific reductase system. Additionally, preliminary tests showed an increased NO evolution from Ctb-expressing *E. coli* presented with nitrite, suggesting that the globin functions as a NO reductase; NO production by purified Ctb from nitrite supports this hypothesis. Finally, the suitability of Ctb-expressing *E. coli* cells as a tool to measure CO-release from CO-releasing molecules (CO-RMs) is also presented. Unravelling the molecular mechanisms of these globins constitutes a key step in the understanding of the NO resistance ability of *C. jejuni*.

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Publications

These publications are directly relevant to this thesis and are enclosed at the end of the manuscript:

- Avila-Ramirez, C*., **Tinajero-Trejo, M***., Davidge, K. S., Monk, C. E., Kelly, D. J. and Poole, R. K. (2013). Do globins in microaerophilic *Campylobacter jejuni* confer nitrosative stress tolerance under oxygen limitation? *Antioxid. Redox Signal*. **18**, 424-31. (***** denotes equal contributions).

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Abbreviations

Caco-2 cells	Colorectal adenocarcinoma cells
CcP	Cytochrome *c* peroxidise
CDT	Cytolethal distending toxin
Cgb	*Campylobacter* single domain haemoglobin
CioAB	*Campylobacter* cyanide-insensitive oxidase
CO-Hb	Carboxy-haemoglobin
CO-Mb	Carboxy-myoglobin
Carboxy-PTIO	2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt, 2-(4-Carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazol-1-yloxy-3-oxide
CO-RM	CO-releasing molecule
CORM-3	$[Ru(CO)_3Cl$ (glycinate)]
Ctb	*Campylobacter* truncated haemoglobin
DETA NONOate	(Z)-1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate
DMSO	Dimethylsulfoxide
FHb	Flavohaemoglobin
GBS	Guillain-Barré syndrome
GSNO	*S*-nitrosoglutathione
HbN	*M. tuberculosis* TrHb1
HbO	*M. tuberculosis* TrHb2
Hmp	*E. coli* flavohaemoglobin
iNOS	Inducible NO synthase
LHb	Leghaemoglobin
M family	Mb-like family of globin proteins
MFS	Miller Fisher syndrome
Mb	Myoglobin

MetMb	Metmyoglobin
Nap	Periplasmic nitrate reductase
NOC-5	3-(Aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene
NOC-7	3-(2-Hydroxy-1-methyl-2-nitrosohydrazino)-N-methyl-1-propanamine
NOD	Nitric oxide dioxygenase
NOS	NO synthase
Nrf	Periplasmic nitrite reductase
NssR	*Campylobacter* nitrosative stress-responsive regulator
O_2-Hb	Oxy-haemoglobin
Pgb	Protoglobin
Phox	Phagocyte NADPH oxidase
PROLI-NONOate	1-(hydroxi-NNO-azoxy)-L-proline
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDgb	Single domain haemoglobin
S family	Sensor globin family
SNAP	*S*-nitroso-N-acetylpenicillamine
SNP	Sodium nitroprusside
SNOs	*S*-nitrosothiols
swMb	Sperm whale myoglobin
TrHb	Truncated haemoglobin
T family	Truncated Mb-fold family
TMAO	Trimethylamine-N-oxide
Vgb	*Vitreoscilla* single domain haemoglobin

CHAPTER 1. Introduction

1.1 Nitric oxide and reactive nitrogen species (RNS) in biology

Nitric oxide (nitrogen monoxide, NO) is a free radical able to react with other existing radicals due to the presence of an unpaired electron (Halliwell and Gutteridge, 2007). The chemistry and biological chemistry of NO and related molecules is highly complex; NO in cellular environments reacts with a number of targets and generates a large number of species that in turn interact with other molecules (reviewed in Lehnert and Scheidt, 2009) .

The NO intracellular toxicity mechanisms are related to the oxidation of the radical and the production of a number of poisonous substances such as the nitrosating agent nitrosonium (NO^+), nitrite (NO_2^-) and peroxynitrite ($ONOO^-$) (Poole and Hughes, 2000). The last arises from the reaction of NO with superoxide (O_2^-) (Hughes, 1999) and, in the cellular environment, $ONOO^-$ reacts with carbon dioxide rendering the adduct ($ONOOCO_2^-$). This product is then broken down via two mechanisms, one evolving carbon dioxide (CO_2) and nitrate (NO_3^-), and the other producing nitrogen dioxide (NO_2) and the carbonate radical ion (reviewed in Bowman *et al*., 2011; Poole and Hughes, 2000).

Although NO is a toxic molecule, it has important functions in biological systems. In 1980, it was discovered that NO is synthesized in mammals as a signalling and immune defence mechanism (Culotta and Koshland, 1992). Since then the transformations of NO and RNS in physiological conditions have been extensively studied. NO is a freely diffusible radical that, *in vivo*, is relatively stable. These characteristics together with the extraordinary high affinity for haem groups make it suitable as a signalling molecule in the cardiovascular and neuronal systems in subnanomolar concentrations. Endothelial cells generate NO that produces relaxation of the vascular smooth muscle, partially related to activation of guanylate cyclase (Murad, 1986). On the other hand, as a response to chronic inflammation and other disease conditions, macrophages can produce up to micromolar concentrations of NO that are bactericidal. For these reasons, tight control of the intracellular concentrations of NO is essential (Singel and Stamler, 2005; Thomas *et al.*, 2008).

Production of NO by NO synthases (NOSs) in a range of cell types results from oxidation of L-arginine to L-citrulline and NO in an NADPH- and O_2-dependent reaction (Stuehr, 1999). In mammals, three NOS isoforms coexist, two constitutively expressed, endothelial (eNOS) and neuronal (nNOS), and an inducible NOS (iNOS) which, in response to infection, produce high levels of NO (reviewed in Alderton *et al*., 2001; Lowenstein and Padalko, 2004).

Key bacterial enzymes such as terminal oxidases (Stevanin *et al.*, 2000) and aconitase (Gardner *et al.*, 1997) are inhibited by high and sustained concentrations of NO generated by the immune system. NO diffuses across the bacterial membrane toward the cytoplasm where it reacts with haems (Hausladen *et al.*, 2001), iron-sulfur (Fe-S) clusters (Cruz-Ramos *et al.*, 2002), and thiols (Hess *et al.*, 2005).

In addition to the toxic effects of NO, it exerts biological roles as a modulator of protein function through the *S*-nitrosylation of specific cysteine thiols in bacteria. Nitrosative stress triggered by the presence of NO and other RNS elicits adaptative responses including the expression of genes related to NO and RNS tolerance and detoxification (Avila-Ramirez *et al.*, 2013; Flatley *et al.*, 2005; Monk *et al.*, 2008; Moore *et al.*, 2004; Mukhopadhyay *et al.*, 2004; Pullan *et al.*, 2007; Richardson *et al.*, 2006 and many others).

Production of endogenous NO, in particular in bacteria that use nitrite as an electron acceptor in the absence of oxygen has been proposed. Certainly, low concentrations of intracellular NO are accumulated as a byproduct of nitrite reduction to ammonia in enteric bacteria, such as *E. coli* (Corker and Poole, 2003; Gilberthorpe and Poole, 2008); however, the physiological role of NO production, if any, is poorly understood. Interestingly, several bacteria possess NOS enzymes (reviewed by Bowman *et al*., 2011); in Gram-positives, NO production has been suggested to confer alleviation of oxidative stress and resistance to antibiotics through chemical modification (Gusarov *et al.*, 2009).

Resistance to NO and RNS in bacteria has been mainly related to the presence of haemoglobins (Poole, 2005) (see section 1.3). However, nitrosative stress tolerance has also been associated with other proteins. For instance, in *E. coli*, the main mechanism for NO detoxification in aerobic conditions is the flavohaemoglobin Hmp (Gardner *et*

al., 1998a). However, in the absence of oxygen, the NO sensor NorR positively controls the expression of the flavorubredoxin protein (NorV) and its reductase (NorW). This pair is the most important NO detoxification mechanism in anaerobic conditions by the reduction of NO to N_2O (Gardner and Gardner, 2002; Hutchings *et al.*, 2002).

1.2 Oxygen and reactive oxygen species (ROS) in biology

The limited tolerance of microorganisms to oxygen is well documented. Anaerobes and microaerophiles are unable to grow in air-saturated conditions and committed aerobes suffer deleterious or lethal effects in hypoxic environments. Dioxygen (O_2) is an inefficient oxidant of organic molecules such as amino acids and nucleic acids due to its stability and moderately weak capacity to accept electrons. However, O_2 readily reacts with organic radicals and transitions metals. On the other hand, ROS such as hydrogen peroxide (H_2O_2), superoxide (O_2^-) and the hydroxide ion (OH^-) are much stronger oxidants (Imlay, 2003).

Oxidative stress has been defined as "a disturbance in the prooxidant-antioxidant balance in favour of the former, leading to potential damage" (Sies, 1991). The rate of H_2O_2 and O_2^- formation is associated with the level of oxidative stress undergone by a microorganism (Imlay, 2003). Intracellular generation of ROS (O_2^-, H_2O_2) comes from the partially reduced oxygen species that are formed during the accidental reduction of oxygen by redox centres of electron-transfer enzymes, especially the ubiquitous flavoenzymes (Fridovich, 1999; Imlay, 2008; Massey *et al.*, 1969).

The irreversible deleterious effects caused by high levels of ROS are related to damage of biomolecules such as oxidation of Fe-S proteins and DNA (Farr *et al.*, 1986; Farr and Kogoma, 1991; Imlay, 2008; Jang and Imlay, 2007). The reaction between H_2O_2 and transition metals such as ferrous iron generates OH^- (Fenton's reaction) that in turn reacts with both base and sugar molecules producing irreversible damage to DNA (Henle *et al.*, 1999; Hutchinson, 1985).

Superoxide is a major antibacterial weapon (Huang and Brumell, 2009; Mastroeni *et al.*, 2000). During bacterial infection, elevated concentrations of O_2^- are produced within the macrophage mainly via the enzymatic complex NADPH oxidase (Phox), (Babior, 1999; Miller, 1997). Chronic granulomatous disease, developed as a result of a genetically

defective production of O_2^-, produces recurrent life-threatening bacterial and fungal infections in humans (van den Berg *et al.*, 2009; Winkelstein *et al.*, 2000).

Specific microbial defences are triggered in response to oxidative stress. In the presence of ROS, up-regulation of the SoxR(S) and OxyR regulons (Aussel *et al.*, 2011; Greenberg *et al.*, 1990; Tsaneva and Weiss, 1990; Zheng *et al.*, 2001) is followed by the induction of several protective proteins, including superoxide dismutase (SOD) and catalase. The preponderant role of these enzymes as bacterial ROS scavengers has been established (Imlay, 2008). For instance, accumulation of O_2^- in *E. coli* lacking SOD under aerobic conditions leads to defective growth (Carlioz and Touati, 1986) and accumulation of H_2O_2 in a catalase/peroxidase mutant produces extensive biochemical damage (Jang and Imlay, 2007; Park *et al.*, 2005).

1.3 Bacterial globins

After a number of reports about the existence of a 'soluble cytochrome *o*' in the obligate aerobe bacterium *Vitreoscilla* (Orii and Webster, 1977; Webster and Orii, 1977; Webster and Orii, 1978; Webster and Orii, 1985), the protein sequence revealed, for the first time, the presence of a dimeric haemoglobin (Wakabayashi *et al.*, 1986). A more complex globin was later described in *E. coli*, the flavohaemoglobin Hmp, a chimeric protein containing an N-terminal globin domain and a C-terminal reductase domain able to bind NADPH and FAD (Vasudevan *et al.*, 1991). Since then, a variety of bacterial haemoglobins have been extensively studied (Forrester and Foster, 2012; Vinogradov *et al.*, 2013; Wu *et al.*, 2003). Vinogradov *et al* (2005) reported the existence of two globin families showing the canonical 3/3 myoglobin-fold (Mb-fold), containing the flavohaemoglobin (FHb) and the globin-coupled sensor (GCS) families, and a third family with a truncated (Tr) Mb-fold (2/2 Mb-fold) with a characteristic vestigial or absent helix A and the presence of a loop instead of helix E (Pesce *et al.*, 2000).

Truncated hemoglobins (trHbs) are composed of 110 to 130 amino acids and are distantly but clearly related to hemoglobin (Hb) and myoglobin (Mb). Examples of TrHbs are found in eubacteria, cyanobacteria, protozoa and plants. According to the amino acid sequences, there are three TrHbs sub-families, and they have been nominated either I, II and III or N, O and P (Vinogradov *et al.*, 2013; Wittenberg *et al.*,

2002). There are notable differences among these groups; the amino acids identity can be as low as 18% (Milani *et al.*, 2003a).

A complete inventory of globins present in the Bacterial kingdom has been recently published (Vinogradov *et al*., 2013). This bioinformatics survey was aimed to provide sequences like-globins in over 2200 bacterial genomes. Approximately half of the genomes contained possible genes encoding globins. Examples of all classes of bacterial haemoglobins, flavohaemoglobins, single domain haemoglobins, truncated haemoglobins and sensor globins were found, and many genomes contained more than one globin-like sequence.

1.3.1 Globin nomenclature

Due to the absence of a general globin phylogeny and an inconsistent identification of bacterial globins in the GeneBank, a more comprehensive nomenclature that includes both prokaryotic and eukaryotic globins was proposed: the 3/3 alpha-helical fold (Mb-fold) contains two families, the Mb-like family (M family) including both flavohaemoglobins (FHb) and single domain haemoglobins (SDgb) and the sensor globin family (S family) comprising protoglobins (Pgbs) and single domain sensor globins (SDSgb). The truncated Mb-fold family (T family) includes TrHb1, TrHb2 and TrHb3 (Vinogradov *et al*., 2013) (Table 1.1).

1.3.2 Globin functions

Even though there are numerous globins in bacteria, the physiological function(s) of these proteins has been studied only in a few examples. Figure 1.1 illustrates the differences between the numbers of globin-like sequences found in the prokaryotic databases and the experimental data reported for individual globins.

1.3.2.1 Crystal structures

From 1161 globin-containing genomes, only 17 crystal structures of individual globins have been solved (Table 1.2). Representative examples for the eight subfamilies are covered. Inference of function from the structural data in isolation is difficult although comparison with other globins whose function is known allows predictions. For instance, the structural homology between the SDgb from *Campylobacter jejuni* (Cgb)

Table 1.1 Proposed globin global nomenclature.

Family	M (Mb-like globins)		S (sensor globins)			T (truncated Mb-fold)		
Mb-fold	3/3			3/3		2/2		
Bacterial subfamily	FHbs (flavo-hemoglobin)	SDgb (single-domain globins)	GCSs (globin-coupled sensors)	Pgbs (proto-globins)	SDSgbs (single domain sensor globins)	TrHb1s N	TrHb2s O	TrHb3s P
Archaea			HemATs	Pgb	SDSgb	TrHb1		
Eukaryote	FHbs	All animal globins			SDSgb	TrHb1	TrHb2	

Taken from Vinogradov *et al*., (2013).

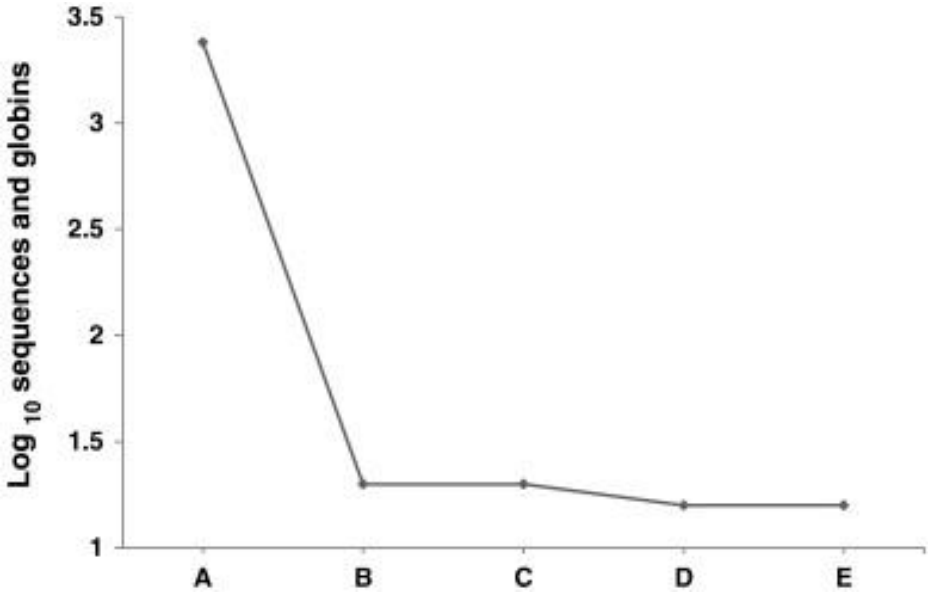


Figure 1.1 Comparison of estimated number of globin-like sequences found in the prokaryotic databases with the experimental data reported for individual globins.

Base 10 logarithm of (A) estimated number of globin-like sequences, (B) number of globins heterologously expressed for studies of function, (C) number of globins in which regulation has been explored (D) number of globins for which structures have been solved, and (E), number of globins studied by mutation/complementation. Taken from Vinogradov *et al*. (2013).

Table 1.2 Bacterial and Archael globins for which the crystal structures have been solved.

Origen of globin	Haemoglobin sub-family	Globin name	References
Ralstonia eutropha (*Alcaligenes eutrophus*)	FHb	FHP	(El Hammi *et al.*, 2011; Ermler *et al.*, 1995a; Ermler *et al.*, 1995b)
Escherichia coli	FHb	Hmp	(Ilari *et al.*, 2002b)
Vibrio cholerae	FHb	HmpA	Unpublished
Vitreoscilla sp	SDgb	Vgb, VHb	(Bolognesi *et al.*, 1999; Tarricone *et al.*, 1997b)
Campylobacter jejuni	SDgb	Cgb	(Shepherd *et al.*, 2010)
Methylacidiphilum infernorum	SDgb	HGbI	(Pechkova *et al.*, 2012)
Mycobacterium tuberculosis	TrHb (TrHb1)	HbN	(Bidon-Chanal *et al.*, 2006; Milani *et al.*, 2001; Milani *et al.*, 2004; Ouellet *et al.*, 2006; Savard *et al.*, 2011)
Mycobacterium tuberculosis	TrHb (TrHb2)	HbO	(Milani *et al.*, 2003b; Ouellet *et al.*, 2007)
Synechocystis sp.	TrHb (TrHb1)	rHb-R, SynHb	(Falzone *et al.*, 2002; Hoy *et al.*, 2004; Hoy *et al.*, 2007; Trent *et al.*, 2004)
Campylobacter jejuni	TrHb (TrHb3)	Ctb	(Nardini *et al.*, 2006)
Bacillus subtilis	TrHb(TrHb2)	Bs-trHb	(Giangiacomo *et al.*, 2005)
Geobacillus stearothermophilus	TrHb (TrHb2)	Gs-trHb	(Ilari *et al.*, 2007)
Thermobifida fusca	TrHb (TrHb2)	Tf-trHb	(Bonamore *et al.*, 2005)
Agrobacterium tumefaciens	TrHb (TrHb2)	At-2/2HbO	(Pesce *et al.*, 2011)
Methanosarcina acetivorans	Pgb	MaPgb	(Nardini *et al.*, 2008)
Bacillus subtilis	GCS	HemAT-Bs	(Zhang and Phillips, 2003)
Geobacter sulfurreducens	GCS globin domain	GsGCS	(Pesce et al., 2009)

(Shepherd *et al.*, 2010) and the globin domain of the FHb from *E. coli* (Hmp) (Ilari *et al.*, 2002b) lead to the proposal of the same molecular mechanism to detoxify NO in both proteins, i.e. a denitrosylase or dioxygenase function (see below). Besides, once the globin function has been revealed by other means, structures are valuable in showing consistencies between protein architecture and biological implications (Vinogradov *et al*., 2013).

1.3.2.2 Studies of globin function by heterologous expression

Heterologous expression is a common practice to over-express recombinant proteins for purification purposes. Interestingly, it is also used as an alternative to study function of proteins from microorganisms where genetic manipulation is difficult or not available (e.g *Vitreoscilla*). A number of bacterial haemoglobins have been heterologously expressed and specific phenotypes found everywhere (Table 1.3). The NO-sensitivity phenotype shown by *E. coli* lacking the FHb (Hmp) has been widely used as a model to study the role of heterologous globins in nitrosative stress resistance. For instance, expression of the *Pseudoalteromonas haloplanktis* truncated haemoglobin (PhHbO) in *E. coli hmp* led to the discovery of its ability to detoxify NO and RNS. A role for PhHbO as a mechanism to protect this Antarctic bacterium from nitrosative stress was suggested. However, this function has not been demonstrated in the actual host (Coppola *et al.*, 2013).

1.3.2.3 Studies of globin function by mutation/complementation

Mutation of encoding-globin genes constitutes perhaps the most robust approach for the study of function. Interestingly, the number of studies where the potential function has been aimed by these means is small (c. 15 globins). In thirteen of these examples, the globins (9 of them FHbs) are implicated in resistance to nitrosative stress (Table 1.4). Only three truncated haemoglobins have been studied by gene mutation, the TrHb3 from *C. jejuni* (Ctb), the TrHb1 from *Synechococcus* sp (GlbN) and the TrHbII from *Pseudoalteromonas haloplanktis.* The former has been implicated in oxygen transfer (Wainwright *et al.*, 2005) while the latter in resistance to NO (Scott *et al.*, 2010).

Table 1.3 Bacterial haemoglobins that have been heterologously expressed for studies of function.

Origin of globin, globin sub-family and globin name	Heterologous host	Suggested function(s)	References
Vitreoscilla sp, SDgb, VHb	*E. coli* WT*, *Bacillus subtilis* WT	Oxygen transfer, NO scavenging	(Dikshit and Webster, 1988; Dikshit *et al.*, 1992; Frey *et al.*, 2002; Kallio and Bailey, 1996; Kallio *et al.*, 2007; Kallio *et al.*, 1996; Khosla and Bailey, 1988; Khosla *et al.*, 1990b; Ramandeep *et al.*, 2001)
R. eutropha, FHb, FHP	*E. coli* WT	Oxygen transfer, increased cell growth in microaerobic conditions	(Frey *et al.*, 2000; Frey *et al.*, 2002)
E. coli, FHb, Hmp	*E. coli* WT	Oxidative stress resistance, NO consumption, increased cell growth in microaerobic conditions	(Bollinger *et al.*, 2001; Frey *et al.*, 2002)
B. subtilis, FHb, HmpBs	*E. coli* WT	NO consumption in cellular extracts	(Bollinger *et al.*, 2001; Frey *et al.*, 2002)
P. aeruginosa, FHb, HmpPa	*E. coli* WT	Oxidative stress resistance	(Bollinger *et al.*, 2001; Frey *et al.*, 2002; Kallio *et al.*, 2007)
Deinococcus radiodurans, FHb, HmpDr	*E. coli* WT	Oxidative stress resistance, increased cell growth in microaerobic conditions	(Bollinger *et al.*, 2001; Frey *et al.*, 2002)
C. jejuni, SDgb, Cgb or CHb	*E. coli* WT	NO scavenging	(Bollinger *et al.*, 2001; Frey *et al.*, 2002)
S. enterica serovar Typhi, FHb, HmpSt	*E. coli* WT	Oxidative stress resistance, NO consumption in cellular extracts, increased cell growth in microaerobic conditions	(Bollinger *et al.*, 2001; Frey *et al.*, 2002)
Klebsiella pneumonia, FHb, HmpKp	*E. coli* WT	NO consumption in cellular extracts	(Bollinger *et al.*, 2001; Frey *et al.*, 2002)

(*continued*)

*Wild type

Table 1.3 Bacterial haemoglobins that have been heterologously expressed for studies of function (*continued*)

Origen of globin, globin sub-family and globin name	Heterologous host	Suggested function(s)	References
M. tuberculosis, TrHb1, HbN	*E. coli hmp*, *M. smegmatis* WT, *Salmonella* serovar *Typhimurium hmp*	NO scavenging	(Lama *et al.*, 2009; Pathania *et al.*, 2002a; Pawaria *et al.*, 2007)
M. tuberculosis, TrHb2, HbO	*E. coli* WT, *E. coli cyoB*, *M. smegmatis* WT, *Salmonella* serovar *Typhimurium hmp*	Oxygen transfer, increased cell growth.	(Liu *et al.*, 2004; Pathania *et al.*, 2002b; Pawaria *et al.*, 2007)
M. tuberculosis, FHb, MtbFHb	*E. coli* WT, *M. smegmatis* WT	D-lactate:phenazine methosulfate reductase activity, oxidative stress resistance	(Gupta *et al.*, 2012; Gupta *et al.*, 2011)
M. leprae, TrHb2, HbO	*E. coli hmp*	NO scavenging	(Fabozzi *et al.*, 2006)
M. smegmatis, TrHb1, HbN	*E. coli hmp*	Low level of NO scavenging	(Lama *et al.*, 2006)
B. halodurans, FHb, HmpBh	*E. coli hmp*	Increased cell growth in microaerobic conditions	(Kallio *et al.*, 2007)
Novosphingobium aromaticivorans, SDgb, NHb	*E. coli hmp*	Unidentified	(Kallio *et al.*, 2007)
Synechocystis, TrHb1, SynHb	*E. coli hmp*	NO scavenging	(Smagghe *et al.*, 2008)
Pseudoalteromonas haloplanktis, TrHb2, PhHbO	*E. coli hmp*	NO scavenging	(Coppola *et al.*, 2013)

Table 1.4 Bacterial haemoglobins in which physiological role(s) have been studied by gene mutation/complementation.

Bacterium, Globin sub-family and globin name	Suggested function(s)	References
E. coli, FHb, Hmp	O_2 dependant NO detoxification to nitrate (alleviation of NO toxicity)	(Corker and Poole, 2003; Gardner and Gardner, 2002; Gardner *et al.*, 1998a; Gardner *et al.*, 1998b; Hausladen *et al.*, 1998; Hernandez-Urzua *et al.*, 2003; Justino *et al.*, 2005; Membrillo-Hernandez *et al.*, 1999; Stevanin *et al.*, 2000; Stevanin *et al.*, 2007; Svensson *et al.*, 2010)
Salmonella enterica serovar Typhimurium, FHb, Hmp	NO detoxification	(Bang *et al.*, 2006; Crawford and Goldberg, 1998b; Gilberthorpe *et al.*, 2007; Gilberthorpe and Poole, 2008; McLean *et al.*, 2010; Park *et al.*, 2011; Stevanin *et al.*, 2002)
Campylobacter jejuni, SDgb, Cgb	NO detoxification	(Avila-Ramirez *et al.*, 2013; Elvers *et al.*, 2004; Pittman *et al.*, 2007)
Campylobacter coli, SDgb, Cgb	NO detoxification	(Elvers *et al.*, 2004)
C. jejuni, TrHb3, Ctb	Oxygen metabolism	(Wainwright *et al.*, 2005)
Ralstonia eutropha, FHb, FHPSt	Accumulation of nitrous oxide during denitrification	(Cramm *et al.*, 1994)
Erwinia chrysanthemi, FHb, HmpX	Implied in survival *in plant* and in synthesis of pectate lyases, NO detoxification	(Boccara *et al.*, 2005; Favey *et al.*, 1995)
B. subtilis, FHb, HmpBs	Anaerobic protection against prolonged nitrosative stress, NO detoxification	(Nakano, 2006; Rogstam *et al.*, 2007)
Synechococcus sp., TrHb1, GlbN	NO detoxification	(Scott *et al.*, 2010)
Staphylococcus aureus, FHb, Hmp	NO detoxification under microaerophilic condition, resistance to azoles	(Goncalves *et al.*, 2006; Nobre *et al.*, 2008; Nobre *et al.*, 2010; Richardson *et al.*, 2006)

(*continued*)

Table 1.4 Bacterial haemoglobins in which physiological role(s) have been studied by gene mutation/complementation. (*continued*)

Bacterium, Globin sub-family and globin name	Suggested function(s)	References
Vibrio fischeri, FHb, Hmp	NO detoxification	(Wang *et al.*, 2010b)
Sinorhizobium meliloti, putative FHb, no name available.	NO detoxification	(Meilhoc *et al.*, 2010)
V. cholerae, FHb, HmpA	NO detoxification	(Davies *et al.*, 2011; Stern *et al.*, 2012)
P. aeruginosa, FHb, HmpPa	NO detoxification	(Arai *et al.*, 2005)
Pseudoalteromonas haloplanktis, TrHb2, PhHbO	Oxidative and nitrosative stress resistance	(Parrilli *et al.*, 2010)

Taken from Vinogradov *et al*., (2013)

1.3.2.4 Globin regulation

Even though there are a number of studies that report transcriptional changes (up-regulation) of bacterial globin genes in specific conditions (e.g. nitrosative stress, oxygen limitation, etc.), in only a half of the cases (c. 9 of 19) transcriptional regulators associated with globin expression are identified (Table 1.5). For instance, in agreement with the role of Hmp in alleviating NO toxicity, up-regulation of *hmp* occurs only in the presence of NO or RNS. Hmp expression in *E coli* and *Salmonella* is mainly controlled at the transcriptional level by the NO-responsive transcription factor NsrR (Filenko *et al.*, 2007b; Spiro, 2007).

NsrR (Bodenmiller and Spiro, 2006) represents the archetype regulator for proteins involved in NO detoxification such as FHbs in enterobacteria. NO in solution and probably the small levels of NO derived from *S*-nitrosothiols (SNOs) or NO_2^- reduction are sensed by this regulator that in turn de-represses its regulated genes. Indeed, in the presence of GSNO, an *nsrR* mutant produces remarkably high levels of Hmp (Gilberthorpe *et al.*, 2007). Constitutive expression of the *E. coli* FHb in the absence of NO results in detrimental production of superoxide anion in a reaction involving oxygen reduction by the heme (McLean *et al.*, 2010; Poole *et al.*, 1997; Wu *et al.*, 2004), revealing the need for tight control of Hmp synthesis.

1.4 The genus *Campylobacter*

Cells of *Campylobacter* (for 'twisted bacteria') are spiral, curved, 0.5 to 5 μm long and 0.2 to 0.8 μm wide. In old cultures, they tend to become coccoid, considered a degenerative feature more than a dormant cellular stage. Most of the species possess a single flagellum at one or both cell poles that enables the characteristic cork-screw-like motion (Debruyne *et al.*, 2008).

After the isolation of a *Vibrio*-like organism from aborted ovine foetuses by McFadyean and Stockman in 1913, followed by the establishment of the genus *Campylobacter* in 1963 (Butzler, 2004; Skirrow, 2006), Véron and Chatelain described four species within the genus *Campylobacter* (Véron and Chateline, 1973), *C. fetus*, *C. coli*, *C. jejuni* and *C. sputorum*. Currently, the *Campylobacteraceae* family includes at least 15 *Campylobacter* species (Debruyne *et al.*, 2008). These microorganisms are found in a

Table 1.5 Bacterial haemoglobins in which regulation has been studied.

Bacterium, globin sub-family and globin name	Regulator(s) involved	Environmental factors and/or compounds involved in up-regulation	References
E. coli, FHb, Hmp	Fnr, MetR, NsrR	Nitrosative stress	(Anjum *et al.*, 1998; Bodenmiller and Spiro, 2006; Cruz-Ramos *et al.*, 2002; Filenko *et al.*, 2007b; Flatley *et al.*, 2005; Justino *et al.*, 2005; Membrillo-Hernandez *et al.*, 1999; Membrillo-Hernández *et al.*, 1998; Membrillo-Hernandez *et al.*, 1997; Mukhopadhyay *et al.*, 2004; Poole *et al.*, 1996)
Vitreoscilla, SDgb, Vgb	CRP, Fnr, ArcA, OxyR and RhyB in *E. coli*	Up-regulation in oxygen-limited conditions in the native host and in *E. coli*, carbon-limited conditions in *E. coli*.	(Boerman and Webster, 1982; Bollinger and Kallio, 2007; Dikshit *et al.*, 1990; Dikshit *et al.*, 1989; Khosla and Bailey, 1988; Khosla and Bailey, 1989; Khosla *et al.*, 1990a; Tsai *et al.*, 1995; Webster and Hackett, 1966; Yang *et al.*, 2005)
Salmonella enterica serovar Typhimurium, FHb, Hmp	Fur, NorR	Nitrosative stress	(Crawford and Goldberg, 1998a; Gilberthorpe *et al.*, 2007; Hernandez-Urzua *et al.*, 2007)
Bacillus subtilis, FHb, Hmpbs	ResD, ResE, Fnr, NsrR	Oxygen limitation, nitrite, NO	(Kommineni *et al.*, 2012; LaCelle *et al.*, 1996; Moore *et al.*, 2004; Nakano, 2002; Nakano *et al.*, 2006; Rogstam *et al.*, 2007)
Staphylococcus aureus, FHb, Hmp	SrrAB	Nitrosative stress, oxygen limitation	(Goncalves *et al.*, 2006; Nobre *et al.*, 2008; Richardson *et al.*, 2006)
Vibrio fischeri, FHb, Hmp	NsrR	NO, initial stage of colonization	(Wang *et al.*, 2010a; Wang *et al.*, 2010b)
V. cholerae, FHb, HmpA	NorR	NO, infant mice and rabbits	(Mandlik *et al.*, 2011; Schild *et al.*, 2007; Stern *et al.*, 2012)
C. jejuni, SDgb, Cgb	NssR	Nitrosative stress	(Avila-Ramirez *et al.*, 2013; Elvers *et al.*, 2005; Monk *et al.*, 2008; Pittman *et al.*, 2007)
C. jejuni, TrHb3, Ctb	NssR	Nitrosative stress	(Avila-Ramirez *et al.*, 2013; Elvers *et al.*, 2005; Monk *et al.*, 2008; Smith *et al.*, 2011)
Pseudomonas aeruginosa, FHb, HmpPa	FhpR	Nitrosative stress	(Arai *et al.*, 2005)
Nostoc spp, TrHb1, GlbN	Unidentified	Oxygen limitation	(Hill *et al.*, 1996)

(*Continued*)

Table 1.5 Bacterial haemoglobins in which regulation has been studied (*continued*)

Bacterium, globin sub-family and globin name	Regulator(s) involved	Environmental factors and/or compounds involved in up-regulation	References
M. tuberculosis, TrHb1, HbN	Unidentified	Stationary phase, nitrite, sodium nitroprusside, hypoxia, intracellular growth in macrophages. Early response to oxidative and nitrosative stress (transcriptional fusions in *M. smegmatis*)	(Couture *et al.*, 1999; Joseph *et al.*, 2012; Pawaria *et al.*, 2008)
M. tuberculosis, TrHb2, HbO	Unidentified	Expressed in all growth phases, up regulated by H_2O_2, hypoxia, intracellular growth in macrophages, nitrite	(Joseph *et al.*, 2012; Pathania *et al.*, 2002b; Pawaria *et al.*, 2008)
M. tuberculosis, FHb, MtbHFb	Unidentified	Early exponential phase, oxygen limitation conditions and GSNO, SNP in microaerophilic conditions. Late exponential and stationary phase in aerobically cultures, H_2O_2, macrophages infection.	(Gupta *et al.*, 2012; Hu *et al.*, 1999)
M. leprae, TrHb2, HbO	Unidentified	Constitutively expressed through the whole growth cycle, up-regulated by nitrosative stress.	(Fabozzi *et al.*, 2006)
Synechococcus sp, TrHb1, no name.	Unidentified	Likely constitutively expressed (unknown)	(Scott *et al.*, 2010)
Sinorhizobium meliloti, putative FHb, no name	Unidentified	NO	(Meilhoc *et al.*, 2010)

(*continued*)

Table 1.5 Bacterial haemoglobins in which regulation has been studied (*continued*)

Bacterium, globin sub-family and globin name	Regulator(s) involved	Environmental factors an/or compounds involved in up-regulation	References
Frankia, putative TrHb1, no name	Unidentified	Nitrosative stress	(Niemann and Tisa, 2008)
Frankia, putative TrHb2, no name	Unidentified	Low oxygen	(Niemann and Tisa, 2008)

variety of niches; from commensals or parasites in domestic animals and humans to free-living environmental organisms.

1.5 *Campylobacter jejuni*

1.5.1 Campylobacteriosis, epidemiology, causes and consequences

In the developed world, *Campylobacter* represents one of the main causes of bacterial gastroenteritis (Friedman *et al.*, 2000; Scallan *et al.*, 2011; Simonsen *et al.*, 2011). Approximately 9.4 million episodes of food-borne infections occur in the United States every year; *Campylobacter* spp. are responsible for 9% of those cases and 15% of the related hospitalisations (Scallan *et al.*, 2011).

In the lower intestine of chickens, *C. jejuni* is found as a commensal. However, it is a common pathogen in humans. Poultry products contaminated during processing represent an important route of transmission (Friedman *et al.*, 2000). An inflammatory response resulting from infection by *C. jejuni* (Bakhiet *et al.*, 2004; Jacobs *et al.*, 1998; Koga *et al.*, 2005; Zheng *et al.*, 2008) is related to pathological symptoms such as abdominal pain, diarrhoea, blood in stool, fever and vomiting. Although, in the majority of the cases, symptoms associated with campylobacteriosis are self-limited and the infection is usually restricted to the intestine, invasion of other tissues, mostly in elderly and immunocompromised patients, ends in significant morbidity and mortality (Allos, 2001; Wassenaar and Blaser, 1999). Furthermore, autoimmune diseases such as Guillain-Barré syndrome (GBS) and Miller Fisher syndrome (MFS), mainly developed as the consequence of gastrointestinal infection (Fisher, 1956; Tam *et al.*, 2007), have been closely related to a previous infection by *C. jejuni*, and the development of inflammatory bowel disease is now recognized as an important risk factor also associated with campylobacteriosis (Garcia Rodriguez *et al.*, 2006).

1.5.2 *C. jejuni* infection and pathogenesis

The human host possesses a number of innate defence barriers designed against pathogenic bacteria. For instance, the acidic environment in the stomach, the peristaltic movements of the gut, the epithelial barrier, the mucosa layer and the innate immune

response represent important challenges that must be overcome by a gastrointestinal pathogen in order to cause infection (Hu and Kopecko, 2008).

On the other hand, bacterial pathogens are able to initiate the disease by developing specific interactions with the mucosal surface of the host such as attachment and/or invasion. Enteritis caused by *Campylobacter* is an acute inflammatory process generally affecting the colon and rectum. Inflammation of the ileum and caecum and mesenteric adenitis are common characteristics associated with bacterial motility and adherence to the surface of mucosa cells, important factors related to colonization of the gut (Blaser and Engberg, 2008; Hu and Kopecko, 2008). Indeed, the inability of flagellar mutants to colonize the gut of animals (Morooka *et al.*, 1985; Newell *et al.*, 1985; Yao *et al.*, 1997) has demonstrated the importance of both motility and chemotaxis as a requirement for bacterial colonization.

Adhesion and invasion are also important factors associated with colonization of the host by *C. jejuni*. Caco-2 and human intestinal epithelial (INT407) cell lines have been exploited as suitable models to mimic the *in vivo* conditions encountered by the bacterium during colonization (Konkel *et al.*, 2001). In this way, a number of adhesion factors have been described; the autotransporter CapA, PEB1, a periplasmic binding protein (Pei and Blaser, 1993), JlpA, a surface exposed lipoprotein (Jin *et al.*, 2001) and CadF, a fibronectin-binding outer membrane protein (Konkel, 1997).

A clear correlation between the ability of *C. jejuni* to invade the intestine and the developing of diarrhoeal disease has been shown in a primate model (Russell *et al.*, 1993). Intracellular bacteria found in tissue cultures and samples from patients have widely demonstrated the invasion capability of *C. jejuni* (reviewed in Dasti *et al.*, 2010). Thus, CadF is involved in two activities; specific binding to the fibronectin of epithelial cells that promotes adhesion, and triggering of signalling pathways that in turn lead to activation of the small Rho GTPases Rac1 and Cdc42. These proteins are suggested to play a significant role in the internalization mechanism of *C. jejuni* (Krause-Gruszczynska *et al.*, 2007) via a microtubule-dependent invasion mechanism (Monteville *et al.*, 2003).

The cytolethal distending toxin (CDT) is the sole toxin identified in the genome of various species of *Campylobacter* including *C. jejuni* (Johnson and Lior, 1988). This

toxin, also present in other Gram-negative pathogenic bacteria such as *E. coli*, *Helicobacter* spp, *Salmonella enterica* serovar Typhi and *Shigella* spp (Haghjoo and Galan, 2004; Thelestam and Frisan, 2004), induces cell distension (identified by the enlargement, swelling, cell cycle arrest and, as a result, cell death), in a variety of mammalian cells such as HeLa and CaCo-2 but shows to be inactive in Vero cells (Smith and Bayles, 2006; Wassenaar, 1997; Whitehouse *et al.*, 1998).

CDT might have a role in *C. jejuni* pathogenesis, specifically supporting invasion (Purdy *et al.*, 2000) and as a modulator of the immune response. Certainly, CDT triggers the production of interleukin (IL)-8 that induces inflammation of the gut via the recruitment of macrophages, dendritic cells and neutrophiles in man but not in chickens (Hickey *et al.*, 1999).

1.5.3 Respiratory metabolism of *Campylobacter*

The bioenergetics and stress responses of *C. jejuni* are poorly understood. An incomplete glycolytic metabolism and the lack of fermentative pathways (Parkhill *et al.*, 2000) make oxidative phosphorylation the major route for energy production in *Campylobacter*. An intricate branched electron transport system involving a variety of terminal reductases and two terminal oxidases with roles in both microaerobic and anaerobic respiration are encoded in the genomic sequence of *C. jejuni* NCTC 11168 (Hitchcock *et al.*, 2010; Parkhill *et al.*, 2000; Sellars *et al.*, 2002; Smith *et al.*, 2000). Figure 1.2 illustrates the complexity of the electron transport pathways of *C. jejuni*. Interestingly, the existence of a single ribonucleotide reductase dependent of O_2 (class II), essential for DNA synthesis, impairs growth of *C. jejuni* under strict anaerobiosis (Sellars *et al.*, 2002).

C. jejuni preferentially grows in an atmosphere containing 3-5% CO_2 and 5-15% O_2 (Ketley, 1997) at 42 °C (Hazeleger, 1998). There is a clear correlation between oxygen availability and growth in cultures of *C. jejuni*, The modifications of the oxygen transfer rates by variations in the volumes of batch cultures, where the oxygen solution rates decrease as the liquid volume increases (Pirt, 1985), is a principle commonly used for studying *C. jejuni* responses under specific oxygen tensions. For instance, in a microaerobic cabinet (10% O_2, 10% CO_2 and 80% N_2 at 42 °C), oxygen transfer

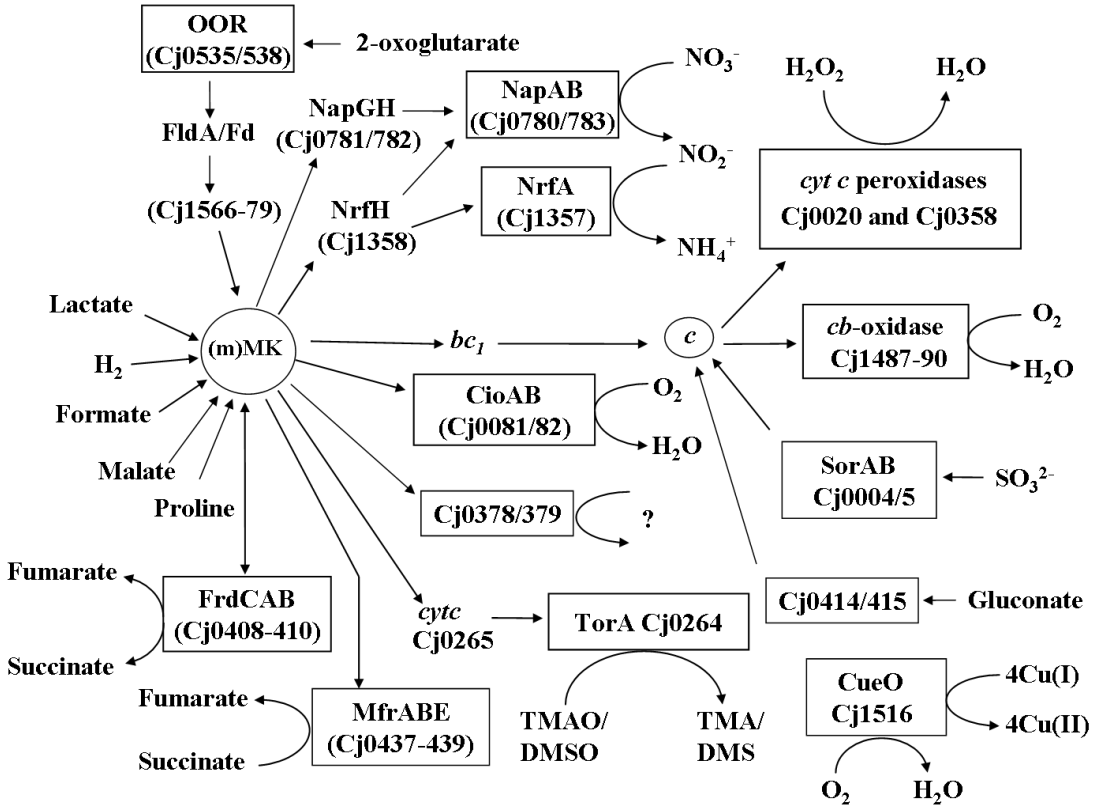


Figure 1.2 The electron transport pathway of *C. jejuni* NCTC 11168.

Organic and inorganic compounds can be used as electron donors by *Campylobacter*. Under oxygen-limited conditions, pathways to a variety of alternative electron acceptors permit growth and energy conservation. Under microaerobic conditions, electrons are transfered from menaquinone to the *cb*-type cytochrome *c* oxidase or to the non-electrogenicquinol oxidase CioAB. Taken from Hitchcock *et al*., (2010).

constants in 250 ml flasks containing water (100, 150 or 200 ml) are 0.43 min^{-1} (microaerobic), 0.16 min^{-1} and 0.06 min^{-1} (oxygen-limited conditions) respectively (Wainwright *et al.*, 2005).

1.5.3.1 Microaerobic respiration

Two terminal oxidases are expressed in *C. jejuni*, a cytochrome *bd*-type quinol oxidase and a *cb*-type cytochrome *c* oxidase (Fouts *et al.*, 2005; Hofreuter *et al.*, 2006; Parkhill *et al.*, 2000); however, spectroscopic signals produced by high-spin hemes *b* and *d*, typical of these oxidases, are not found in *C. jejuni* cells. Up-regulation of the *cydAB* operon is associated with survival in 5% oxygen (v/v) and formate respiration. CydAB has shown to be associated with resistance to cyanide and, for this reason, it was renamed CioAB (cyanide-insensitive oxidase). Increased expression of *cioAB* at higher oxygen tensions, a relatively low affinity for oxygen (K_m = 0.8 µM) and a V_{max} of > 20 nmol mg^{-1} s^{-1} have been reported. The *cb*-type cytochrome *c* oxidase, encoded by *ccoNOQP*, is a cyanide-sensitive complex playing a major role in respiration in conditions of microaerobiosis. It shows a higher oxygen affinity (K_m = 0.04 µM) and a V_{max} of 6 to 9 nmol mg^{-1} s^{-1} (Jackson *et al.*, 2007). The presence of this oxidase has been suggested to be essential for viability due to unsuccessful attempts to isolate mutants. Under oxygen-limited conditions, *C. jejuni* grows in media supplemented with nitrate, nitrite, fumarate, DMSO or TMAO. This indicates the presence of alternative pathways for electron acceptor-dependent energy conservation (Sellars *et al.*, 2002).

1.5.4 Sources of nitrosative stress during *Campylobacter* infection and colonization

In addition to NO coming from the action of NOS (Section 1.1), *Campylobacter* faces a variety of sources of nitrosative stress that depend on the environmental niche. Additional production of NO independent of the specific host defence response probably arises from other nitrogenous species, such as nitrite in the oral cavity (RauschFan and Matejka, 2001) and on the skin (Suschek *et al.*, 2006). Dietary nitrite reacts with stomach acid producing NO, and this process is exacerbated via the reduction of dietary nitrate to nitrite by the oral microflora (Olin *et al.*, 2001).

Moreover, consumption of meat and meat products containing nitrates as a preservative increases the exposure of *Campylobacter* to sources of nitrosative stress.

1.5.5 Inhibition of respiration by NO and RNS in *Campylobacter*

The microaerobic respiration of *Campylobacter* is inhibited by NO; however, the bacterium possesses a range of respiratory complexes able to process sources of nitrosative stress: both periplasmic nitrite (Nrf) and nitrate (Nap) reductases are encoded in the genome of *C. jejuni* NCTC 11168 (Pittman and Kelly, 2005; Sellars *et al.*, 2002). NrfA, a pentahaem cytochrome *c* nitrite reductase, is the terminal enzyme in the dissimilatory reduction of nitrite to ammonia (Pittman and Kelly, 2005; Sellars *et al.*, 2002). The *nap* operon in *C. jejuni* is formed by *napAGHBLD*; NapA and NapB constitue the two subunits of the periplasmic machinery. NapA (~90 kDa) plays a role as the catalytic subunit reducing nitrate to nitrite and contains a [4Fe-4S] group and a *bis*-molybdenum guanosine dinucleoside cofactor. NapB (~16 kDa) is a di-haem *c*-type cytochrome (Butler *et al.*, 2001). Even though *napC*, a gene encoding a tetra-haem cytochrome that, in *E. coli* couples nitrate reduction to quinol oxidation, is absent in the *C. jejuni nap* operon (Brondijk *et al.*, 2004), this bacterium possesses a putative *napC* gene that may be related to the nitrite reductase system (reviewed by Pittman and Kelly, 2005).

1.6 The single domain haemoglobin of *Campylobacter*, Cgb.

1.6.1 Functional characterisation

Since the report of the presence of a globin-like sequence in the genome of *C. jejuni* NCTC 11168 (Parkhill *et al.*, 2000) (Cj1586), similar to the sequence encoding the *Vitreoscilla* haemoglobin Vgb (Wakabayashi *et al.*, 1986), important progress has been made towards the understanding of the physiological role, structural features and the regulatory mechanism behind the globin known as Cgb (for *Campylobacter* globin). Cgb belongs to the Mb-like haemoglobin family, being a member of the single-domain haemoglobin sub-family (SDgb) (Vinogradov *et al*., 2013). Cgb comprises 140 residues (MW= 16.1 kDa). Even though it lacks the reductase domain present in the flavohaemoglobins, this protein shares a high level of sequence homology with the globin domains of the FHbs from *E. coli*, *Salmonella enterica* serovar Typhimurium

and *B. subtilis* (33, 34 and 39% respectively). Besides, Cgb is a homologue of the SDgb from *Vitreoscilla* (Vgb) (42% amino acid identity) (Elvers *et al.*, 2004).

Infection and pathogenesis of *C. jejuni* depend in part on its ability to cope with the toxic environment imposed by the presence of NO and RNS in the host. Survival of *C. jejuni* under conditions of nitrosative stress has been mainly related to the expression of two haemoglobins (Cgb and Ctb).

The first indication of the contribution of Cgb to resistance against nitrosative stress was reported in a study aimed at testing the protection offered by the heterologous expression of a variety of bacterial haemoglobins in *E. coli*. A significant improvement in growth was shown in cultures of Cgb-expressing cells compared to the parental strain in the presence of sodium nitroprusside (SNP) (Frey *et al.*, 2002), a clinically important NO donor used as a vasodilator (Miller and Megson, 2007; Wang *et al.*, 2002). Currently, it is generally accepted that resistance to NO and nitrosative stress agents in *Campylobacter* is linked to the expression of Cgb. A number of studies support this: (i) in the presence of the nitrosative agent *S*-nitrosoglutathione (GSNO), the growth of *C. jejuni* is impaired by mutation of *cgb* (Avila-Ramirez *et al.*, 2013; Elvers *et al.*, 2004); (ii) strains lacking *cgb* have a significantly diminished tolerance to GSNO, SNP, and NO (Elvers *et al.*, 2004; Wainwright *et al.*, 2005); (iii) Cgb protects cellular respiration of *C. jejuni* cells from NO-mediated respiratory inhibition by consumption of NO (Avila-Ramirez *et al.*, 2013; Elvers *et al.*, 2004; Monk *et al.*, 2008); (iv) Infected colorectal adenocarcinoma cells (Caco-2) with a *cgb* defective strain accumulate higher levels of NO than uninfected cells or cells infected with the parental strain (Elvers *et al.*, 2004); (vi) the expression of *cgb* is triggered by GSNO, NOC-18, SNP, spermine NONOate, nitrate and nitrite; and (vii) Cgb is a member of a small regulon controlled by the positive transcription factor NssR under nitrosative stress conditions (see section 1.8) (Elvers *et al.*, 2005; Pittman *et al.*, 2007).

Little is known about the reaction intermediates involved in the Cgb-mediated detoxification of NO. On the other hand, the flavohaemoglobin Hmp in *E. coli*, a homologue of Cgb, has been widely studied. The NO detoxification mechanism via a dioxygenase (NOD) (Gardner *et al.*, 2006; Gardner *et al.*, 2000; Gardner *et al.*, 1998b) or denitrosylase activity (Hausladen *et al.*, 2001; Hausladen *et al.*, 1998) involves the

conversion of NO and O_2 to the harmless ion nitrate. Structural studies of the haem pocket suggest a similar function mediated by Cgb (Lu *et al.*, 2007b) via the general reaction:

$$CgbFe(II) + O_2 + NO \longrightarrow CgbFe(III) + NO_3^- \qquad \text{(Eq. 1)}$$

If the conversion of NO to nitrate is catalysed by Cgb *in vivo*, two conditions must be fulfilled; the first is oxygen availability and the second is the presence of a reductase system able to efficiently regenerate the ferrous haem cofactor (Fe(II)) subsequent to oxidation by nitric oxide. In *Campylobacter*, the correlation between resistance to nitrosative stress and oxygen availability has been certainly demonstrated. Important differences are shown in resistance to NO and GSNO under microaerobic or oxygen-limited conditions. For example, better protection of respiration and growth from NO inhibition is shown in cultures pre-treated with GSNO at higher rates of oxygen diffusion, and NO consumption is also more efficient (Avila-Ramirez *et al.*, 2013; Monk *et al.*, 2008). An NO detoxification mechanism dependent on O_2 for Cgb seems likely; the expression of Cgb maximally occurs in the presence of oxygen, (Avila-Ramirez *et al.*, 2013; Elvers *et al.*, 2005; Elvers *et al.*, 2004; Monk *et al.*, 2008; Wainwright *et al.*, 2005). However, nitrate production by Cgb either *in vitro* or *in vivo* has not been demonstrated so far. Indeed, over-expression of the globin did not increase the NO consumption activity of *E. coli* wild type soluble cell extracts and no differences in nitrate production compared to the control were reported (Frey *et al.*, 2002), although it is plausible that the effect of Cgb could be masked by the presence of the flavohaemoglobin Hmp in the system.

1.6.2 Cgb reduction: the redox partner mystery

The production of NO_3^- from the reaction of NO and O_2 catalysed by single-domain globin proteins implies the oxidation of the haem, which requires re-reduction for subsequent enzymatic turnover. Therefore, the existence of reductase proteins acting as partners of SDgbs has been speculated upon for a long time. The ability of Vgb and other bacterial haemoglobins to associate with the cytoplasmic membrane (Park *et al.*, 2002), and their participation in oxygen transfer (Dikshit *et al.*, 1992) has led some to suggest the respiratory chain as the electron source for Cgb reduction.

Flavohaemoglobins evade the problem of haem reduction by containing a reductase domain: intra-protein electron transfer from the reductase domain (or FNR, ferredoxin-NADP reductase-like domain) to the N-terminal haem domain in an NAD(P)H-dependent reaction via a noncovalently bound FAD allows the reduction of the ferric haem (Fe(III)) (Gardner *et al.*, 1998a; Hausladen *et al.*, 1998; Hernandez-Urzua *et al.*, 2003). Substantial differences between Cgb and the FHbs suggested that Cgb may not interact with the same type of reductase as the flavohaemoglobins. Indeed, the conserved residue Lys-84 responsible for the formation of a salt bridge between the domains in the flavohaemoglobins (Ermler *et al.*, 1995a) is absent in Cgb.

The gene (*cj1585c*) adjacent to *cgb* encodes a lactate dehydrogenase enzyme that has been suggested as a candidate for a redox partner for Cgb (Thomas *et al.*, 2010) and spectroscopic characterisation supports this hypothesis. However, even though *cj1585c* is up-regulated in response to NO, this occurs only in oxygen-limited conditions, which does not promote the *cgb* induction (Avila-Ramirez *et al.*, 2013) (see section 1.8). Consequently, a role of Cj1585c in an oxygen-dependent detoxification mechanism seems unlikely.

1.6.3 Structural characterisation

Flavohemoglobins possess an N-terminal globin domain (a 3/3 α-helical Mb-fold) and a C-terminal domain with sites for binding FAD and NAD(P)H (Ermler *et al.*, 1995a; Ilari *et al.*, 2002a; Vasudevan *et al.*, 1991). On the other hand, single domain globins also have the Mb-fold haem domain but lack a C-terminal domain (Tarricone *et al.*, 1997a; Tarricone *et al.*, 1997b). Globin subunits are constituted by a 6-8 α-helical segments fold around a haem cofactor. The structure of Cgb matches the general globin fold where a central iron atom allows haem coordination to a His residue.

The structure of cyanide-bound Cgb solved by X-ray crystallography (resolution of 1.35 Å) shows a classic three-on-three α–helical globin architecture (Shepherd *et al.*, 2010) (Fig. 1.3) with helices labelled A to H in sequence order, in accordance with standard globin nomenclature. Structural homology between Cgb and Vgb, the globin domain of Hmp and sperm whale myoglobin (swMb) was reported. While Vgb is a dimer (Tarricone *et al.*, 1997b), Cgb was purified and crystallised as a monomer.

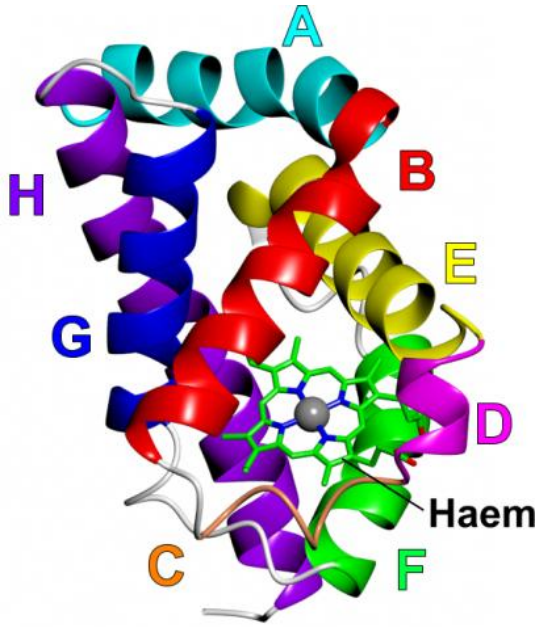


Figure 1.3 Backbone topology of *Campylobacter jejuni* haemoglobin Cgb

The 3-over-3 α-helical fold of Cgb with the haem cofactor is shown. Helices are labelled in accordance with conventional globin nomenclature. Taken from Shepherd *et al*., (2010) (PDB ID: 2WY4).

The homodimeric structure of Vgb is due to the juxtaposition of helices H and F from each subunit forming a loose four-helix bundle holding together by van der Waals contacts in a hydrophobic zipper and two water molecules between Pro72 and Asp-139 and Ala73 and Asp139 (Tarricone *et al.*, 1997b). In Cgb, several charged residues occupy the region producing steric clashes that might impair the homodimeric structure of Vgb (Shepherd *et al.*, 2010).

Cgb displays higher ligand affinities than myoglobin. The dissociation constants for O_2 binding are 6 nM for Cgb (Lu *et al.*, 2007b) and 0.86 μM for swMb (Gibson *et al.*, 1986), respectively. The character of the residues in the B10 and E7 positions are important for modulating ligand binding. In mammalian globins, the E7 position is generally occupied by a histidine. For instance, in myoglobin, the haem-bound dioxygen is stabilised by the H-bonding from HisE7 and the B10 position is usually occupied by hydrophobic amino acids. In Cgb, the B10 and E7 residues are occupied by tyrosine and glutamine (as in Hmp and Vgb); however, in Cgb but not in Hmp or Vgb, a GlnE7 is stabilized by a hydrogen bonding network reminiscent of cytochrome *c* peroxidase (CcP). This structure is in agreement with the suggested role for Cgb as an NO dioxygenase proceeding via a peroxidase-like mechanism (Lu *et al.*, 2007b). Indeed, peroxidases and oxidases have a much more polar character compared to mammalian globins. Besides, an additional hydrogen bonding network in the proximal pocket might impose imidazolate character upon the histidine F8 that has been also implicated in the Cgb catalysis mediated by the peroxidase-like mechanism. These characteristics are central for the 'push-pull' model for peroxidase-like enzymes (Poulos, 1996), allowing the catalysing cleavage of the O-O bond that is critical for the isomerisation of peroxynitrite, an intermediate in the conversion of NO and O_2 to NO_3^- during the NOD reaction (Lu *et al.*, 2007b; Mukai *et al.*, 2001):

$$Fe^{2+}\text{-O-O} + \cdot N{=}O \rightarrow [Fe^{3+}\text{-O-O-}NO^-] \rightarrow Fe^{3+} + NO_3^- \quad \text{(Eq. 2)}$$

1.7 The truncated haemoglobin of *Campylobacter jejuni*, Ctb

1.7.1 Functional characterisation

In addition to the single domain haemoglobin Cgb, Elvers *et al* (2004) identified a second haemoglobin-like protein (Cj0465c) in the genome of *C. jejuni* NCTC 11168

(Parkhill *et al.*, 2000). The truncated globin named Ctb (*Campylobacter* truncated globin) is classified within the poorly explored sub-family III (or P) of the truncated haemoglobins (TrHb3) (Pesce *et al.*, 2000; Vinogradov *et al.*, 2005) and is constitutively expressed at low levels in *C. jejuni*. However, its expression is increased in an NssR-dependent manner under conditions of nitrosative stress (GSNO and SNAP) (see section 1.8) (Wainwright *et al.*, 2005).

Even though Ctb has been extensively characterised (Bolli *et al.*, 2008; Lu *et al.*, 2007a; Nardini *et al.*, 2006; Wainwright *et al.*, 2006), its physiological function remains unclear. Some of the approaches aiming at elucidating the role of Ctb include testing its capacity to improve microaerobe cellular growth and its ability to offer cellular protection against toxic oxygen tensions and nitrosative stress conditions.

The *Vitreoscilla* globin (Vgb) improves microaerobic growth in *E. coli* and other bacterial and eukaryotic species (reviewed in Frey *et al.*, 2011). When the growth profile of a *C. jejuni ctb*-lacking strain and the wild type were compared at different oxygen concentrations, a slower growth rate of the mutant was reported during the stationary phase in microaerobic conditions (Wainwright *et al.*, 2005), suggesting a role of Ctb in oxygen transfer. However, in oxygen-limited conditions, there were no differences.

The findings on the influence of Ctb upon oxygen consumption are as follows: (i) respiration rates of *C. jejuni* cells decreases 50% in a *ctb* mutant compared to wild type; (ii) the K_M values for oxygen in wild type and the globin mutants (*ctb*, *cgb* or *cgb ctb* lacking strains) are all comparable but the *ctb* mutant showed a greater V_{max} determined by the deoxygenation of oxy-LHb (Contreras *et al.*, 1999; D'mello *et al.*, 1994; D'mello *et al.*, 1995; D'mello *et al.*, 1996; Smith *et al.*, 1990) (Wainwright *et al.*, 2005) and (iii) the *in vivo* O_2 consumption rate of the *ctb* mutant decreases below ~1 µM but increases in the range between 1 µM and air saturation (Wainwright *et al.*, 2005). Given that high external oxygen tensions are toxic for *Campylobacter*, consumption of O_2 by Ctb might confer protection during microaerobic growth.

Based on the data above, a role for Ctb in supporting microaerobic growth and moderating respiration in *C. jejuni* seems plausible. However, the physiological relevance of these observations is not immediately clear. For example, under conditions

of nitrosative stress, the *ctb* gene is up-regulated but it is not induced by oxidative stress or variations in oxygen concentrations (Wainwright *et al.*, 2005). Certainly, Ctb production was induced by GSNO and SNAP, as confirmed by western-blotting, but paraquat or peroxides failed to influence its expression.

1.7.2 Structural characterisation

Ctb was the first class III truncated haemoglobin to be structurally characterised. A dimeric cyanide-bound structure, solved via X-ray crystallography (resolution of 2.15 Å) (Nardini *et al.*, 2006) revealed a 2-over-2 α–helical sandwich, in agreement with all truncated globins characterised to date (Fig. 1.4). A distinct feature of Ctb, compared to previously characterised TrHb, was the absence of the conserved Gly-Gly sequence motif found at the AB inter-helical turning point and the C-terminal to the E helix, apparently indispensable for the adoption of the 2-over-2 fold in TrHbs 1 and 2.

The matrix tunnel or cavity system that in the TrHb1 and 2 allows ligand migration to and from the distal pocket is not present in the Ctb structure. However, three conserved residues (B10Tyr, G8Trp, and E15Trp) participate in a hydrogen-bonding network with an active site water molecule. The Ctb crystal structure showed the E7His residue in 'open' and 'closed' conformations, suggesting a possible gating system for ligand entry/exit, as occurs in myoglobin (Nardini *et al.*, 2006).

An atypically larger stretching mode at 514 cm^{-1} reported from resonance Raman data for the Fe-CO derivative of Ctb compared to other TrHbs suggests distinctive roles for B10Tyr, E7His and G8Trp residues in ligand stabilisation (Wainwright *et al.*, 2006). The exceptionally high affinity of Ctb for oxygen (K = 222 μM^{-1}, compared to K= 1.1 μM^{-1} for swMb, defined as the ratio of k_{on} *versus* k_{off}) might be explained by the presence of these residues forming an intertwined hydrogen bond network contributing to the stabilisation of the haem-bound oxygen (Lu *et al.*, 2007a). Data obtained by combining resonance Raman techniques with molecular dynamics led to the conclusion that, when oxygen is bound, Ctb could exist in one of two conformations, and both conformers G8Trp may be stabilising the interactions (Arroyo Manez *et al.*, 2011).

It has been proposed that Ctb may not function in oxygen transport or storage due to its high affinity for O_2. Since the structure of the distal haem pocket of Ctb resembles that

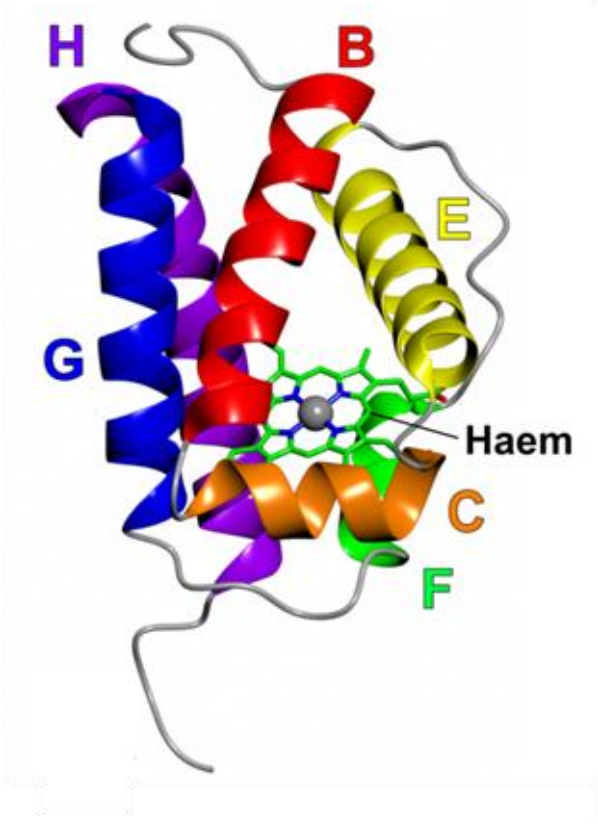


Figure 1.4 Backbone topology of *Campylobacter jejuni* truncated haemoglobin Ctb.

The 2-over-2 α-helical fold of Ctb with the haem cofactor is shown. Helices are labelled in accordance with conventional globin nomenclature. Taken from Nardini *et al*., (2006) (PDB ID: 2IG3).

of cytochrome *c* peroxidase (CcP) it is hypothesised that Ctb may play a role in oxygen chemistry (Lu *et al.*, 2007a). However, mutation of *ctb* does not produce sensitivity to peroxides (Wainwright *et al.*, 2005), suggesting a function for Ctb different from peroxide decomposition.

1.8 Regulation of globin expression in *Campylobacter*

1.8.1 Changes in gene expression elicited by nitrosative stress: the role of NssR

The inducibility of the *cgb* gene was reported for the first time in *Campylobacter coli*. Cells carrying a vector containing *astA*, a reporter gene, under control of the *cgb* promoter showed induction by SPN and GSNO but not by methyl viologen (Hendrixson and DiRita, 2003). This result, supported by immunoblotting tests using Cgb polyclonal antibodies, led to the suggestion that haemoglobin expression was specifically induced under conditions of nitrosative stress (Elvers *et al.*, 2004).

A screening of the *C. jejuni* genome sequence (Parkhill *et al.*, 2000) based on analogues of sensors and regulators from other bacteria, showed the presence of three potential transcription factors that may sense NO and, as a consequence, induce the expression of Cgb (Elvers *et al.*, 2004): Fur, a Fe^{2+} cofactor-containing an iron sensor that, in *C. jejuni*, is related to iron acquisition, non-iron ion transport and flagellar biogenesis (Butcher *et al.*, 2012); PerR, a metalloregulator implicated in peroxide stress responses (Butcher *et al.*, 2012; Mongkolsuk and Helmann, 2002) and Cj0466, a member of the Crp-Fnr superfamily of transcription regulators (Korner *et al.*, 2003).

Mutants of either *fur* or *perR* retained the Cgb expression profile found in the parental strain after exposure to GSNO. However, the *fur* mutant showed an increased sensitivity to GSNO (Elvers *et al.*, 2005); chemical interaction between exogenous RNS and endogenous ROS was proposed. Indeed, hypersensitivity to nitrosative stress in the absence of Fur has been associated with the derepression of the iron assimilation system producing, as a consequence, oxidative stress in *E. coli* (Mukhopadhyay *et al.*, 2004). Conversely, the insensitivity of a *C. jejuni fur* mutant to inhibition by NO was reported more recently. In this case, the Fur-regulated genes were suggested to play a role in protection against nitrosative stress, associated with an augmented iron acquisition that, in turn, allows the repair of damaged Fe-S and haem proteins (Monk *et al.*, 2008).

Although these results seem to be contradictory, compounds with different biological properties were used in each case (GSNO, a nitrosative agent, and NO respectively). It is not clear whether or not these differences can cause such dissimilar results.

Deletion of the *cj0466* sequence in the *C. jejuni* genome abolished the expression of Cgb upon exposure to GSNO; given that, under nitrosative stress conditions, the globin expression appeared to be dependent on Cj0466, the protein was designated NssR (Nitrosative stress sensing Regulator) (Elvers *et al.*, 2005). However, an augmented sensitivity to methyl viologen of an NssR-lacking strain may indicate an additional role for this regulator. Indeed, sensitivity to methyl viologen is related to superoxide production in *Campylobacter* (Purdy *et al.*, 1999).

A transcriptional analysis comparing microarray data from microaerobic batch cultures of *C. jejuni* wild type in the absence and presence of GSNO showed the up-regulation of eight genes: sequences encoding the single domain globin Cgb (*cj1586*), the truncated globin Ctb (*cj0565c*), a probable peptide ABC transport system permease protein (*cj1582c*), four probable integral membrane proteins (*cj0830, cj0851c*, *cj0313* and *cj0430*), and a hypothetical product with unidentified function (*cj0761*) were reported (Elvers *et al.*, 2005). However, the scope of the NssR-dependent response was later defined from transcriptional data comparing GSNO-treated cultures of the parental strain and the *nssR* mutant; *cgb*, *ctb*, *cj0761* and *cj0830* showed up-regulation and the transcription was confirmed by RT-PCR (Elvers *et al.*, 2005).

A more detailed study of transcriptional changes elicited by GSNO was performed in continuous cultures (Monk *et al.*, 2008). This time, 97 genes (from a total of 1632 genes arrayed) were up-regulated (≥ 2-fold) and the presence of the NssR regulon confirmed. Among the genes observed were: *cgb* (320-fold), *ctb* (63.8-fold), *cj0761* (49.7-fold) and *cj0830* (12.3-fold). Besides, the presence of both Cgb and Ctb was demonstrated in cultures treated with GSNO by proteomic analysis (Monk *et al.*, 2008). Interestingly, a modest up-regulation of *nssR* (2.2-fold) led to the suggestion of the presence of a regulatory mechanism associated with the expression of NssR under nitrosative stress conditions. Other genes induced in this condition were *cj0757*, *cj0758* and *cj0759* whose products are homologues of HrcA, GrpE and DnaK respectively, proteins implicated in the heat-shock response (Parkhill *et al.*, 2000), *cj0311* (Ctc) involved in

general stress response (Volkert *et al.*, 1994), and TrxA and TrxB (a thioredoxin and its reductase) related to oxidative stress tolerance. Additionally, nine genes from a group of 18 genes implicated in iron transport showing transcriptional changes under low iron conditions were also reported (Holmes *et al.*, 2005). The up-regulation of iron acquisition genes as a consequence of nitrosative stress has been demonstrated in a variety of other bacteria (Hernandez-Urzua *et al.*, 2007; Moore *et al.*, 2004; Mukhopadhyay *et al.*, 2004; Richardson *et al.*, 2006). Derepression of genes under control of Fur is due to NO binding to the ferrous haem of the transcriptional regulator.

The differences in the chemical properties and biological interactions of NO donors (NOCs) and SNOs such as GSNO (a nitrosative agent) has been recently reviewed (Bowman *et al.*, 2011). SNOs together with derivative species have biologically relevant functions other than the simple release of NO (Hess *et al.*, 2005). GSNO is moderately stable and, for this reason, is used extensively in bacterial growth experiments. However, this compound is not ideal for the purpose of studing the physiological effects of NO. For example, transfer of the ion nitrosonium (NO^+) from GSNO to membrane thiols is suggested in *Bacillus* (Morris and Hansen, 1981); however, other reports show that toxicity is associated with active transport. Indeed, a SNO-derived nitrosated dipeptide (*S*-nitroso-L-cysteinylglycine) is transported inwards via the Dpp-encoded dipeptide permease in *E. coli*, and, as a result, it produces intracellular transnitrosation reactions (Jarboe *et al.*, 2008; Laver *et al.*, 2012). Interestingly, comparison of the *C. jejuni* transcriptional profiles upon exposure to GSNO (Monk *et al.*, 2008) or to a combination of NOCs (NOC-5 and NOC-7) revealed common features. The NssR regulon (including the Cgb and Ctb globins), heat shock proteins and regulators were similarly affected by either GSNO or NO (Smith *et al.*, 2011). On the other hand, in *E. coli*, transcriptional responses tested in continuous cultures upon addition of GSNO or NOCs revealed some similarities but numerous important differences (Pullan *et al.*, 2007).

Since release of NO from GSNO has been demonstrated (Singh *et al.*, 1996), it seems probable that the transcriptional response of *C. jejuni* to GSNO was related in part to the NO released from the *S*-nitrosothiol. It has been proposed that the induction of Hmp mediated by the transcriptional regulator NsrR in *E. coli*, might be the consequence of the sub-micromolar concentrations of NO released from GSNO. For example, less than

5 µM NO is released from 500 µM GSNO (Jarboe *et al.*, 2008). In the transcriptomic studies of *C. jejuni*, cultures were treated with GSNO (250 µM) (Monk *et al.*, 2008) or NOC-5 plus NOC-7 (10 µM each) (Smith *et al.*, 2011). It seems unlikely that a NO concentration under 2.5 µM (putatively released from GSNO) was responsible for the majority of the transcriptional changes, although the extremely high affinity of bacterial globins for NO might induce their expression in the presence of micromolar concentrations.

1.8.2 Influence of the oxygen availability on the transcriptional responses to nitrosative stress.

Variations in oxygen tension produce notable differences in the transcriptional profile of *C. jejuni* to NO (Avila-Ramirez *et al.*, 2013). Genes induced by NOCs in microaerobic conditions are not up-regulated in oxygen-limited conditions. In the latter, only 11 genes are induced and members of the NssR regulon were not found. Interestingly, the *cj1585c* gene, situated next to *cgb*, was marginally up-regulated under oxygen-limited but not in microaerobic cultures. The product of *cj1585c*, a lactate dehydrogenase, has been suggested as the redox partner of Cgb; however, given that *cj1585c* induction does not occur simultaneously with the expression of Cgb (the absence of the globin under nitrosative stress conditions in oxygen-limited cultures was demonstrated by immunoblotting), this proposal seems rather unlikely. The findings above raise several new questions about the ability of *C. jejuni* to survive in the host during infection and pathogenesis where variations in oxygen levels may be expected.

1.8.3 The transcriptional regulator NssR

NssR is a member of branch E of the Crp-Fnr superfamily (Korner *et al.*, 2003; Matsui *et al.*, 2013), being the only transcription factor involved in NO regulation in this branch. Since some regulators from the Crp-Fnr family are not directly activated through the interaction with the signal molecule, but their expression is induced by an independent sensor system (Fischer, 1994), the same mechanism has been suggested for NssR. However, a role as a NO sensor and regulator seems more plausible for NssR, as its expression is scarcely augmented in conditions of nitrosative stress (Elvers *et al.*, 2005).

The region -35 upstream of the -10 TATA box sequence for σ^{70} has been shown to be not conserved in the *C. jejuni* genome (Petersen *et al.*, 2003). A σ^{70} recognition site, a -10 motif lacking the -35, was found in the regions upstream of *cgb, ctb, cj0830, cj0761* and *nssR*. In addition, an Fnr-like binding sequence upstream of the -10 was identified in all genes (Elvers *et al.*, 2005). The consensus sequence (TTAAC-N_4-GTAA) shows similarities with the proposed recognition sequences of the NO-sensing regulator Nnr in *Paraccocus pantotrophus* (TTAAC-N4-GTCAA) (Korner *et al.*, 2003) and the regulator of virulence gene expression PrfA from *Listeria monocytogenes* (TTAACA-N_2-TGTTAA) (Saunders *et al.*, 2000). Thus, the four genes regulated by NssR share the same architecture that is consistent with a class II Fnr-dependent promoter (Guest *et al.*, 1996).

Comparison of the wild type and an altered sequence (TTAACacaaGTCAA and CTAACacaaGTCAG respectively) in transcriptional fusions to *lacZ* demonstrated the specificity in the recognition of the Fnr-like sequence by NssR in the *cgb* promoter. In cultures containing GSNO, *lacZ* expression was highly induced in the non-modified promoter whereas the mutant sequence fully prevented the NssR recognition (Elvers *et al.*, 2005).

The influence of NssR not just in the regulation of *ctb* expression but also in its own expression has been suggested since the truncated globin and the *nssR* genes are divergently transcribed, sharing the potential NssR-binding sequence (Elvers *et al.*, 2005). Indeed, the modest induction of NssR shown in continues cultures challenged with GSNO (Monk *et al.*, 2008) might represent an auto-regulation mechanism.

In the absence of nitrosative stress, NssR binds to a 32 bp region in the *ctb* promoter ($K_{d(app)}$ ~50 nM) (Smith *et al.*, 2011), explaining the low but constitutive expression of the truncated globin in the absence of GSNO (Elvers *et al.*, 2005). Nonetheless, gel shift experiments showed that the affinity of NssR for the *ctb* promoter was not enhanced in the presence of GSNO or NOCs (Smith *et al.*, 2011). This is a significant result since it suggests that NssR might differ from other well characterised transcription factors within the Crp-Fnr family which, in the presence of their associated signal molecules, exhibit increased DNA-binding compared to the absence of the molecule ($K_{d(app)}$ in the nM range and in the µM range respectively) (Green *et al.*, 2001). Hence, it appears

possible that NssR remains permanently bound to the promoter regions of the genes within the regulon, and that, upon exposure to NO, conformational changes facilitate the binding of the transcriptional machinery.

A mechanism implying the nitration of one of the numerous tyrosine residues or nitrosylation of the sole cysteine present in the NssR structure has been proposed (Smith *et al.*, 2011). Nitration involves the modification of peroxynitrite, a compound resulting from the reaction between NO and superoxide. Production of superoxide by oxy-ferrous Ctb is a possibility given that production of this compound has been demonstrated in other bacterial haemoglobins (Membrillo-Hernandez *et al.*, 1996); however, in a *ctb* mutant, Cgb expression is increased in oxygen limited conditions. This result cannot be explained by the same hypothesis (Smith *et al.*, 2011).

NssR activity may require iron as a cofactor either for interaction with NO or, after interaction with NO, for reconstitution of the transcription factor. Certainly, the presence of a 4Fe-4S group is implicated in the NO-response of other regulators within the Fnr family (Korner *et al.*, 2003). However, in NssR, the cysteine signature for binding the haem is not present (Monk *et al.*, 2008). The NssR sensing mechanism remains obscure.

1.9 The nitrite reductase of *Campylobacter* (NrfA)

A constitutively expressed pentahaem cytochrome *c* nitrite reductase (NrfA) mediates the dissimilatory reduction of nitrite to ammonia in *Campylobacter* (Pittman and Kelly, 2005; Sellars *et al.*, 2002). The ability of NrfA homologues to reduce not only nitrite but also NO in a range of bacteria has been shown *in vitro* (Bamford *et al.*, 2002; Costa *et al.*, 1990). For instance, NrfA reduces NO to ammonia under anaerobic conditions in *E. coli* (Costa *et al.*, 1990; Poock *et al.*, 2002; van Wonderen *et al.*, 2008), suggesting a function as an anaerobic NO detoxification mechanisms in addition to the flavorubredoxin NorV and the flavohaemoglobin Hmp (Poock *et al.*, 2002).

The function of NrfA as a protection mechanism against nitrosative stress in *C. jejuni* has been investigated by testing a number of single mutants affected in key genes of the *nrf* and *nap* operons. NapA is the only nitrate reductase in *C. jejuni*: the stoichiometric production of nitrite from nitrate observed in the parental strain is abolished by deletion

of *napA* (Pittman *et al.*, 2007). Similarly, nitrite consumption depends solely on NrfA and its electron-donor NrfH. Significant protection elicited by NrfA against nitrosative stress has been shown in *C. jejuni*. Indeed, an *nrfA*-lacking strain growing in microaerobiosis is hypersensitive to spermine NONOate, SNAP and GSNO; cellular respiration is severely inhibited and NO consumption is decreased when compared to the parental strain. These findings support the role of the nitrite reductase in resistance to NO and RNS in *C. jejuni* (Pittman *et al.*, 2007).

Even though a direct comparison between the level of protection to nitrosative stress associated with Cgb and NrfA has not been performed, under the same conditions, *cgb* or *nssR*-lacking strains exhibit a severe inhibition of growth while an *nrfA* mutant only decreased the growth rate in the presence of nitrite when compared to the parental strain (Pittman *et al.*, 2007). Hence, it has been suggested that, in *Campylobacter*, NrfA might play a role as a constitutive defence against nitrosative stress allowing survival until the Cgb expression is triggered (Pittman *et al.*, 2007). However, in oxygen-limited conditions, where the NssR-dependent Cgb up-regulation does not take place, a *nrfA* mutant fails to show increased sensitivity to GSNO (the growth of the wild type and the mutant were comparable) (Avila-Ramirez *et al.*, 2013). This interesting finding might mean that neither Cgb nor NrfA function as a defence against nitrosative stress under oxygen limited conditions.

1.10 NO resistance in oxygen-limited conditions is independent of NssR.

It has been proposed that the adaptative response to conditions of nitrosative stress under oxygen-limited conditions is not dependent on NssR, Cgb or NrfA (Avila-Ramirez *et al.*, 2013). Indeed, cultures pre-treated with GSNO in microaerobiosis show an NssR-dependent induction of Cgb that protects respiration from inhibition by NO (Avila-Ramirez *et al.*, 2013; Elvers *et al.*, 2004). On the other hand, GSNO pre-treatment of cultures in oxygen limitation elicits a smaller but still significant protection against NO-mediated inhibition of respiration that is not found in cells untreated with the *S*-nitrosothiol (Avila-Ramirez *et al.*, 2013). The protection mechanism(s) against NO and RNS in oxygen limitation has proved to be independent of NssR (see section 1.8.2), suggesting the existence of an alternative inducible NO detoxification mechanism not related to either members of the NssR regulon or the constitutively

expressed enzyme NrfA. This putative system may be physiologically important during *Campylobacter* infection, where oxygen availability can be extremely low.

1.11 Cgb and Ctb: an integrated response?

Integrated function of globins co-existing in the same microorganism has not been previously described. However, since *ctb* and *cgb* belong to the same regulon (being expressed during exposure to NO and RNS via NssR) (Elvers *et al.*, 2005), and considering that the function of Ctb in conditions of nitrosative stress is not evident (Wainwright *et al.*, 2005), a mechanism involving coordinated responses of Ctb and Cgb might be possible.

Even though the *Campylobacter* globins are not expressed in nitrosative stress conditions under oxygen limitation (Avila-Ramirez *et al.*, 2013), expression of Cgb in a *ctb*-lacking strain has been recently reported in microaerobiosis and O_2 limitation (Smith *et al.*, 2011). This result led to the speculation that Ctb functions as a modulator of NO availability, controlling, consequently, NssR activity. That is, under microaerobic conditions, where the Ctb ferrous haem is bound to oxygen (FeII-O_2) (Wainwright *et al.*, 2006), the binding of NO is likely to be hindered and NO is available for the NssR activation. On the other hand, in oxygen limitation conditions, the binding of NO to the Ctb ferrous haem is facilitated by the poor oxygen availability, thus preventing the induction of the NssR-dependent genes (e.g. Cgb) (Smith *et al.*, 2011). However, more experimental approaches are needed to prove this hypothesis.

The interaction of the *C. jejuni* globins during nitrosative stress may be also supported by the following data: (i) the viability of cultures of *C. jejuni* lacking Cgb is diminished under nitrosative stress conditions (Elvers *et al.*, 2004); however, the effect of the *cgb* mutation is reverted by deletion of the *ctb* gene, the growth being comparable to the *ctb* mutant or the parental strain (Wainwright *et al.*, 2005); (ii) in agreement with this, cultures of a strain lacking both *cgb* and *ctb* genes are less sensitive to GSNO than the *cgb* mutant, suggesting that the absence of Ctb partially suppresses the effect of the *cgb* mutation (Avila-Ramirez *et al.*, 2013) and (iii) microaerobic cultures of the *ctb* mutant grow slower than the parental strain at the final stage of the exponential phase in the

absence of nitrosative stress; however, mutation in the second globin results in similar growth profiles of the double mutant and the wild type (Wainwright *et al.*, 2005).

1.12 Scope of this thesis

C. jejuni has been demonstrated to be a suitable model for studies to explore functional activities of the Cgb and Ctb globins *in vivo*. Certainly, inference of function(s) has been possible by testing globin mutants, and important findings about the regulation of the Cgb and Ctb expression under nitrosative stress conditions have been achieved. Besides, *in vitro* approaches including structural, biophysical and mechanistic characterisations of the purified globins, have also contributed substantially to the current understanding of these proteins. However, many questions, mainly related to the molecular mechanism behind the NO resistance offered by Cgb in *Campylobacter* and the physiological significance of Ctb remain unanswered.

Since further investigation of globin function in *Campylobacter* is restricted by complications in complementation by transformation of plasmids, the present work is aimed at studying the Cgb and Ctb activities through the expression of the globin genes in an engineered heterologous host, namely an *E. coli* mutant lacking the flavohaemoglobin Hmp and being, as a consequence, hypersensitive to NO and RNS. This approach is directed to confirm the Cgb activity as a NO and RNS detoxification mechanism. If this is the case, it will be possible to use this model for exploring the possible source(s) of electrons (redox partner(s)) implied in the reconversion of the ferrous haem required for binding ligands. Besides, attempts to elucidate functions of Ctb in the heterologous host will be performed as a tool to better understand its function in *Campylobacter*.

CHAPTER 2. Materials and Methods

2.1 Bacteriological methods

2.1.1 Strains and plasmids

E. coli and *C. jejuni* strains are described in Tables 2.1 and 2.2 respectively. Plasmids are described in Table 2.3.

2.1.2 Culture media

Media were sterilised by autoclaving for 15 min at 121 ºC and 15 p.s.i. The chemicals were purchased from Sigma, unless otherwise stated. When required, chemicals and solutions were sterilised by filtration using Millipore filters with a pore size of 0.45 µm (Sambrook and Russell, 2001).

2.1.2.1 Luria Bertani broth and solid medium (LB)

Tryptone (10 g), 5 g yeast extract (both from Oxoid) and 10 g NaCl were dissolved in 1 l H_2O and the pH adjusted to 7.0. To make solid medium, 15 g agar were added.

2.1.2.2 2 X TY broth and solid medium

Tryptone (16 g), 10 g yeast extract and 5 g NaCl were dissolved in 1 l H_2O and the pH adjusted to 7.0. For solid medium, 12 g agar was added before autoclave.

2.1.2.3 Mueller Hinton broth (Oxoid)

Dried Mueller Hinton broth (21 g) was dissolved in 1 litre H_2O.

2.1.2.4 Columbia Blood agar (Oxoid)

Dried Columbia agar (39 g) was dissolved in 1 litre H_2O and autoclaved. The medium was cooled down to 55 °C prior to the addition of 20 ml (5% (v/v)) defibrinated horse blood.

2.1.2.5 Brain heart infusion broth (Oxoid)

Dried brain heart infusion (37 g) was dissolved in 1 l H_2O.

2.1.2.6 Succinate minimal medium (SMM)

$Na_2HPO_4 \cdot 12H_2O$ (10.96 g), 2.7 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 5 g sodium succinate and 10

Table 2.1 *E. coli* strains

Strain	Genotype	Source/Reference
MG1655	Wild type *F*- lambda- *ilvG- rfb-50 rph-1*	*E. coli* Genetic Stock Culture Collection; Yale University
RKP4960	BL21 (DE3), pLysS	Invitrogen
RKP3036	MG1655 *hmpA*::Tn5 Kan	(Blattner et al., 1997)
RKP116	RKP3036, pPL341	This study
RKP 3919	RKP3036, pBAD/HisA	This study
RKP 3920	RKP3036, pLW1	This study
RKP 3921	RKP3036, pMT1	This study
RKP5867	JW2536 K-12 (BW25113) *hmpA::*Tn5 Kan	NBRP (NIG, Japan): *E. coli*
RKP117	MG1655 *hmpA::*Tn5 Kan mutation transduced from RKP5867	This study
RKP139	AV055, MG1655 *hmpA* (RKP117 but the Kan marker removed)	Constructed by Angie Vreugdenhil, University of Amsterdam.
RKP3940	AG200, K-12 (AB1157) *ygaA::lac* Cm (*ygaA* now named *norR*)	(Gardner et al., 2002)
RKP3941	AG400, K-12 (AB1157) *ygbD::lac* Cm (*ygbD* now named *norW*)	(Gardner et al., 2002)
RKP5877	MG1655 *norR*::Cm, mutation transduced from RKP3940	This study
RKP5873	RKP3036 *norR*::Cm, mutation transduced from RKP3940	This study
RKP5878	MG1655 *norW*::Cm, mutation transduced from RKP3941	This study
RKP5872	RKP3036 *norW*::Cm, mutation transduced from RKP3941	This study
RKP5884	RKP5873, pPL341	This study
RKP5885	RKP5873, pBAD/HisA	This study

(*Continued*)

Table 2.1 *E. coli* strains (*continued*)

Strain	Genotype	Source/Reference
RKP5886	RKP5873, pMT1	This study
RKP5887	RKP5872, pPL341	This study
RKP5888	RKP5872, pBAD/HisA	This study
RKP5889	RKP5872, pMT1	This study
RKP120	AV033 MG1655 *ubiCA*::Kan	Constructed by Angie Vreugdenhil, University of Amsterdam.
RKP122	RKP139 (AV060) *ubiCA*::Kan	Constructed by Angie Vreugdenhil, University of Amsterdam.
RKP127	RKP122, pPL341	This study
RKP128	RKP122, pBAD/HisA	This study
RKP129	RKP122, pMT1	This study
RKP130	RKP122, pLW1	This study

Table 2.2 *C. jejuni* strains

Strain	Genotype	Source/Reference
NCT11168	Parental strain	Dr. S. F. Park, University of Surrey
RKP1336	11168 *cgb*::Kan	(Elvers *et al.*, 2004)
RKP1386	11168 *ctb*::Kan	(Wainwright *et al*., 2005)
RKP1389	11168 *ctb*::Kan, *cgb*::Tet	(Wainwright *et al.*, 2005)
-	11168 *cj1585C*::Kan	Kindly given by Prof. David Kelly (Thomas *et al*., 2010)

Table 2.3 Plasmids

Plasmid	Description	Source/Reference
pPL341	pBR322 with *hmp*$^{+}$ under control of its own promoter and Amp^{R} marker.	(Vasudevan *et al.*, 1991)
pRKP1097	pET16b (Qiagen) containing the *cgb* gene cloned in *Xho*I site.	(Pickford *et al.*, 2008)
pLW1 (pBAD-*ctb*)	380 bp PCR product carrying the *ctb* gene in pBAD/HisC between *Nco*I and *Hin*dIII.	(Wainwright *et al*., 2005)
pMT1 (pBAD-*cgb*)	830 bp segment containing the coding region of *cgb* was cut from pRKP1097 with *Nco*I and *Xho*I and cloned into pBAD/HisA cut with the same restriction enzymes.	This study

ml trace elements solution were dissolved in water. After autoclaving, the medium was supplemented with 1 ml $MgCl{\cdot}6H_2O$ (1 M) previously sterilized by filtration (Poole, 1989). For the trace elements solution preparation, 5 g EDTA disodium salt were dissolved in 1 l H_2O and the pH adjusted to 7.0 followed by the addition of 0.05 g $FeCl_3$, 0.05g ZnO, 0.01g $CuCl_2$, 0.01g $CoC1_2.6$ H_2O and 0.01 g H_3BO_3 (Poole and Haddock, 1974).

2.1.2.7 SOB medium

Tryptone (20 g), 5 g yeast extract and 0.5 g NaCl were dissolved in 950 ml H_2O. KCl 10 ml (250 mM) was added and the pH adjusted to 7.0. The volume was made up to 1 l. Agar, 15 g, was added to make solid medium. After autoclaving, the medium was cooled to 55 ºC, and 5 ml of a sterile $MgCl_2$ solution (2M) was added (Sambrook and Russell, 2001).

2.1.2.8 TB soft agar

Tryptone (8 g) and 5 g NaCl were dissolved in 1 l H_2O and the pH adjusted to 7.0, then 7 g agar were added.

2.1.2.9 Phage lysate plates (PL)

Tryptone (4.0 g), 2.5 g yeast extract, 2.5 g NaCl, 1 g glucose and 6.0 g agar were dissolved in 500 ml H_2O. After autoclaving, the medium was cooled to 55 ºC and 5 ml $CaCl_2$ (0.5 M), 5 ml $MgSO_4$ (1 M) and 0.5 ml $FeCl_3$ (10 mM) added. The plates were stored immediately at 4 ºC.

2.1.2.10 P1 plates

Tryptone (4.0 g), 2.5 g yeast extract, 2.5 g NaCl, 1 g glucose and 6.0 g agar were dissolved in 500 ml H_2O. After autoclaving, the medium was cooled to 55 ºC and 5 ml $CaCl_2$ (0.5 M) added. The plates were stored immediately at 4 ºC.

2.1.2.11 TY broth

Tryptone (8 g), 5 g yeast extract and 5 g NaCl were dissolved in 1 l H_2O.

2.1.3 Strain storage

For long term storage, strain stocks were maintained at -70 ºC in 15% glycerol (v/v) in brain heart infusion broth for *C. jejuni* strains and 15% (v/v) glycerol in LB for *E. coli* strains.

2.1.4 Maintenance of bacteria

E. coli strains were stored on LB plates (supplemented with antibiotics) at 4 ºC.

2.1.5 Antibiotic media supplements

Media were supplemented where indicated with vancomycin (10 μg/ml), kanamycin (35 μg/ml), ampicillin (100 μg/ml), chloramphenicol (25 μg/ml) and tetracycline (10 μg/ml), (all final concentrations).

2.1.6 Culture techniques

2.1.6.1 *E. coli* aerobic growth conditions

E. coli cells were taken from glycerol stocks and spread on LB or 2 X TY plates containing antibiotics and grown overnight at 37 ºC. Starter cultures were prepared by transferring single colonies into LB or 2 X TY (5 ml) contained in 20 ml tubes. Cultures were grown overnight at 37 ºC, 240 rpm and used to inoculate secondary cultures (1% (v/v)) contained in either 20 ml tubes or 250 ml arm-side flasks and incubated at 37 °C, 240 rpm.

2.1.6.2 *E. coli* anaerobic growth conditions

Starter cultures were prepared as in section 2.1.6.1 but anaerobic cultures were grown in 8 ml screw cap tubes filled with LB containing a glass bead to facilitate resuspension and were incubated statically at 37 °C.

2.1.6.3 *C. jejuni* growth conditions

Standard *C. jejuni* cultures were performed as described previously using a MACS-VA500 microaerophilic cabinet (Don Whitley Science) at 80% N_2, 10% O_2 and 10% CO_2 at 42 ºC (Elvers *et al.*, 2004). Cells taken from glycerol stocks were spread on Columbia blood plates and incubated for 48 h. Starter cultures were prepared by harvesting cells from 1 plate with 3 ml Mueller-Hinton broth and the suspension was added to the same medium (50 ml) supplemented with vancomycin (plus additional antibiotics when the strain had resistance markers) in a 125 ml baffled flask. After 16 h incubation in a rotatory shaker (Mini Orbital Shaker S05, Stuart Scientific) at 150 rpm, the OD at 600 nm was adjusted to 0.6. This was used to inoculate secondary cultures (5% (v/v)) in Mueller-Hinton broth (100 ml) added with antibiotics in a 250 ml baffled flask unless otherwise stated.

2.1.6.4 Susceptibility tests

Starter cultures (1% (v/v)) were used to inoculate tubes containing LB medium (2 ml) plus antibiotics and, when indicated, supplemented with arabinose (0.02%), $FeCl_3$ (13 μM) and δ-aminolevulinic acid (1 mM). Increasing concentrations of DETA NONOate or GSNO were added and the cultures incubated for an additional 18 h. Growth was determined as the optical density at 600 nm by using a spectrophotometer (Jenway).

2.1.6.5 Growth curves

Starter cultures (1% (v/v)) were used to inoculate LB or 2 X TY (10 ml) fresh medium plus antibiotics and, when indicated, supplemented with arabinose (0.02%), $FeCl_3$ (13 μM) and δ-aminolevulinic acid (1 mM). Cultures were added with DETA NONOate (1 mM), GSNO (3 mM) or H_2O_2 (3 mM) and incubated at 37 °C, 240 rpm in 250 ml flasks fitted with side arms. Growth measurements were recorded every hour using either the colorimeter or the spectrophotometer at 600 nm. For *Campylobacter* growth curves, starter cultures (5% (v/v)) were used to inoculate Mueller-Hinton broth (100 ml) with antibiotics in a 250 ml baffled flask and 500 μM DETA NONOate or 400 μM GSNO were added.

2.1.7 Overexpression of Cgb and Ctb in *E. coli*

For *in vivo* tests, Cgb and Ctb were over-expressed as described previously (Pickford *et al.*, 2008). Starter cultures of *E. coli* carrying pLW1 or pMT1 (see Table 2.1) were used to inoculate LB or 2 X TY broth media containing ampicillin (plus additional antibiotics when the strains had resistance markers) and supplemented with arabinose (0.02%), $FeCl_3$ (13 μM) and δ-aminolevulinic acid (1 mM) and incubated at 37 ºC, 240 rpm. For purification purposes, starter cultures of *E. coli* BL21 (DE3), pLysS freshly electrotransformed with pRKP1097 or pLW1 were used to inoculate (1% (v/v)) 5 x 2 l flasks containing 500 ml LB each added with ampicillin. Cultures were incubated at 37 ºC, 240 rpm to an OD at 600 nm of 0.4-0.6. Cultures were then induced with IPTG (1 mM) or arabinose (0.02%) respectively and supplemented with δ-aminolevulinic acid (1 mM) and $FeCl_3$ (13 μM). After a further 4 h incubation, cells were harvested by centrifugation at 10,000 rpm for 20 min and pellets stored at -70 ºC until required.

2.1.8 Culture turbidity measurements

Measurements of optical density were done using a Jenway 7305 spectrophotometer at 600 nm in cuvettes with a 1 cm path length against a medium blank or in a Klett Summerson photoelectric colourimeter (Klett Manufacturing Co, New York, N. Y) with a no. 66 red filter when 250 ml flasks fitted with side arms were used.

2.1.9 Preparation of cell suspensions

Batch cultures (45 ml) grown in LB or 2 X TY were added with antibiotics, and for expression of heterologous globins, supplemented with arabinose (0.02%), $FeCl_3$ (13 μM) and δ-aminolevulinic acid (1 mM) and incubated overnight at 37 °C, 240 rpm. Cultures were centrifuged at 5,500 rpm at 4 °C for 10 min and pellets resuspended in buffer Tris-HCl 50 mM (pH 7.4) (approximately 8 ml) followed by standardization of the OD at 600 nm.

2.1.10 Isolation of bacterial membranes

This method was modified from Poole and Haddock (1974). Overnight starter cultures were diluted 1:100 in 2 X TY medium (250 ml) in 1 l baffled conical flasks and incubated in standard conditions for 6 h. Cultures were centrifuged at 10,000 rpm for 20 min and cell pellets resuspended in Tris-HCl 50 mM, 2 mM $MgCl_2$, 1 mM $MgCl_2$ (pH 7.4) (10 ml). Cells were disrupted by sonication (Soniprep 150) (4 x 20 s bursts at an amplitude of 10 μm) on ice, and centrifuged for a further 30 min to separate cell debris and undisrupted cells. Membranes were separated from the supernatant by ultracentrifugation at 40,000 rpm for 1 h. The supernatant was discarded and membranes resuspended in buffer (200 μl) and aliquots (40 μl) stored at -80 °C.

2.1.11 Preparation of soluble extracts

For preparing *E. coli* soluble extracts, overnight cultures were diluted 1:100 (200 ml final volume) in LB in 1 l conical flasks and incubated for a further 6 h at 37 °C, 240 rpm. For *C. jejuni* extracts, overnight cultures (50 ml in 100 ml conical flasks) grown in Mueller-Hinton broth (MH) at 42°C were diluted 1:3 (150 ml final volume) with MH broth in 250 ml baffled flasks and incubated for an additional 9 h. Cells were harvested by centrifugation and resuspended in Tris-HCl 50 mM (pH 7.4) (10 ml). Cell suspension was sonicated (Soniprep 150) (4 x 20 s bursts at an amplitude of 10 μm) on ice, and then centrifuged at 10,000 for 20 min to separate intact cells and cell debris.

Supernatant was ultracentrifuged at 40,000 rpm for 1 h to separate membranes and the supernatant (soluble content) was stored up to 48 h at 4 °C.

2.2 Buffers and solutions

Chemicals were purchased from Sigma, unless otherwise stated. When required, chemicals and solutions were sterilised by filtration using Millipore filters with a pore size of 0.45 µm (Sambrook and Russell, 2001).

2.2.1 Phage dilution buffer

Trisma base (0.61 g), 1.23 g $MgSO_4 \cdot 7H_2O$, 0.37 g $CaCl_2 \cdot 2H_2O$ and 1.46 g NaCl were dissolved in 450 ml H_2O and the pH adjusted to 7.5 with HCl. The solution was made up to 500 ml.

2.2.2 Tris-HCl 50 mM, pH 7.4

Tris-HCl (7.88 g) was dissolved in 700 ml H_2O. The pH was adjusted and the solution made up to 1 l.

2.2.3 Tris-acetate-EDTA (TAE) 50 X buffer

Tris base (242 g), 57.1 ml glacial acetic acid, 100 ml EDTA (0.5 M, pH 8.0) dissolved in H_2O and made up to 1 l (Sambrook and Russell, 2001).

2.2.4 Antibiotic selection

All antibiotics were added as a stock solution to liquid agar and liquid media at 50 ºC at 1/1000 dilution. The amounts added to make 1 ml stock solutions were as follows: tetracycline 5 mg (in ethanol), kanamycin 10 mg, ampicillin 50 mg, chloramphenicol 34 mg (in ethanol) and vancomycin 10 mg. Stocks were stored at -20 °C.

2.2.5 Preparation of *S*-nitrosoglutathione (GSNO)

A 500 ml beaker was filled with ice and water and placed on a stirring plate in a fume cupboard. To a 100 ml conical flask, covered with foil and placed into the beaker with a magnetic stirrer, were added 3.08 g L-glutathione (reduced) and 18 ml ice cold water. The mixture was stirred until glutathione was dissolved. HCl (concentrated, 0.83 ml) and 0.69 g $NaNO_2$ were added and the solution stirred until it turned pink (40 min approximately). The solution was stirred for a further 10 min upon addition of 20 ml

ice-cold acetone (20 ml) and filtered through filter paper on a Buchner funnel using a concentrator pump. The precipitate was washed 5 times with 2 ml ice-cold H_2O, 3 times with 10 ml ice cold acetone and 3 times with 10 ml diethyl ether and dried in a vacuum desiccator protected from light (Hart, 1985). Solid GSNO was stored in aliquots at -70 ºC. To make a stock solution, a small amount of solid GSNO was dissolved in H_2O at 55 ºC protected from light. GSNO displays an absorption maximum at 545 nm, with an extinction coefficient of 15.9 M^{-1} cm^1. The GSNO solution was prepared immediately prior to the experiments and the stock concentration determined spectrophoyo metrically.

2.2.6 Glycerol 10% (v/v)

Glycerol (126 g) (density 1.6 g/cc) was added to 900 ml H_2O.

2.2.7 DETA NONOate stock solution

DETA NONOate, ((Z)-1-[2-(2-Aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (Enzo)) (25 mg) was dissolved in 1 ml NaOH (0.1 M) to obtain a 153 mM stock and stored at -20 °C protected from direct light.

2.2.8 PROLI-NONOate stock solution

A small amount of PROLI-NONOate (1-(hydroxi-NNO-azoxy)-L-proline)) powder (Cayman Chemicals) was dissolved in 10 ml NaOH (0.01 M). PROLI NONOate displays an absorption maximum at 252 nm, with an extinction coefficient of 8,400 M^{-1} cm^1. The stock concentration was determined spectrophotometrically and aliquots (500 µl each) stored at -70 °C protected from direct light.

2.2.9 Markwell assay reagents

Reagent A was a solution of 20 g Na_2CO_3, 4 g NaOH, 1.6 g sodium tartrate and 10 g SDS dissolved in H_2O and made up to 1 l. For regent B, 4g $CuSO_45H_2O$ were dissolved in H_2O and made up 100 ml. Reagent C was made by diluting reagent A with reagent B (1:100) and mixed. Folin-Ciocalteau phenol reagent was prepared by diluting commercial reagent with mQH_2O (1:1) within an hour before use.

2.2.9.5 Protein standard (BSA)

Bovine serum albumin standard was prepared fresh at 200 µg/ml final concentration.

2.2.10 Tris-glycine running buffer

Tris base (3.02 g) and 18.8 g glycine were dissolved in 990 ml H_2O and added to 10 ml SDS (10% (w/v)).

2.2.11 SDS loading buffer (2X)

Tris-HCl (100 mM) pH 6.8, 200 mM DTT, SDS (4% (w/v)), bromophenol blue (0.2% (w/v)) and glycerol (20%) in 20 ml final volume.

2.2.12 Western blot solutions

Transblot buffer was made by dissolving 14.4 g glycine, 15.15 g Tris base, 200 ml methanol and 3 ml SDS (10% (w/v)) in 1 l H_2O. Tris-buffered saline with 0.1% Tween (TBS-T) was a solution of 2.24 g Tris-HCl (pH 7.5), 8 g NaCl and 100 µl Tween 20 dissolved in 1 l distilled water.

2.2.13 Preparation of CORM-3

A stock solution (10 mM) was prepared by dissolving 3.1 mg of CORM-3 ([Ru(CO)(3)Cl(glycinate)], synthesized as described previously (Clark *et al.*, 2003)) in 1 ml H_2O. The solution was kept on ice during the experiment and stored at 4 °C for up to 48 h.

2.3 Genetic methods

2.3.1 Plasmid DNA purification

Plasmid DNA purification was made using the QIAprep Spin Miniprep Kit, according to the manufacturer's instructions (Qiagen). Overnight cultures (5 ml) were centrifuged at 5,000 rpm for 5 min and the pellet resuspended in 250 µl buffer P1 and transferred to a microcentrifuge tube. Buffer P2 (250 µl) was added and the tube was inverted 5 times to mix. Buffer N3 (350 µl) was added and the contents were mixed by inverting the tube 5 times. The tube was centrifuged at 13,000 rpm for 10 min and the supernatant transferred to the QIAprep spin column and centrifuged for 60 s. The column was washed with 750 µl buffer PE and centrifuged for 60 s twice. The DNA was eluted from the column by adding 50 µl H_2O and centrifuging for 2 min.

2.3.2 Restriction enzyme digestions

Restriction enzymes were purchased from Promega. The manufacturer's instructions were followed with regard to reaction buffers and incubation temperatures.

2.3.3 Ligation

The restricted DNA fragment and vector (1:1, 10 ng each) were ligated with T4 DNA ligase (1 µl) (Promega) and ligase buffer (1 µl) (Promega) and the mixture (10 µl final volume) was incubated overnight at room temperature. Ligation mixture (1 µl) was used to transform electrocompetent cells. The pMT1 construction was verified by restriction with *Nco*I and *Xho*I and by sequencing (Cogenics Technologies, Inc.) using universal primers (pBAD F and R primers).

2.3.4 Agarose gel electrophoresis of DNA

Plasmid DNA and restriction enzyme digests were routinely analysed by electrophoresis through a 1.0% agarose gel in TAE buffer supplemented with 0.5 µg/ml ethidium bromide (Sambrook and Russell, 2001). Loading buffer (5X) (Bioline) was added to DNA samples, and 10 to 100 ng of DNA was loaded per lane. HyperLadder I (BIOLINE) (200 ng) was run as a marker alongside the samples to estimate the sizes of DNA fragments. DNA was visualized by exposure to a source of 254 nm ultraviolet light.

2.3.5 Construction of deletion mutants.

E. coli strains carrying multiple gene deletions were constructed by P1*vir* phage transduction. The *hmp ubiCA* mutant was constructed by transferring the *hmp* mutation from JW2536 (Baba *et al.*, 2006) into MG1655 where first the kanamycin marker was removed as described (Datsenko and Wanner, 2000) and then the *ubiCA* deletion transferred (Table 2.1). The *norR* and *norW* mutant genes were transferred from strains AG200 and AG400 respectively (Gardner *et al.*, 2002) into MG1655 and MG1655 *hmp* (RKP3036) (Table 2.1).

2.3.6 Generalized transduction with bacteriophage P1 *vir*

2.3.6.1 Preparation of lysates

Lysates were prepared according to a published method (Miller, 1972). Donor cells were grown overnight at 37 ºC in TY medium supplemented with $CaCl_2$ (5 mM). P1 *vir*

stock was diluted from 10^8 to 10^3 PFU/ml; each was mixed with 100 µl culture and incubated at 37 ºC for 20 min. Pre-warmed (37 ºC) TB (1ml) and 1.5 ml warm (55 ºC) TB soft agar were mixed and added to the phage/cell mix and poured onto phage lysate plates. The plates were incubated at 37 ºC in a "wet box" (with a humid atmosphere) until plaques had taken on a "lacy" appearance, after which the plates were chilled at 4 ºC for half an hour and an overlay of cold phage dilution buffer added. Plates were stored at 4 ºC overnight and the overlying liquid harvested using a Pasteur pipette. Lysates were stored at 4 °C and for long storage a few drops of chloroform were added.

2.3.6.2 Generalized transduction

Recipient cells were grown overnight at 37 ºC in 2.5 ml TY supplemented with $CaCl_2$ (5 mM). P1 *vir* stock from a lysate preparation (100 µl) was mixed with the same volume of cells and incubated at 37 ºC for 20 min. The mixture was spread onto P1 plates containing antibiotics plus $Na_4P_2O_7$ (0.125 mM) and incubated overnight at 37 ºC (Miller, 1972). After overnight incubation, potential transductants were re-plated onto selective medium and the phenotype verified.

2.3.7 Electrotransformation of *E. coli*

Electrotransformation of electrocompetent cells was performed according to the manufacturer's instructions (MicroPulser™ electroporation apparatus operating instructions and applications guide, Bio Rad).

2.3.7.1 Preparation of electrocompetent cells

LB broth (500 ml) was inoculated with an overnight culture (1% inoculum) and cells incubated at 37 °C, 240 rpm until 0.5-0.7 OD at 600 nm was reached. The culture was chilled on ice for 20 min and cells harvested in a pre-chilled container by centrifugation at 5,500 rpm for 15 min at 4 °C. The supernatant was discarded and the pellet resuspended in 500 ml 10% ice-cold glycerol and centrifuged for 15 min at 4 °C. The same was repeated twice but adding 250 and 20 ml 10% ice-cold glycerol respectively. Finally, the cell pellet was resuspended in 2 ml of 10% ice-cold glycerol and stored at -70 °C in small aliquots for up to 6 months.

2.3.7.2 Electrotransformation

Electrocompetent cells were thawed on ice. A 1.5 ml tube and a 0.1 electroporation cuvette (Bio Rad) were also chilled on ice. Cells (40 µl) were mixed with 1 µl plasmid DNA in the 1.5 ml tube and incubated for 1 min on ice. The MicroPulse^{TM} was set to "Ec1" and the mixture transferred to the electroporation cuvette. The cuvette was seated in the chamber and pulsed once. After the cuvette was removed from the chamber, 1 ml LB at 37 °C ml was added and the suspension transferred immediately to a 1.5 ml tube. After 1 h incubation at 37 °C (240 rpm), a sample of the suspension (100 µl) was spread on LB plates containing antibiotic to select transformants.

2.4 Biochemical methods

2.4.1 Protein quantification

2.4.1.1 Markwell assay

This is a method modified from the Lowry protocol (Markwell *et al.*, 1978). Samples were diluted as required with H_2O before the assay. Freshly made reagent C (see section 2.2.9) (3 ml) was mixed with the protein samples (1 ml) by vortexing and incubated at room temperature for 1 h. Folin-Ciocalteau phenol reagent was added (0.03 ml) to the samples with whirlimixing and samples incubated at room temperature for a further 45 min. The absorbance was recorded at 660 nm. Dilutions of a BSA stock were made from 0 to 200 µg protein/ml for the standard curve. The assay was perfomed with three different volumes of sample in triplicate (e.g. 10, 50 and 100 µl sample).

2.4.1.2 Bradford assay

BioRad protein assay (Bradford assay) was performed in cuvettes according to the manufacturer's instructions. Diluted protein assay solution (1 ml) was added followed by the protein sample. A BSA standard curve using concentration from 0 to 21 µg/ml protein was also performed and the absorbance recorded at 595 nm.

2.4.2 SDS-polyacrylamide gel electrophoresis (PAGE)

A BioRad MiniProtean gel tank with self-poured polyacrylamide gels was used. Precast gels 15% (BioRad) were electrophoresed in Tris-glycine running buffer. Protein assay (Bradford) was performed for cell free extracts and samples diluted with H_2O to

standardize the protein concentration across the samples. Samples (20 µl) were resuspended in 2X SDS loading buffer and incubated at 100 ºC for 15 min and run on SDS-PAGE gels. Gels were performed in duplicate; one was unstained and used for Western blotting and the other stained with Coomassie blue to check equal amounts of protein across the samples.

2.4.3 Western blot

An unstained SDS-PAGE gel carrying the samples (10 µg) was used to transfer the proteins onto a nitrocellulose membrane (Hybond-C Extra membrane (Amersham Bioscience)) by using a BioRad Mini Trans-Blot Electrophoresis cell containing ice-cold transblot buffer according to the manufacturer's instructions. Samples were transferred from the gel to the membrane at 400 mA, 4 ºC for 90 min. The membrane was immersed in 5% casein (w/v) (prepared in TBS-T) and incubated overnight at room temperature. The membrane was washed with TBS-T (1 x 15 min followed by further 3 x 5 min). The Cgb and Ctb antibodies were diluted in 5% casein ((w/v) 1:2000 and 1:1000 respectively) and the membrane incubated for 1 h before a further round of washings was performed. ECL peroxidase-labelled secondary antibody (2 µl) (Amersham Biosciences) was added to 25 ml 5% casein and the membrane incubated for 1 h prior to a third wash step. ECL Western blotting detection reagents 1 and 2 (Amersham Biosciences) were mixed according to the manufacturer's instructions and the mixture poured onto the membrane followed by 5 min incubation. Finally, the membrane was covered in clingfilm and overlaid with Hyperfil ECL high performance chemiluminiscence film (Amersham Biosciences) for 1 to 5 min in the dark room and the film developed using Industriex developer (Kodak).

2.5 Polarographic measurements of oxygen and nitric oxide

2.5.1 Respiration rates of cell and membrane suspensions

The oxygen electrode was calibrated with air-saturated Tris-HCl 50 mM (pH 7.4) (assuming an oxygen concentration at air saturation of 200 µM O_2) and anoxia was achieved by addition of sodium dithionite (a few grains) (Gilberthorpe and Poole, 2008). Cell suspensions (see section 2.1.9) diluted 1:1 with Tris-HCl 50 mM buffer (pH 7.4) (2 ml final volume) or membranes suspensions (20 µl) were added to a chamber fitted with a Clark-type polarographic oxygen electrode (Rank Brothers) (Gilberthorpe

and Poole, 2008). Tests were performed at a polarizing voltage of 0.6 V at 37 °C with constant stirring and a sealing lid. DataTrax software (World Precision Instruments, Inc.) was used to record the data. Glycerol solution (5 mM final concentration) or succinate (20 mM final concentration) were added to the chamber with a Hamilton syringe in order to promote respiration of cells and membranes respectively. Protein concentration was determined by the Markwell assay and rates were expressed as nmol O_2/min mg protein.

2.5.2 Determination of NO consumption of cell suspensions

NO consumption was determined by using a modified Clark-type oxygen electrode system (Rank Brothers) harbouring an NO electrode (Precision Instruments ISO NOP sensor (2-mm diameter)) by closing the chamber with a tight lid, with the NO sensor inserted through a tailor-made hole in the lid (Mills *et al.*, 2001). The NO electrode was calibrated according to the manufacturer (Corker and Poole, 2003). Cell suspensions (section 2.1.9) were diluted 1:1 with Tris-HCl buffer in the chamber (2 ml working volume) at 37 °C. PROLI NONOate aliquots (1 μM final concentration) were added to determine NO consumption.

2.5.3 Simultaneous measurement of NO and O_2 consumption of cellular suspensions.

The method is based on (Gilberthorpe *et al.*, 2007). The experiments were carried out in an O_2 electrode chamber (Rank Brothers) harbouring an NO sensor (section 2.5.3). Glycerol (5 mM) was added to promote respiration (measured as in section 2.5.1). PROLI NONOate solution was added using a Hamilton syringe (1 μM final concentration in each case) when the O_2 concentration reached 70, 40, 20 and 0%. Initial respiratory rate, the inhibited rate, and the rate when oxygen uptake was spontaneously reinitiated were calculated. The period of inhibition of respiration was calculated as the period between addition of the NO solution (when respiration becomes inhibited) and the point where oxygen uptake was reinitiated. Protein was determined by the Markwell assay.

2.5.4 Determination of NO evolution from cellular suspensions

Consumption of O_2 and NO production were followed simultaneously as described in section 2.5.4 but the method was based on (Corker and Poole, 2003). Cell suspension was diluted 1:1 with buffer in the chamber (2 ml working volume). Glycerol (15 mM)

was added to promote respiration and after O_2 was depleted, $NaNO_2$ (25 mM final concentration) was added by injection with a Hamilton syringe and concentration of O_2 and NO production were followed. Carboxy-PTIO (10 mM final concentration) was injected either when NO production reached a plateau or after 40 min incubation. Concentration of total protein was quantified by the Markwell assay.

2.6 Protein purification

2.6.1 Ctb purification

Purification of Ctb was performed as described (Pickford *et al.*, 2008). A frozen cell pellet obtained from 2.5 l of culture (see section 2.1.7) was defrosted and resuspended in Tris-HCl 50 mM buffer (pH 7.0) (40 ml) followed by sonication (3 x 20 sec in a MSE Soniprep 150 at maximum power). Cell debris was removed by centrifugation at 10,000 rpm for 20 min. The supernatant (red-brown in colour) was loaded onto a DEAE Sepharose Fast Flow 30-ml column (Pharmacia Biotech) previously equilibrated with Tris-HCl 50 mM (pH 7.0) connected to an Akta Purifier (GE Healthcare Bio-Sciences, Amersham Biosciences Ltd., UK). After washing the column with the same buffer (40 ml) the sample was eluted with a NaCl gradient (0-0.5 M) in buffer. Red-coloured fractions were concentrated to 5 ml in a Vivaspin 20 concentrator (Vivascience) (cutoff of 5 kDa) and used for a further purification by gel filtration. The sample was loaded onto a Superdex-200 column (16 x 60 cm, GE Healthcare Bio-Sciences, Amersham Biosciences Ltd., UK) previously equilibrated with Tris-HCl 50 mM (pH 7.0), 0.2 M NaCl and eluted with the same buffer (1 ml/min). Red fractions were mixed and SDS-PAGE was used to verify the presence and purity of Ctb (14.06 kDa monomer) and small aliquots stored at -70 °C.

2.6.2 Cgb purification

The Cgb purification protocol was modified from (Pickford *et al.*, 2008). A frozen cell paste obtained from 2.5 l of culture (see section 2.1.7) was defrosted and suspended in Tris-HCl 50 mM buffer (pH 9.0) (40 ml) and disrupted by sonication (3 x 20 sec in a MSE Soniprep 150 at maximum power). Cell debris was removed by centrifugation at 10,000 rpm for 10 min. The supernatant fraction was applied to a 25 ml DEAE-Sepharose FF column. Proteins were eluted using a 250 ml NaCl gradient from 0 to 0.3 M in the same buffer. Red fractions eluting at about 65 mM NaCl were combined and

0.32 ml of 4 M ammonium sulfate was added to each ml of the sample to bring the ammonium sulfate concentration to about 1.2 M. The sample was then applied on a 5 ml Hi-Trap Phenyl-HP column (GE Healthcare) and eluted by 50 ml of a reverse gradient of ammonium sulfate (1.2 M to 0 in 50 mM Tris-HCl buffer pH 8.0). Coloured fractions eluting at about 0.9 M ammonium sulfate were combined; a volume of the sample was reduced to 2 ml using a VivaSpin 20 concentrator (Sartorius) and applied on a 16 x 60 cm HiLoadSuperdex200 column (GE healthcare) equilibrated in buffer 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl. Gel filtration was performed in the same buffer. Coloured fractions eluted from the column at 90 ml and corresponded to Cgb in the monomeric state. SDS-PAGE was used to verify the presence and purity of Cgb (16.08 kDa monomer) and small aliquots stored at -70 °C.

2.7 Spectroscopic techniques

2.7.1 Reducibility of Cgb and Ctb in cell-free extracts

Optical spectra were recorded using an Olis RSM 1000 spectrophotometer. Spectra of purified Cgb (5 µM) or Ctb (4.8 µM) in *E. coli* or *C. jejuni* soluble extracts or Tris-HCl 50 mM (pH 7.4) buffer with or without NADH (10 mM) were recorded every minute against a baseline of buffer (Tris-HCl, 50 mM, pH 7.4) at room temperature until a stable spectrum was attained. PROLI NONOate (5 and 10 µM) was added followed by gentle mixing and changes recorded every min for 40 min. Redox forms were mainly identified from the α,β region of the spectra by reference to published data (Shepherd *et al.*, 2011; Wainwright *et al.*, 2006).

2.7.2 Optical spectroscopy of intracellular Ctb

Absorption spectra of whole cells of *E. coli hmp* overexpressing Ctb were recorded using an SDB-4 dual-wavelength scanning spectrophotometer at room temperature (Kalnenieks *et al.*, 1998) in native and reduced states. Reduction was achieved either by addition of sodium dithionite (a few grains) or glucose (15 mM). Optimal respiratory capacity of cell suspensions was verified polarographically (see section 2.5.1) prior to the spectrometric tests by addition of glucose in a closed chamber and, when anoxic conditions were reached, the chamber was opened and the time for re-oxygenation measured to ensure anaerobiosis during the tests. Respiration was considered optimal when open-lid suspensions showed re-oxygenation after 30 min incubation in the open

system. Native and reduced samples with sodium dithionite or glucose were bubbled with CO gas for 2 min and spectroscopic changes recorded immediately. When CORM-3 (300 µM) was added to the reduced samples, changes were recorded every min for 10 min and every 5 min for an additional 20 min. Difference spectra (CO-reduced minus reduced) were plotted. Cells carrying the empty vector were reduced with dithionite and bubbled with CO gas and the difference spectrum was plotted for comparison. The Ctb absolute spectrum (reduced and CO-reduced) were obtained by subtraction of the absorbance values from the samples carrying the empty vector. Data were analyzed using Excel 2007 and Sigma Plot 11.0.

2.7.3 Determination of haem content in cellular suspensions (alkaline pyridine assay)

This method was performed as described previously (Poole *et al.*, 1986)**.** Cell suspension (see section 2.1.9) was sonicated (Soniprep 150) (4 x 20 s bursts at an amplitude of 10 µm) on ice, and then centrifuged at 12,000 rpm for 10 min to separate intact cells and cell debris. Supernatant (0.6 ml) was mixed with the same volume of pyridine/NaOH regent (NaOH (0.4 M) and pyridine (4.2 M)) in a 1 ml quartz cuvette to promote haemochrome formation. For the oxidised sample, a few grains of ferricyanide were added and, after mixing, the sample was scanned in the range 500-600 nm using a Johnson Foundation SDB3 dual-wavelength spectrophotometer at room temperature (Kalnenieks *et al.*, 1998). For the reduced sample, a few grains of sodium dithionite were added to a fresh sample and scanned as before. Reduced minus oxidised spectra were plotted and haem concentration was determined. Haem *b* displays an absorption maximum at 556 nm, with an extinction coefficient of 19 mM^{-1} cm^{1}.

2.7.4 Determination of NO production from nitrite by Ctb

This method was modified from (Salhany, 2008) as described by (Pedersen *et al.*, 2010). Purified Ctb (3.7 µM) in Tris-HCl 50 mM (pH 7.4) buffer was reduced with sodium dithionite (10 mM final concentration) followed by the addition of sodium nitrite (0.5 mM) and changes in the spectrum were recorder with a Cary 50 Conc UV-visible spectrophotometer (Varian) before addition of nitrite and after 20 min incubation with nitrite at room temperature. Spectra were recorded against a buffer baseline and the formation of FeII-NO Ctb was followed at 419, 540 and 565 nm (Shepherd *et al.*, 2011).

2.7.5 Kinetics of NO-reduced Ctb formation from nitrite

The conditions were similar to section 2.7.4 but Ctb (10 μM) was reduced with sodium dithionite (10 mM) and treated with sodium nitrite (0.5 mM). Changes in absorbance were recorded before addition of nitrite and then every 0.3 min for 40 min after addition of nitrite. Differences in absorbance (434 minus 426 and 420 minus 426) were plotted.

Chapter 3. Responses to nitrosative and oxidative stress of an *E. coli* NO-sensitive strain expressing the *C. jejuni* haemoglobins Cgb and Ctb.

3.1 Introduction

E. coli possesses two main NO-detoxifying mechanisms. In aerobic conditions, the flavohaemoglobin Hmp, the best characterised bacterial haemoglobin, which consists of a globin and a reductase domain (Mukai *et al.*, 2001), catalyses the conversion of NO and O_2 to the innocuous ion nitrate by either a dioxygenase (NOD) (Gardner *et al.*, 2006; Gardner *et al.*, 2000; Gardner *et al.*, 1998b) or denitrosylase activity (Hausladen *et al.*, 2001; Hausladen *et al.*, 1998). In anaerobic environments, the NO sensor NorR positively regulates the expression of the flavorubredoxin protein (NorV) and its reductase partner (NorW); the pair NorVW detoxifies NO by reduction to N_2O (Gardner and Gardner, 2002; Gardner *et al.*, 2003; Hutchings *et al.*, 2002). Even though, in the absence of O_2 Hmp is also able to reduce NO, this reaction proceeds at a very low rate (Mills *et al.*, 2001).

Resistance to nitrosative stress by *C. jejuni* has been mainly attributed to the presence of the single-domain haemoglobin Cgb, a homologue of the Hmp haem domain (33% identity) (Elvers *et al.*, 2004). Structural and functional studies suggest that Cgb catalyses a NO detoxification reaction (Lu *et al.*, 2007b), proceeding by a NOD or a denitrosylase mechanism (Shepherd *et al.*, 2011). There is strong evidence supporting the NO and RNS detoxification as the main function of Cgb in *C. jejuni*. For instance, Cgb expression, mediated by the transcription regulator NssR, is induced under nitrosative stress conditions (Elvers *et al.*, 2005), cells expressing Cgb by exposure of cultures to sub-inhibitory concentrations of GSNO, exhibit clear NO consumption and undisrupted respiration compared with the *cgb* mutant or untreated cells, and cultures of a *cgb* mutant are hypersensitive in the presence of NO and GSNO (Elvers *et al.*, 2004).

The confirmation that the NO detoxification mechanism is mediated by Cgb *in vivo* is complicated by difficulties expressing proteins in *trans* in *C. jejuni*. Additionally, studies *in vitro* using purified protein are limited by the lack of the reductase domain in

Cgb that, in Hmp, allows the restoration of the ferrous state required for ligand-binding activity (Hernandez-Urzua *et al.*, 2003).

Heterologous expression to investigate the ability of Cgb to confer resistance to nitrosative stress in *E. coli* has been previously reported (Frey *et al.*, 2002). That study showed an improvement of the growth profile of a Cgb-expressing *E. coli* strain under nitrosative stress conditions compared to the wild type. However, when the NO uptake of soluble protein fractions from the strain expressing Cgb and the control were compared, there were no differences. However, since a wild type strain was used as the model, the inevitable presence of Hmp in the tested conditions makes it difficult to distinguish between the effects due to Hmp *per se*, and the possible additional effect produced by the expression of the heterologous globin.

Under nitrosative stress conditions, *C. jejuni* expresses a second globin (Ctb) also under control of NssR (Elvers *et al.*, 2005). This protein is classified as a member of group III of the truncated haemoglobins (TrHb3) (Wittenberg *et al.*, 2002). The structural and kinetic characteristics of Ctb have been extensively studied (Bolli *et al.*, 2008; Lu *et al.*, 2007a; Nardini *et al.*, 2006; Wainwright *et al.*, 2006) and, due to the exceptionally high oxygen affinity of Ctb (dissociation kinetics 0.0041 s^{-1}) (Lu *et al.*, 2007a) together with evidence of its role as an O_2 regulator (Wainwright *et al.*, 2005), involvement of this globin in oxygen chemistry has been suggested. However, the physiological significance of Ctb expression under nitrosative stress conditions has not been unravelled.

In order to produce data that allowed a better understanding of the molecular mechanism(s) and physiological functions of the *C. jejuni* haemoglobins, the main objective of the present chapter is to investigate the effects of the heterologous expression of the *C. jejuni* globins in *E. coli* under nitrosative and oxidative stress conditions. An NO-sensitive *E. coli* strain (*hmp* mutant) expressing Cgb or Ctb is used as the biological model.

3.2 Results

3.2.1 Expression of the *C. jejuni* haemoglobins Cgb and Ctb in an *E. coli hmp* mutant strain.

In order to test the effect of the Cgb and Ctb haemoglobins in the response to nitrosative stress in the heterologous host *E. coli*, an expression vector (pBAD/HisA (Invitrogen)) was used to clone the reading frame of the *cj1586* (*cgb*) gene under control of an arabinose-inducible promoter. The construction was verified by sequencing and designated pMT1 (Fig. 3.1A). A similar plasmid but containing the reading frame of the *cj0465c* (*ctb*) gene was used for the expression of the truncated haemoglobin Ctb (pLW1) (Wainwright *et al.*, 2006) (Fig. 3.1B).

In an approach to test the ability of Cgb and Ctb to protect the heterologous host *E. coli* from nitrosative stress, an *E. coli* NO-sensitive strain (*hmp* mutant) was transformed with the plasmid pMT1, pLW1 or the empty vector pBAD/HisA and the expression of the *C. jejuni* haemoglobins in cultures was verified on SDS polyacrylamide gels and by Western blot. Total protein of cellular extracts from cultures grown in the presence or absence of the inducer arabinose (0.02%) was assayed. Even though it was not possible to observe the presence of Cgb in a conventional SDS-polyacrylamide gel, expression of this globin was demonstrated by the detection of a band with an approximate weight of 14 KDa (Cgb molecular weight, 16.08 KDa) using polyclonal antibodies against the globin (Fig. 3.1C). Higher concentrations of arabinose failed to improve the expression of Cgb (not shown). On the other hand, Ctb (molecular weight 14.8 KDa) was over-expressed at much higher levels. A band of approximately 14 KDa was clearly observed in the SDS polyacrylamide gel and the Western blot analysis using anti-Ctb antibodies confirmed the globin identity (Fig. 3.1D).

3.2.2 Expression of the *C. jejuni* haemoglobins Cgb and Ctb does not alter the growth profile of an *E. coli hmp* mutant strain.

As a preliminary characterisation, toxic effects or modifications on the growth profile of the *E. coli hmp* mutant by the expression of the *C. jejuni* haemoglobins were investigated. No significant differences in the growth capacity were observed when

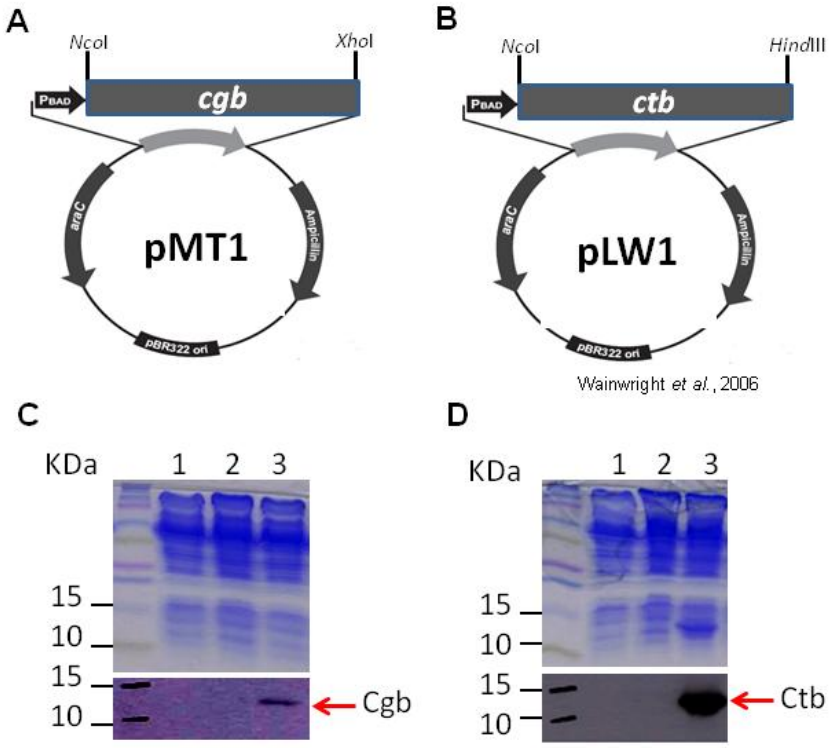


Figure 3.1 Expression of the *C. jejuni* haemoglobins in an *E. coli hmp* mutant strain.

Immunoblotting of cellular extracts from cultures growing in LB supplemented with arabinose were tested for the expression of Cgb and Ctb in an *hmp* mutant strain transformed with the expression vector pMT1 (A) or pLW1 (B) respectively. Extracts of the *hmp* mutant transformed with the empty vector (lane 1, C and D), pMT1 or pLW1 without arabinose (lane 2, C and D respectively) were tested as a control. Anti *C. jejuni* Cgb or Ctb antibodies were used for detection of Cgb and Ctb (lanes 3, C and D respectively). SDS-polyacrylamide gels containing total protein are shown for comparison purposes (C and D upper panels).

aerobic cultures of the *hmp* mutant transformed with pMT1 or pLW1 were grown in the presence or absence of the inductor arabinose or compared with control cultures of the same strain but complemented *in trans* with the *hmp* gene under control of its own promoter (pPL341) (Vasudevan *et al.*, 1991), or transformed with the empty vector (pBAD/HisA) (Fig. 3.2). This result supported the suitability of this heterologous system to further investigate the effects of Cgb and Ctb in *E. coli* in the presence of nitrosative and oxidative stress agents.

3.2.3 Cgb complements the resistance phenotype of an *E. coli hmp* mutant strain to nitrosative stress.

In order to study the ability of Cgb to restore the resistance to nitrosative stress, impaired by the absence of Hmp in *E. coli*, susceptibility tests and growth curves were performed. First, to measure susceptibility, aerobic cultures of the *hmp* mutant carrying pMT1 were grown overnight in the presence or absence of arabinose and with increasing concentrations of the NO donor DETA NONOate or the nitrosative agent GSNO. Concentrations of 1 mM DETA NONOate and 3 mM GSNO prevented the growth of cultures in the absence of arabinose as in the case of cells carrying the empty vector. On the other hand, cultures in which the Cgb expression was promoted by the addition of arabinose (0.02%) showed resistance to the toxic compounds even at concentrations as high as 2 and 5 mM DETA NONOate and GSNO respectively. The optical densities recorded after 24 h incubation for the Cgb-expressing cultures were comparable to those of control cultures (complemented strain with pPL341) (Fig. 3.3A and B). Secondly, when the growth was followed in cultures after addition of 1 mM DETA NONOate or 3 mM GSNO, the *hmp* mutant expressing Cgb showed a similar resistance profile to the complemented strain (Fig. 3.3C and D).

The results shown above confirm the previously suggested role of Cgb in the resistance to nitrosative stress, and constitute the first evidence of the ability of this globin to complement the nitrosative stress resistance in *E. coli* in the absence of the flavohaemoglobin Hmp.

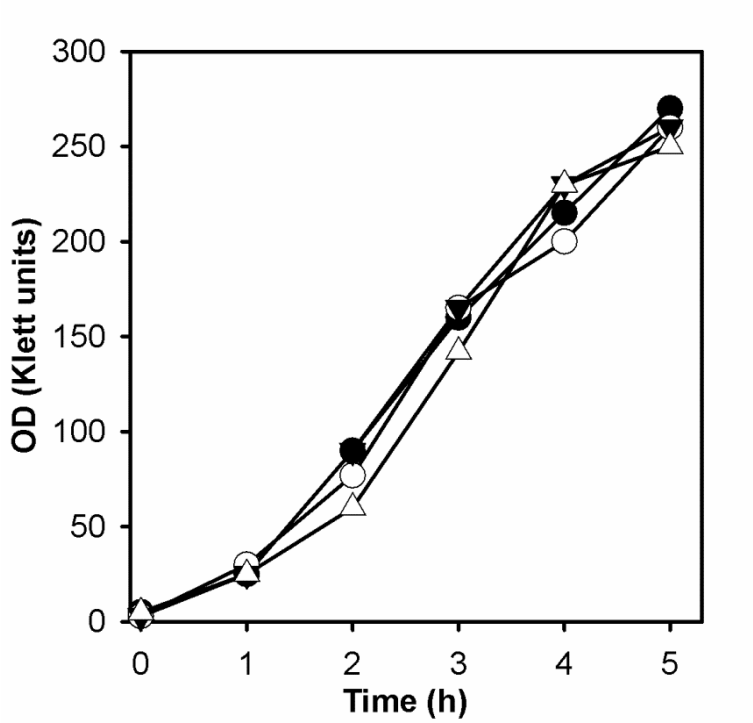


Figure 3.2 Growth profile of *E. coli* expressing Cgb or Ctb.

Cultures of an *E. coli hmp* mutant strain harbouring pMT1 (▼) or pLW1 (Δ) were grown aerobically at 37 °C in LB supplemented with arabinose. Optical density was recorded every hour. Cultures of the same strain but carrying the empty vector (○), or pPL341 (●), were tested as a control. Similar results were obtained in three independent experiments. Taken from Tinajero-Trejo *et al*. (2013).

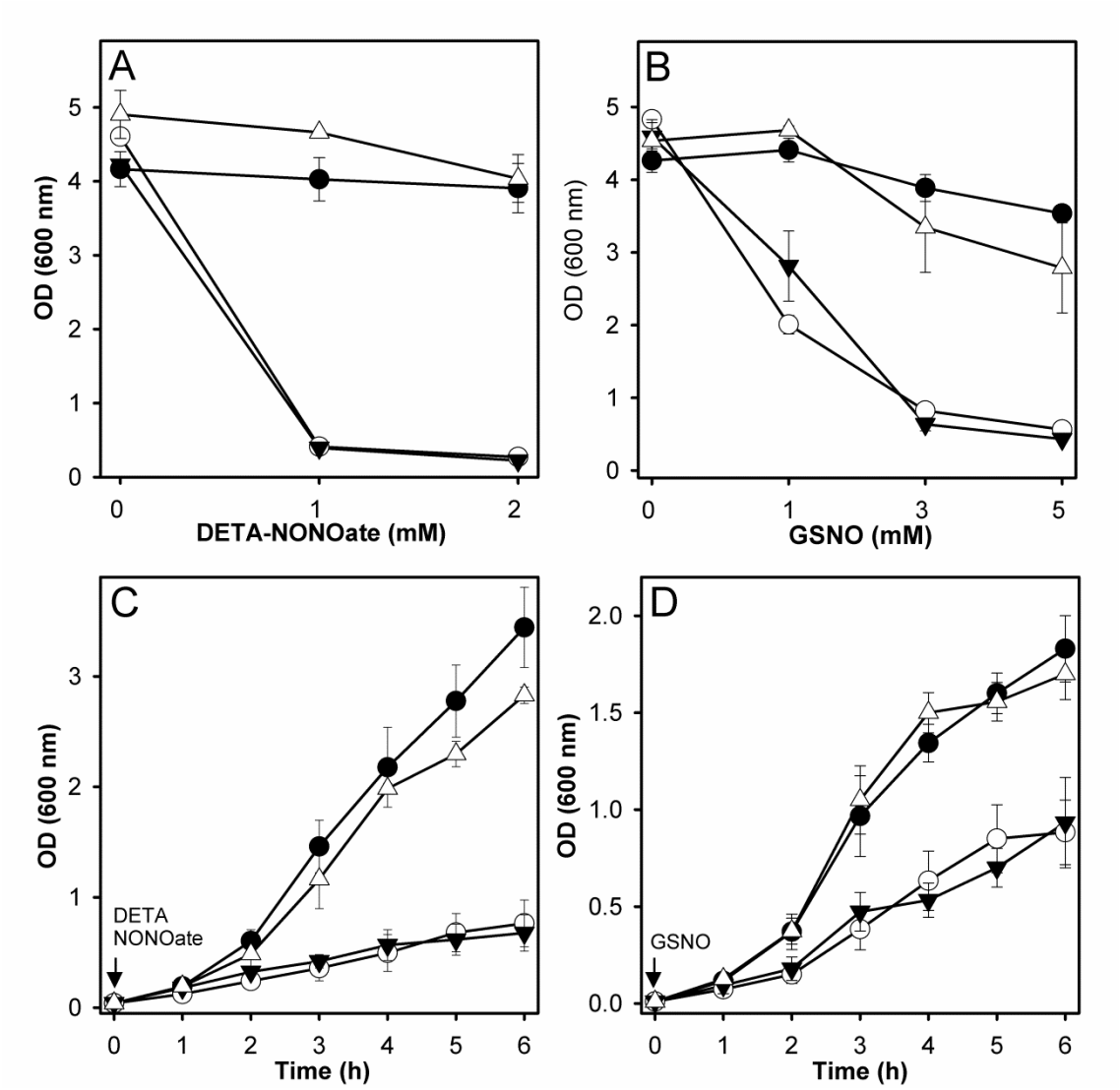


Figure 3.3 Susceptibility tests and growth curves of *E. coli* expressing Cgb under nitrosative stress conditions.

Aerobic cultures of the *hmp* mutant carrying the plasmids pPL341 (●), pBAD/HisA (○), or pMT1 (Δ) were grown in LB supplemented with arabinose. The indicated concentrations of (A) DETA NONOate or (B) GSNO were added and cultures incubated for 24 h at 37 ºC; OD was recorded at 600 nm. In (C), 1 mM DETA NONOate or (D) 3 mM GSNO were added (arrow) and the OD recorded every hour. The *hmp* mutant carrying pMT1 but in the absence of arabinose was used as a control (▼). Bars represent the standard deviation of three independent experiments. Taken from Tinajero-Trejo *et al*. (2013).

3.2.4 Ctb fails to complement the resistance to nitrosative stress in an *E. coli hmp* mutant strain.

It has been established that the truncated haemoglobin Ctb from *C. jejuni* constitutes one of the members of the nitrosative stress-response regulon controlled by the transcription factor NssR in *C. jejuni* (Elvers *et al.*, 2005). However, its role in the resistance to NO and RNS remains unclear.

In order to assess the ability of Ctb to support the growth of *E. coli* under nitrosative stress conditions, similar experiments to those carried out for Cgb (section 3.2.3) were performed. The *E. coli hmp* mutant transformed with the plasmid pLW1 was grown in the presence of arabinose to express the truncated haemoglobin. Ctb failed to support the growth of the NO-sensitive *E. coli* strain either upon addition of DETA NONOate or GSNO regardless of the concentrations added (Fig. 3.4A and B). Even though cultures were incubated for a period of time before NONOate (1 mM) or GSNO (3 mM) were added (until 0.2 OD at 600 nm was reached), increase in cellular mass did not produce differences in the growth profile of the strain expressing Ctb compared to the cultures grown in the absence of arabinose or carrying the empty vector (Fig. 3.4C and D).

In order to investigate whether Ctb may offer a modest protection against NO, cultures of the Ctb-expressing strain were treated with low concentrations of DETA NONOate (0.25 and 0.5 mM). However, in these conditions, the strain carrying the empty vector grew at the same level as those expressing Ctb (or Hmp) after 24 h incubation, indicating absence of NO toxicity and, consequently, making it difficult to test this hypothesis (not shown).

The inability of Ctb to support the growth of *E. coli* under nitrosative stress conditions is in agreement with previous studies suggesting the involvement of this globin in function(s) different from NO detoxification in *C. jejuni* (Smith *et al*., 2011).

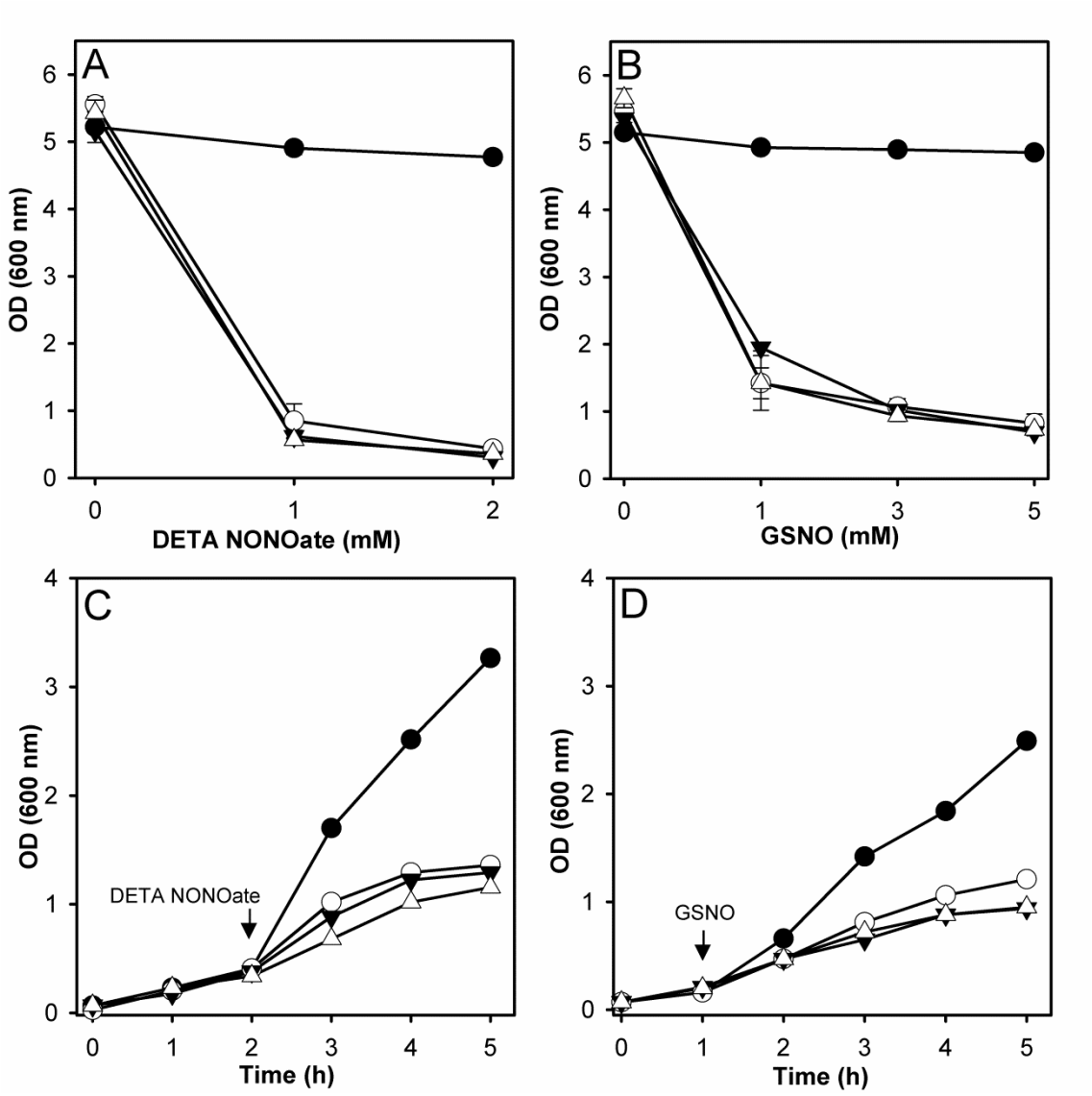


Figure 3.4 Susceptibility tests and growth curves of *E. coli* expressing Ctb under nitrosative stress conditions.

Aerobic cultures of the *hmp* mutant carrying the plasmids pPL341 (●), pBAD/HisA (○), or pLW1 (Δ) were grown in LB supplemented with arabinose. The indicated concentration of (A) DETA NONOate or (B) GSNO were added and cultures incubated for 24 h at 37 ºC; OD was recorded at 600 nm. In (C), 1 mM DETA NONOate or (D) 3 mM GSNO were added (arrow) and the OD recorded every hour. The *hmp* mutant carrying pLW1 but in the absence of arabinose was used as a control (▼). Bars represent the standard deviation of three independent experiments. Taken from Tinajero-Trejo *et al*. (2013).

3.2.5 Expression of Cgb and Ctb in *E. coli* does not exacerbate oxidative stress.

Hmp generates the superoxide anion *in vitro*, and oxidative stress is caused by the over-expression of this flavohaemoglobin *in vivo* in the absence of nitrosative stress (Membrillo-Hernandez *et al.*, 1996; Mills *et al.*, 2001). To investigate whether the *C. jejuni* haemoglobins could produce deleterious effects related to generation or augmentation of oxidative stress, cultures of the *E. coli hmp* mutant expressing Cgb (pMT1) or Ctb (pLW1) were treated with increasing concentrations of H_2O_2 and the growth profiles were compared with cultures of the complemented strain (pPL341) or carrying the empty vector (Fig. 3.5). Concentrations of 1 and 2 mM H_2O_2 failed to produce harmful effects on the growth of the cultures regardless of the presence of either the *C. jejuni* globins or Hmp (Fig. 3.5A and B). In agreement with the oxidative stress produced by the over-expression of Hmp, the complemented strain (pPL341) was fully inhibited upon addition of 3 mM H_2O_2. However, neither the expression of Cgb nor of Ctb affected the resistance of the *hmp* mutant in this condition (Fig. 3.5C). When cultures were challenged with 4 mM H_2O_2, a slight inhibition of growth was experienced in the strains expressing Cgb and Ctb. However, the strain carrying the empty vector was also inhibited, suggesting a cytotoxic effect due to the presence of H_2O_2 *per se* and not related to the presence of the heterologous globins (Fig. 3.5D).

3.2.6 The respiratory profile of an *E. coli hmp* mutant is not altered by the expression of Cgb or Ctb.

It has been shown that *E. coli* defective in cytochrome *o* and *d* terminal oxidases is able to recover the ability to grow on non-fermentable substrates such as malate and succinate by the expression of the *Vitreoscilla* single-domain haemoglobin (Vgb). Moreover, membrane vesicles prepared from the same strain consume oxygen with succinate as the electron donor (Dikshit *et al.*, 1992).

In order to test the effect of the *C. jejuni* globins on *E. coli* respiration, O_2 consumption of *hmp* mutant cell suspensions expressing Cgb or Ctb was measured polarographically and rates compared with those determined for the complemented strain (pPL341) or the strain carrying the empty vector (Table 3.1). Neither Cgb nor Ctb produced a significant

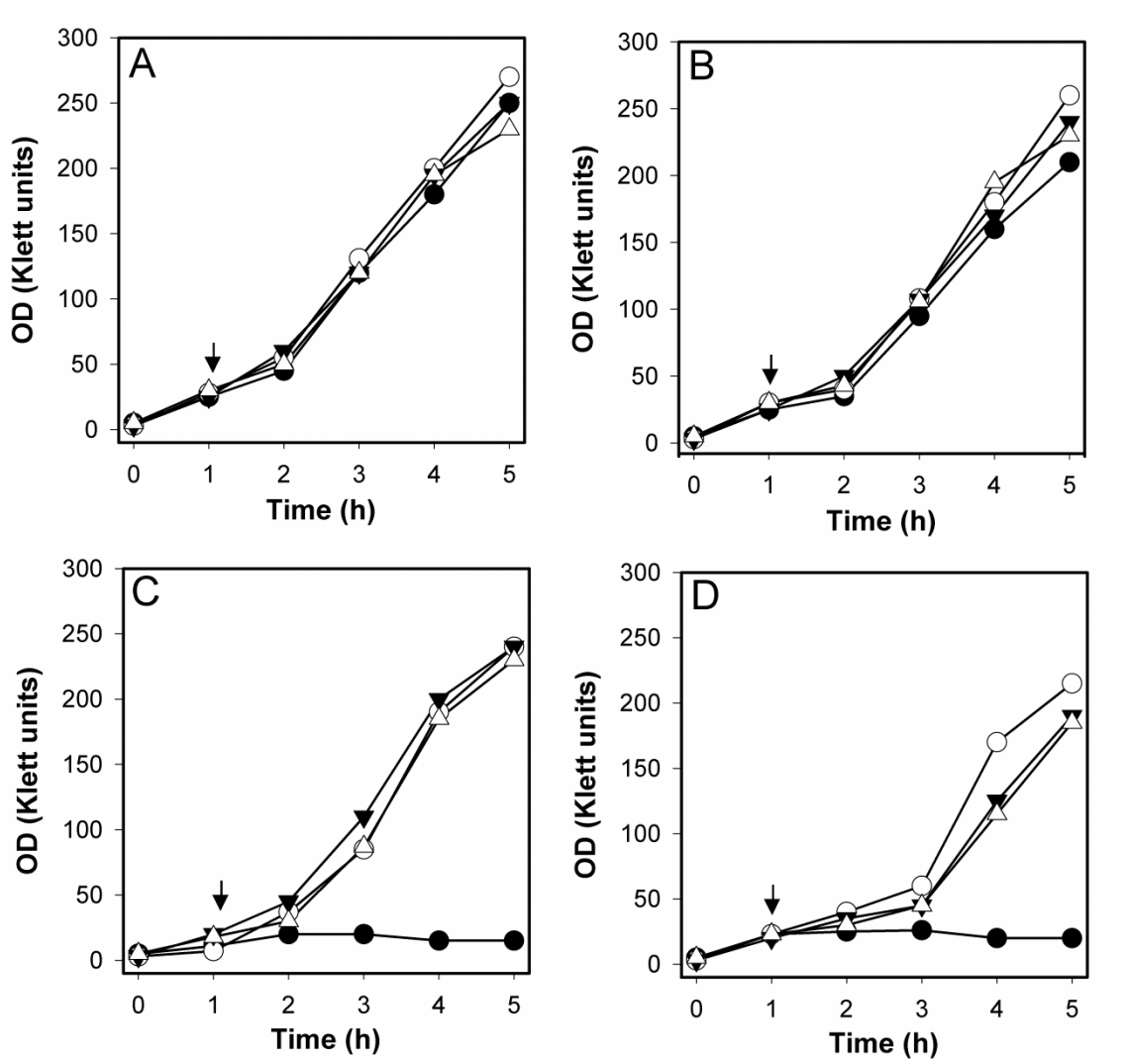


Figure 3. 5 Growth curve of *E. coli* expressing Cgb or Ctb in the presence of H_2O_2.

Aerobic cultures of the *hmp* mutant carrying the plasmid pMT1 (▼), or pLW1 (Δ) were grown in LB supplemented with arabinose and incubated at 37 ºC, 240 rpm and OD was recorded every hour. When cultures reached 30 klett units 1 (A), 2 (B), 3 (C) or 4 mM (D) H_2O_2 was added (arrows). The *hmp* mutant carrying pPL341 (●), or pBAD/HisA (○) were used as a control. Similar results were obtained in two independent experiments. Taken from Tinajero-Trejo *et al*. (2013).

Table 3.1 Oxygen consumption rates of an *E. coli hmp* mutant expressing Cgb or Ctb.

Strain	Respiration rate* [nmol O_2 (min^{-1} mg $protein^{-1}$)]
E. coli hmp, pPL341	53.83 ± 3.9
E. coli hmp, pBAD/HisA	19.31 ±1.06
E. coli hmp, pMT1	16.19 ± 1.03
E. coli hmp, pLW1	18.07 ± 1.06

*Cell suspensions of an *E. coli hmp* mutant expressing Hmp (pPL341), Cgb (pMT1), Ctb (pLW1) or carrying the empty vector (pBAD/HisA) were added with 5 mM glycerol to promote respiration and the oxygen consumption was measured polarographically. Protein concentration was determined by the Markwell assay (Markwell *et al.*, 1978). Respiration rates are averages of three independent experiments. Values of ± the standard deviation are shown.

difference in the respiration rates when compared with the strain harbouring the empty vector. However, the complemented strain showed a 3-fold increase in the O_2 consumption rate. This result can be explained as a consequence of the oxidative burst due to the over-expression of Hmp.

The fact that the oxygen uptake rates of the *E. coli hmp* mutant were not modified by the expression of Cgb or Ctb suggest that, at least in the tested conditions, these heterologous globins have no effect on the respiratory capacity of *E. coli*.

3.2.7 Cgb and Ctb moderately consume NO protecting the respiration of an *E. coli hmp* mutant.

The ability of Cgb to complement the NO resistance phenotype of the *E. coli hmp* mutant strongly supports the activity of the globin in the uptake and detoxification of NO not only in *C. jejuni* but also in the heterologous host *E. coli* (section 3.2.3).

In order to further investigate the ability of Cgb to consume NO, the protection from respiratory inhibition of *E. coli hmp* cell suspensions expressing the globin was tested upon addition of NO. Uptake of O_2 and NO were followed polarographically in a chamber equipped with an O_2 and a NO electrode working simultaneously. After addition of glycerol to promote cellular respiration, aliquots of 1 µM PROLI NONOate (a fast NO releaser, half-life of 1.8 s) were added subsequently at 75, 50, 25, and 0% O_2 concentration, and the respiration rates were calculated before and after the NONOate additions (Fig. 3.6). Control samples of cell suspensions expressing Hmp did not show inhibition of the respiration regardless of the O_2 concentration, and no NO accumulation was detected during the aerobic phase of the experiment (Fig. 3.6A). This is consistent with the previously characterised ability of Hmp to protect respiration from inhibition by NO (Hernandez-Urzua *et al.*, 2003). On the other hand, cells harbouring the empty vector showed a clear inhibition and a very poor recovery upon addition of PROLI NONOate aliquots. Moreover, NO was accumulated in high concentrations reaching up to 1.4 µM at the end of the test (Fig. 3.6B). This result demonstrated the suitability of the system to test the ability of Cgb to protect the highly inhibited respiration by NO in the absence of Hmp.

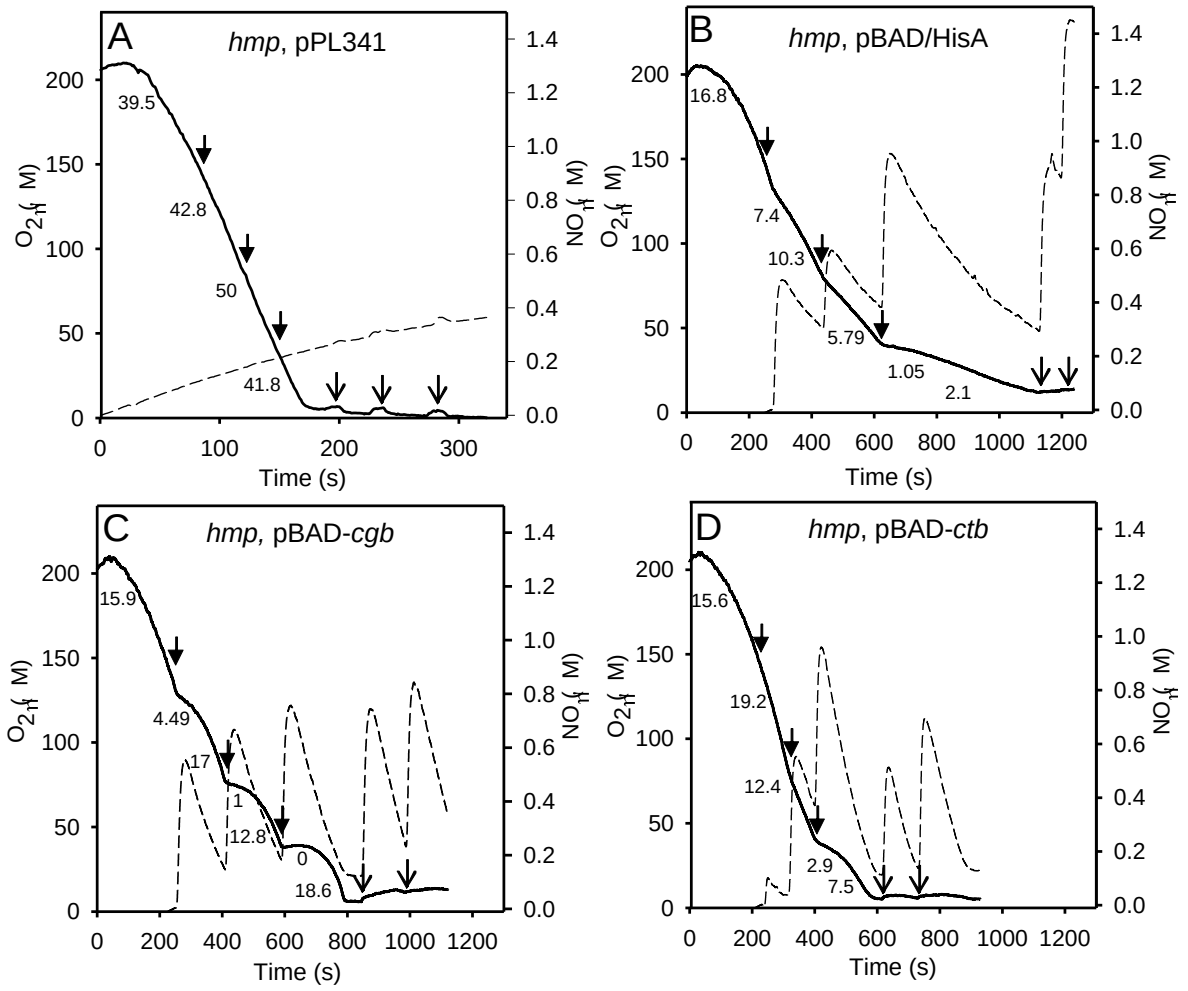


Figure 3.6 Protection of the respiration and NO consumption of *E. coli hmp* mutant cell suspensions by the expression of Cgb or Ctb.

Cells from overnight cultures grown in the presence of arabinose were resuspended in 50 mM Tris-HCl buffer (pH 7.4). Glycerol was added to promote respiration and O_2 (solid line) and NO consumption (dashed line) were polarographically recorded at the same time. Respiration rates were calculated at different O_2 tensions before and after additions of 1 μM PROLI NONOate and expressed as nmol O_2/(min mg protein) (shown on traces). Additions of PROLI NONOate in aerobic (closed arrows) or anaerobic (open arrows) conditions are indicated. Taken from Avila-Ramírez *et al*. (2013).

Cell suspensions of the *hmp* mutant expressing Cgb showed severe inhibition of respiration immediately after addition of the PROLI NONOate aliquots. However, rapid recovery was shown and rates were comparable to that calculated at the beginning of the test, (before the first PROLI NONOate addition). Besides, even though NO presence was clearly recorded upon each NONOate addition, traces showed a rapid disappearance of the accumulated NO in a way that coincided with the recovery of the respiration rate (Fig. 3.6C), suggesting NO consumption by the Cgb-expressing cells. This result demonstrates a moderate capability of Cgb to consume and detoxify NO that results in protection of the respiration from inhibition by NO. Interestingly, NO uptake was observed even after the depletion of the oxygen in the chamber, suggesting that the ability of this globin to consume NO is not necessarily dependent on the oxygen content.

As mentioned before in this chapter, the up-regulation of Ctb under nitrosative stress conditions in *C. jejuni* leads to the hypothesis that the globin might play a role in NO detoxification; however, the growth tests of the *E. coli hmp* mutant expressing Ctb failed to support this proposal. On the other hand, when the ability of Ctb to protect the *E. coli hmp* cell respiration from NO toxicity was tested, a moderate protection was shown. As with Cgb, the NO-consumption was independent of the O_2 presence, being comparable in aerobic and anaerobic conditions (Fig. 3.6D).

Considering that the Ctb expression level demonstrated by Western blot analysis was substantially higher than that of Cgb in *E. coli* (see Fig. 3.1), the protection of respiration in the cells expressing Ctb may be the result of unspecific NO binding to the Ctb haem cofactor instead of a globin-based detoxification mechanism. In order to investigate this, the intracellular concentration of Cgb and Ctb were determined by the haem assay (alkaline pyridine) and found to be 0.68 µM and 2.6 µM respectively in the volume of cell suspensions contained in the chamber during the experiments. Since five sequential aliquots of 1 µM PROLI NONOate were added during the tests and 2 moles of NO are released per molecule of PROLI NONOate, protection based on unspecific NO-haem binding was ruled out and the involvement of a specific NO detoxification mechanism not only for Cgb but for Ctb-expressing cells seems plausible.

The results described in this section constitute the first report of the ability of Cgb to consume NO with the concomitant respiratory protection in a heterologous host. At the same time, an unexpected capability of Ctb to interact and perhaps detoxify NO in *E. coli* is also reported.

3.2.8 The *C. jejuni* haemoglobins Cgb and Ctb fail to support the anaerobic growth of an *E. coli* NO-sensitive strain under nitrosative stress conditions.

The unexpected capability of *E. coli hmp* cells expressing Cgb and Ctb to consume NO under anaerobic conditions (see Fig. 3.6C and D) suggests a possible O_2-independent detoxification mechanism mediated by these globins. Such a mechanism might be similar to the NO reductase activity described for the flavohaemoglobin Hmp in anaerobiosis (Gardner and Gardner, 2002).

To further investigate the role of Cgb and Ctb in detoxifying NO in anoxic environments, an *E. coli hmp norR* mutant strain was constructed. The *norR hmp* deletions produce a NO hypersensitivity phenotype in both aerobic and anaerobic conditions in *E. coli*. Indeed, the absence of the positive regulator NorR prevents the induction, under nitrosative stress conditions, of the *norVW* operon, thereby preventing the NO reduction mediated by NorV and its redox partner NorW (Gardner and Gardner, 2002).

An *E. coli* K12 *norR* mutant containing an insertion of a chloramphenicol resistance cassette (Gardner *et al*., 2002) was used as a donor to replace the wild type *norR* gene in the genome of the *E. coli hmp* mutant and its isogenic strain (MG1655) (see Table 2.3). Transduction based on the P1 *vir* bacteriophage system rendered double mutants (*hmp norR*) that were selected by their resistance to kanamycin (*hmp* mutation) and chloramphenicol. After the absence of the *norR* gene was verified by PCR, anaerobic cultures of the isogenic strain were compared to cultures of the *norR* and the *hmp norR* mutant growing in the presence of 500 μM DETA NONOate. Even though the single mutant was clearly inhibited by NO, the double mutant showed an even higher inhibition related, perhaps, to the loss of the reductase activity mediated by Hmp in the absence of O_2. Neither the expression of Cgb nor Ctb protected the growth of the *hmp norR* mutant from the NO toxicity (Fig. 3.7). Indeed, the inhibition of the double mutant

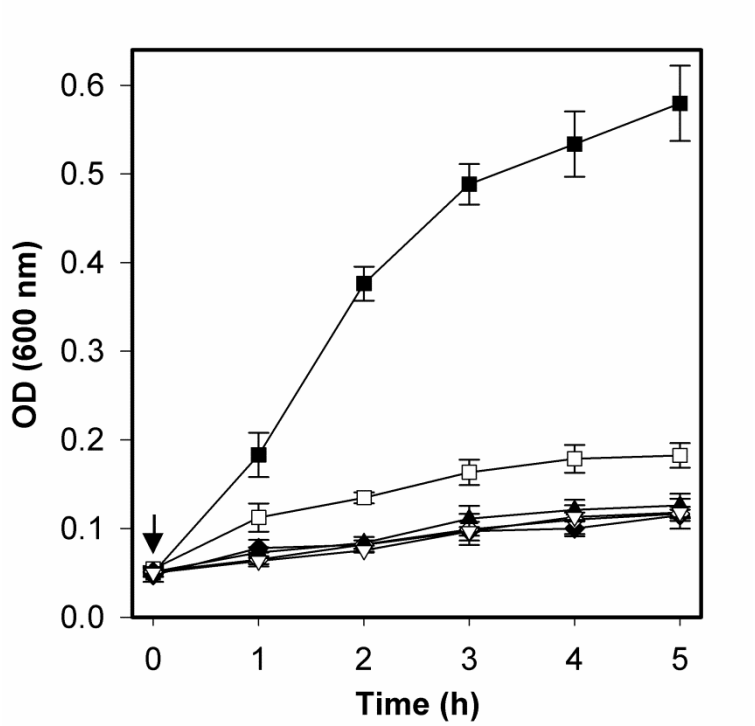


Figure 3.7 Anaerobic growth curve of an *E. coli hmp norR* expressing Cgb or Ctb under nitrosative stress conditions.

Cultures of an *E. coli hmp norR* mutant expressing Cgb (pMT1) (Δ) or Ctb (pLW1) (▼) by the addition of arabinose were grown in LB at 37 ºC under anaerobic conditions. DETA NONOate (0.5 mM) was added (arrow) and the OD recorded at 600 nm every hour. *E. coli* wild type (■), *norR* mutant (□), *hmp norR* carrying the empty vector (○), or expressing Hmp (pPL341) (●) were tested as a control. Bars represent standard error of three independent experiments. Taken from Tinajero-Trejo *et al*. (2013).

expressing the globins or harbouring the empty vector was comparable. Surprisingly, complementation of the *hmp* gene in the double mutant by transformation with pPL341 failed to reach the same level of growth as the *norR* mutant. This might relate to the deleterious effects of the over-expression of Hmp in *E. coli* (Mills *et al.*, 2001).

The results above strongly suggest that the ability of Cgb to detoxify NO *in vivo* is dependent on the presence of O_2. However, it is possible that the flavorubredoxin reductase (NorW) is playing a role as the reductase partner of Cgb in *E. coli*. Indeed, NorW has been successfully used as the reductase of the human neuroglobin *in vitro* (Giuffre *et al.*, 2008). As a *norR hmp* mutant was used for the anaerobic tests, the induction of *norW* was prevented by the absence of NorR. Thus, the inability of Cgb to support the growth of the double mutant might be related to the lack of NorW and not to the absence of O_2. This hypothesis is further investigated (see Chapter 5).

3.3 Discussion

Strains of *E*. coli lacking the flavohaemoglobin Hmp and consequently sensitive to NO and RNS have proved to be a useful model to study resistance to nitrosative stress related to the expression of heterologous haemoglobins. For instance, the restoration of the NO resistance of *E. coli hmp* achieved by the expression of the TrHb1 (HbN) from *M. tuberculosis* (Pathania *et al*., 2002a) and, more recently, by the expression of the TrHb2 (PhHbO) from the Antartic bacterium *P. haloplanktis* (Coppola *et al.*, 2013), led to the suggestion of the involvement of these globins in such a function in their native hosts. Indeed, inactivation of the PhHbO-encoding gene in *P. haloplanktis*, produced sensitivity to nitrosative and oxidative stress, in agreement with the findings reported by the heterologous expression (Parrilli *et al.*, 2010). By using the same model (*E. coli hmp*), functions for a number of other globins have been suggested, most of them associated to nitrosative stress (Vinogradov *et al*., 2013) (Table 1.3, Chapter 1).

The availability of crystal structures for both Cgb (Shepherd *et al.*, 2010) and Ctb (Nardini *et al.*, 2006), together with a detailed spectroscopic and kinetic characterisation (Bolli *et al.*, 2008; Lu *et al.*, 2007b; Lu *et al.*, 2007c; Pickford *et al.*, 2008; Shepherd *et al.*, 2011; Wainwright *et al.*, 2006) and extensive functional studies based on globin mutants *in vivo* (Avila-Ramirez *et al.*, 2013; Elvers *et al.*, 2004; Pittman *et al.*, 2007; Smith *et al.*, 2011; Wainwright *et al.*, 2005) locate the *C. jejuni* globins within the selected group of bacterial globins, lead by Hmp (Forrester and Foster, 2012), that have been most comprehensively studied. However, difficulties in complementation by transformation of plasmids and, consequently, expression of proteins in *C. jejuni* restrict additional exploration related to function and confirmation of functions *in vivo*. For this reason, the heterologous expression of Cgb and Ctb represented a convenient next step in the characterisation of these proteins.

The ability of Cgb to complement the loss of the nitrosative stress resistance phenotype by the absence of the flavohaemoglobin Hmp in *E. coli* constitute perhaps the most direct evidence of the globin function so far. Indeed, the growth of the Cgb-expressing or the complemented cultures with Hmp was comparable (Fig. 3.3), supporting the role of Cgb as an efficient NO and RNS detoxification mechanism in the heterologous host. This finding is in agreement with the evidence of the nitrosative stress sensitivity shown

by the deletion of the *cgb* gene in *C. jejuni* (Avila-Ramirez *et al.*, 2013; Elvers *et al.*, 2004). Thus, these results together strongly support a prominent role for this single-domain globin as a primary defence against nitrosative damage in the native host.

Since Cgb shares a high level of homology with the globin domain of Hmp from *E. coli* (33%) (Elvers *et al.*, 2004), a similar molecular mechanism involving the conversion of NO and O_2 to NO_3^- is likely. Indeed, a denitrosylase or dioxygenase activity has been suggested (Shepherd *et al.*, 2011). This proposal implies two conditions, the existence of efficient reductases in both the heterologous and the native host and the availability of O_2. Interestingly, the protection of respiration against NO inhibition offered by Cgb in *E. coli hmp* cells clearly correlates with the consumption of NO (figure 3.6C). However, the ability of Cgb to consume NO does not seem to be O_2-dependent since the consumption patterns were comparable in the presence and absence of O_2. This behaviour suggests the capability of this globin to detoxify NO by a mechanism different from the production of NO_3^-; reduction of NO to N_2O is a possibility.

The capability to detoxify NO under anaerobic conditions appears to vary among globins from different organisms. For instance, as mentioned before, the FHb Hmp from *E. coli* plays a major role as a NO detoxifier in aerobic conditions but only a minor one as a NO reductase under anaerobiosis (Mills *et al.*, 2001). In agreement, the TrHb2 (PhHbO) from *P. haloplanktis* was shown to consume NO in an O_2-dependent manner when the globin was heterologously expressed in *E. coli*, losing its function in the absence of O_2 (Coppola *et al.*, 2013). On the other hand, the fungal FHb from *Saccharomyces cerevisiae* substantially contributes to NO consumption in both aerobic and anaerobic conditions (Liu *et al.*, 2000). And, although the evidence is still scarce, *in vitro* studies support a more efficient NO reduction activity of fungal FHbs compared to their very well characterised orthologues in bacteria (e.g. *E. coli*) (reviewed by Forrester and Foster, 2012).

The data above suggest that reduction more than denitrosylation (or dioxygenation) might be the reaction mediated by Cgb. This hypothesis could explain why consumption of NO seems to be independent of O_2, at least in *E. coli* (Fig. 3.6C). However, since studies in *Campylobacter* have demonstrated a correlation between globin expression and O_2 availability under conditions of nitrosative stress, an O_2-dependent NO

detoxification reaction for this single-domain globin has been generally proposed. Indeed, Cgb is maximally expressed in the presence of oxygen (Avila-Ramirez *et al.*, 2013; Elvers *et al.*, 2005; Elvers *et al.*, 2004; Monk *et al.*, 2008; Wainwright *et al.*, 2005). Besides, the inability of Cgb to complement the NO resistance phenotype of the *E. coli hmp norR* mutant under anaerobic conditions (Fig. 3.7) supports the O_2-dependence of the Cgb function. However, this result is not conclusive, since a role of NorW, the transcription of which depends on NorR, has been suggested as the heterologous reductase partner of Cgb in *E. coli* (see Chapter 5).

The data presented here do not necessarily rule out the possible function of Cgb as a NO reductase. For instance, during infection and pathogenesis, when *Campylobacter* faces nitrosative stress, it is possible that the NssR-dependent genes (including Cgb) are induced in a complex pattern due to the expected variations in oxygen availability. Thus, once Cgb is expressed, a stable protein might perhaps play a role detoxifying NO independent of the oxygen tension. However, experimental approaches are needed to support this proposal.

The inability of Ctb to support the growth of the *E. coli hmp* in the presence of NO and GSNO (Fig. 3.4) is in agreement with previous studies in *Campylobacter* where a strain defective in the truncated haemoglobin failed to show increased sensitivity to nitrosative stress. Indeed, cultures of the *C. jejuni ctb* mutant grew at a rate comparable to the isogenic strain in the presence of GSNO while a *cgb* mutant was severely inhibited (Avila-Ramirez *et al.*, 2013; Wainwright *et al.*, 2005). Moreover, the enhanced expression of Ctb, triggered by the presence of NO or RNS appears to play a deleterious role in the absence of Cgb in *C. jejuni* (in a *cgb* mutant). It has been shown that the hypersensitivity of a *cgb* mutant to nitrosative stress, measured as loss of viability in the presence of NO (Wainwright *et al.*, 2005) or as impairment of growth by GSNO (Avila-Ramirez *et al.*, 2013) is alleviated by the deletion of the truncated globin, suggesting that the absence of Ctb partially suppresses the effect of the *cgb* mutation.

In the present study, the ability of Ctb to protect respiration of *E. coli hmp* from NO inhibition has been demonstrated. This function appears to be, as in the case of the Cgb-expressing cells, an O_2-independent NO consumption mechanism (Fig. 3.6D). Indeed, NO uptake was not diminished by the depletion of O_2 in the chamber. However, since

the growth of the heterologous host was not protected from the NO and GSNO toxicity by the expression of Ctb (Fig. 3.4), and there is no evidence in *Campylobacter* to support the role of this globin in protection against nitrosative stress, we believe that the function of Ctb observed in *E. coli* might have no physiological significance.

The *Vitreoscilla* globin (Vgb) enhances microaerobic growth of *E. coli* and other bacterial and eukaryotic species (reviewed by Frey *et al*., 2011) by supposedly transfer O_2 to the terminal oxidases (Park *et al.*, 2002; Ramandeep *et al.*, 2001; Webster, 1987). In agreement with this function, the up-regulation of the globin-encoding gene in oxygen-limitation has been documented in the native host (Boerman and Webster, 1982) and in *E. coli* (Dikshit *et al.*, 1990). On the other hand, the structural and kinetic characteristics of Ctb and its involvement in controlling the intracellular oxygen tensions shown in *C. jejuni* (Wainwright *et al.*, 2005) suggest a more direct role of this globin in O_2 chemistry. However, the question of why Ctb is over-expressed under conditions of nitrosative stress but not by oxidative stress or variations in oxygen concentrations remains unanswered.

During purification, Ctb is found in the soluble fraction of *E. coli* cells (Pickford *et al.*, 2008), suggesting that the globin is not bound to cellular membranes and consequently might not be able to improve the O_2 uptake of the heterologous host. Growth of the *E. coli hmp* mutant expressing either Ctb or Cgb was comparable to control cultures carrying the empty vector or complemented *in trans* with *hmp* (Fig. 3.2). Besides, when the respiration rates of Ctb-expressing cells were compared to the control harbouring the empty vector or to cells expressing Cgb, no differences were found either (Table 3.1). It is possible however that, by limiting the O_2 availability during growth (e.g. microaerobic or anaerobic conditions), the expression of Ctb in *E. coli* produced differences. Preliminary work testing the growth of the *E. coli hmp norR* mutant expressing the *C. jejuni* globins in anaerobic conditions failed to show any improvement in the profile of Ctb or Cgb-expressing cultures compared to the controls (Hannah Southam personal communication). Thus, herein it is concluded that the role in supporting microaerobic growth and moderating respiration described for Ctb in *C. jejuni*, is not transferable to the heterologous host *E. coli*.

3.4 Conclusions

The single domain haemoglobin Cgb, but not the truncated haemoglobin Ctb, from *C. jejuni* confers tolerance to NO and RNS when expressed in the heterologous host *E. coli* lacking the flavohaemoglobin Hmp. This finding confirms the role of Cgb as a NO detoxification mechanism in the native host. Besides, the ability of both Cgb and Ctb to protect respiration from NO inhibition, and their ability to consume NO in aerobic and anaerobic conditions is demonstrated, suggesting that the molecular mechanism to detoxify NO might be independent of O_2 availability. However, the failure of Cgb to support the growth of an *E. coli hmp norR* mutant in the absence of O_2 argues against this proposal. Neither Cgb nor Ctb expression affected the respiration capacity or the response to oxidative stress of the heterologous host.

Chapter 4. Reducibility of the Cgb and Ctb haem cofactors in *E. coli* and *C. jejuni.*

4.1Introduction

During aerobic NO detoxification, FHbs catalyse the formation of the harmless ion nitrate. This reaction implies the oxidation of the haem group that in turn must be re-reduced in order to complete the following detoxification cycle. FHbs solve the problem of haem reduction by encoding a reductase domain: an intra-protein electron transference from the reductase domain (or FNR, ferredoxin-NADP reductase-like domain) to the N-terminal haem domain in an NAD(P)H-dependent reaction via a noncovalently bound FAD allows the reduction of the ferric haem (FeIII) (Gardner *et al.*, 1998a; Hausladen *et al.*, 1998; Hernandez-Urzua *et al.*, 2003).

The absence of a reductase domain in the structures of a growing number of bacterial SDgbs and TrHbs that have been implicated in nitrosative stress resistance (see Chapter 1, Tables 1.3 and 1.4) implies the interaction of such proteins with cognate reductases *in vivo* in order to promote haem reduction after oxidation by NO. However, unlike eukaryotic globins such as Mb in which the reduction of the haem is catalyzed by a NADH-dependent myoglobin reductase in a reaction mediated by cytochrome b_5 in muscle (Livingston *et al.*, 1985), the associated reductases for bacterial haemoglobins lacking the flavo-domain have not been identified.

The use of visible absorption spectra has allowed the observation of the intracellular redox state of eukaryotic and prokaryotic globins heterologously expressed in *E. coli*. Thus, the haem iron atom of human neuroglobin is found in the ferrous form (Dewilde *et al.*, 2001; Trandafir *et al.*, 2004; Van Doorslaer *et al.*, 2003), while the TrHb2 (PhHbO) from *P. haloplanktis* (Coppola *et al.*, 2013) and Ctb from *C. jejuni* (Wainwright *et al.*, 2006) are in the oxy-ferrous form (FeII-O_2). These results suggest the presence of a reductase or a reducing environment in the *E. coli* cells that is equally efficient for maintaining the reduced form of these, independently of differences among globin structures. Moreover, reduction of oxidised neuroglobin, cytoglobin and horse muscle myoglobin is facilitated by the presence of NADH in either soluble cellular

fractions of *E. coli* or extracts from brain and liver, pointing to the possibility of a non-specific reduction mechanism (Trandafir *et al.*, 2007).

The ability of Cgb to efficiently support the growth of a NO-sensitive *E. coli* strain under nitrosative stress conditions and the NO consumption of Cgb and Ctb-expressing *E. coli* cells (Chapter 3) implies the existence of an associated reductase(s) or the adequate intracellular environment to facilitate the reduction of the haem groups required to bind the ligands. Both Cgb and Ctb have been purified and their spectroscopic characteristics described (Table 4.1). By using these data, it is possible to follow the changes in the redox state of the haem groups of the *C. jejuni* globins. Thus, the objective of the present chapter is to examine the redox forms of the Cgb and Ctb haems in the presence of cellular extracts of *E. coli* and *C. jejuni*, testing the reducibility of the globins in the presence and absence of reducing equivalents (NADH). Furthermore, the re-reducibility of the globins after oxidation by the addition of NO is also investigated.

Table 4.1 Electronic absorbance characteristics of the *C. jejuni* globins

Globin	Redox form	Absorption maxima (nm)		Extinction coefficient (mM^{-1} cm^{-1})	References
		Soret region	α, β region		
Cgb	Ferrous (FeII)	434	555	13.6	(Pickford *et al.*, 2008; Shepherd *et al.*, 2011)
	Oxy-ferrous (FeII-O_2)	411	540, 575		
	Ferric (FeIII)	398	505, 640		
	Ferrous-NO (FeII-NO)	419	540, 565		
	Ferric-NO (FeIII-NO)	520	532, 566		
Ctb	Ferrous (FeII)	432	566	23.47	(Pickford *et al.*, 2008; Shepherd *et al.*, 2011; Wainwright *et al.*, 2006)
	Oxy-ferrous (FeII-O_2)	414	542, 578		
	Ferric (FeIII)	410	512, 542, 582, 640		
	Ferrous-NO (FeII-NO)	419	540, 565		
	Ferric-NO (FeIII-NO)	-	-		
	Ferrous-CO (FeII-CO)	421	538, 569		

4.2 Results

4.2.1 NADH promotes reduction of Cgb in cellular extracts of *E. coli*.

In order to test the reducibility of Cgb in a cellular milieu, purified Cgb (5 μM) was added to soluble extracts of an *E. coli hmp* mutant in the presence of an excess of NADH (10 mM) and changes in the optical spectra were recorded using an Olis RSM 1000 spectrophotometer. Due to difficulties in identifying clearly the redox forms from the absorption maxima of the spectra in the Soret region, the α, β region was used instead. Within this region, the absorption maxima did not deviate more than 2 to 3 nm from previously described values (Pickford *et al.*, 2008; Shepherd *et al.*, 2011; Wainwright *et al.*, 2006). The absolute spectra recorded against a base line of NADH-containing extracts showed a mixture of FeII-O_2 and FeIII redox forms immediately after the addition of the globin to the NADH-containing extracts (421, 542, 575 nm) (Fig. 4.1A, red dashed line). However, after 15 min incubation at 37 °C, a stable reduced form (FeII) (434, 558) (Fig. 4.1A, blue solid line) was observed. On the other hand, addition of the globin to cellular extracts in the absence of NADH did not produce changes in the redox state of the haem, remaining as a mixture of FeII-O_2 and FeIII forms even after 20 min incubation (Fig. 4.1B, red dashed and solid blue lines respectively). This result suggests that the reduction of Cgb in *E. coli* is dependent on the presence of NADH.

To investigate whether the reduction of Cgb in NADH-containing cellular extracts was due to the interaction of components of the cellular milieu mediated by the presence of reducing equivalents, or a consequence of the presence of the latter only, the reducibility of Cgb was tested in buffer containing NADH. After 20 min incubation, the spectrum shifted from FeII-O_2/FeIII to FeII-O_2 according to the absorption maxima observed in the α, β region (542, 573 nm) (Fig. 4.1C, blue solid line). However, the peak expected at 411 nm was absent in the Soret region. Instead, peaks at 421 and 645 nm were recorded, indicating the presence of oxidised haem (FeIII) forms (Table 4.1). This suggests that the presence of NADH in the absence of cellular components has a poor effect as a reductant, and it is concluded that the reduction of the Cgb haem requires the presence of both cellular extracts and NADH.

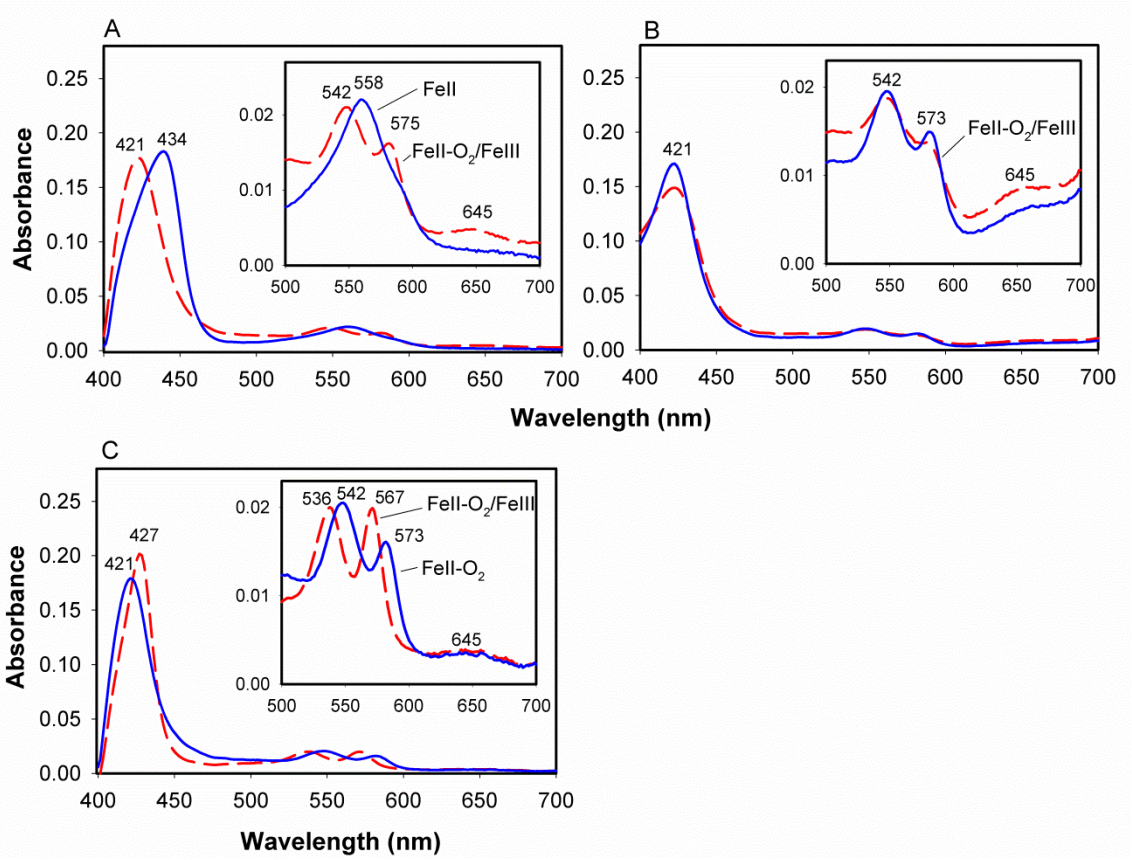


Figure 4.1 Redox state of the Cgb haem cofactor in *E. coli* extracts.

Purified Cgb (5 µM) was mixed with soluble extracts of *E. coli hmp* mutant in 50 mM Tris-HCl buffer (pH 7.4), containing NADH (10 mM) at 37 °C, and absorption spectrum was recorded immediately after addition of the protein (red dashed line) and after 15 min incubation (blue solid line) (**A**). Changes in absorbance immediately (blue solid lines) and after 20 min incubation in the presence of soluble extract without NADH (**B**) and in buffer containing NADH (**C**) are shown as controls. Soluble extracts containing NADH, soluble extracts or buffer added with NADH were used as a baseline (**A**, **B** and **C** respectively). Tests were repeated three times with similar results. Modified from Tinajero-Trejo *et al*. (2013).

The reducibility of Cgb in *E. coli* extracts in the presence of NADH suggests the presence of a reductase system in the heterologous host. Therefore, in order to test the efficiency of the putative reduction system, addition of an equimolar aliquot of PROLI NONOate with regards to the globin concentration (5 μM) was added to promote oxidation and changes in the redox form towards the re-reduction were followed (Fig. 4.2). Since PROLI NONOate releases 2 moles of NO per mole of parent compound in the tested conditions (Saavedra *et al.*, 1996), NO is expected to be in excess. Cgb was incubated for 15 min in *E. coli hmp* soluble extracts containing NADH to promote reduction and the spectrum was recorded (427, 532, 566 nm) (Fig. 4.2A, red dashed line). Upon addition of PROLI NONOate, a shift in the absorption maxima from FeII to the characteristic NO-ferric form (FeIII-NO) was immediately observed (Fig. 4.2A, green short dashed line). However, after 10 min incubation, a mixture of the FeII and FeII-O_2 forms was recorded (Fig. 4.1A, blue solid line). Furthermore, a second addition of a higher concentration of PROLI NONOate (10 μM) produced a similar effect (Fig. 4.1B) suggesting that the reductase(s) or the reductant environment is able to efficiently promote the turnover of the haem during several oxidation/reduction cycles.

On the other hand, addition of PROLI NONOate (5 μM) to Cgb in buffer plus NADH, produced a FeIII-NO spectrum followed by a rapid change to a transient oxidised form (~FeIII) (413, 505, 645) (Fig. 4.2, blue solid line). Surprisingly, the haem signal was lost after 15 min incubation (Fig. 4.2, gray solid line), suggesting the decomposition or loss of the haem group. The basis of this is unclear.

The findings above are indicative of the presence of an NADH-dependent reductant system in the heterologous cellular milieu that efficiently reduced the Cgb haem following oxidation by NO.

4.2.2 NADH promotes reduction of Cgb in cellular extracts of *C. jejuni*.

In order to investigate whether the Cgb reduction observed in cellular extracts of *E. coli* was also produced in extracts of the native host, the globin (5 μM) was added to soluble fractions prepared from cultures of a *C. jejuni cgb ctb* mutant strain in the presence of NADH (10 mM). As in the *E. coli* extracts, the spectrum of a mixture of FeII-O_2 and

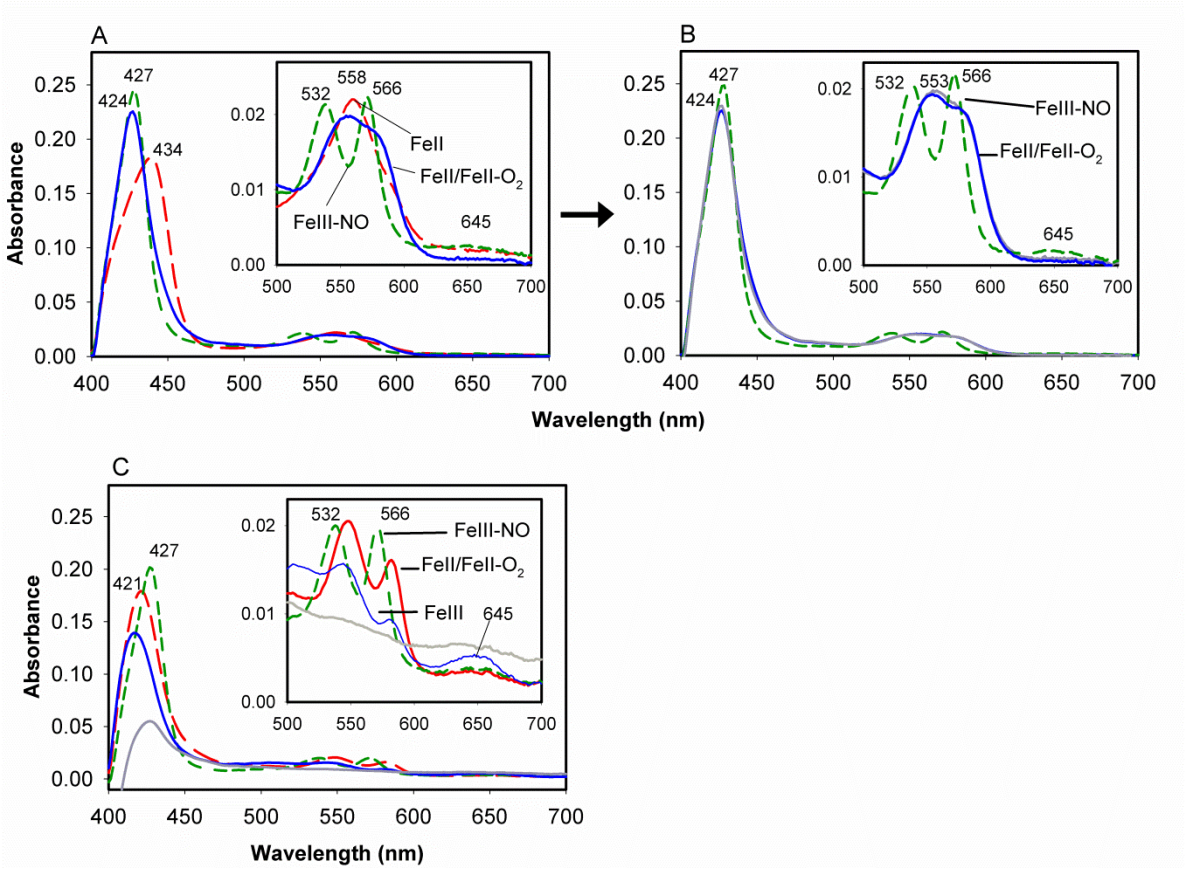


Figure 4.2 Redox state of the Cgb haem cofactor in *E. coli* extracts and turnover after oxidation by NO.

Purified Cgb (5 µM) was mixed with soluble extracts of *E. coli hmp* mutant in 50 mM Tris-HCl buffer (pH 7.4), containing NADH (10 mM) at 37 °C. Arrow (**A** to **B**) indicates subsequent steps performed with the same sample. (**A**) After reduction of Cgb (red long dashed line), PROLI NONOate (5 µM) was added and changes in the absorption were recorded immediately (green dashed line) and after 10 min incubation (blue solid line). (**B**) Additional PROLI NONOate (10 µM) was added to the sample (blue solid line) and the changes recorded immediately (green dashed line) and after 2 min incubation (gray solid line). (**C**) Changes in the absorbance of Cgb in buffer containing NADH is shown as a control. Soluble extracts containing NADH (**A** and **B**) or buffer containing NADH (**C**) were used as a baseline. The tests were repeated three times with similar results. Modified from Tinajero-Trejo *et al*. (2013).

FeIII redox forms was recorded immediately after the addition of the globin (419, 542, 577, 645 nm) (Fig. 4.3A, red dashed line). However, in this condition, incubation of Cgb led to incomplete reduction; a stable mixture of FeII and Fe-O_2 was observed after 30 min incubation (427, 548, 568 nm) (blue solid line). On the other hand, incubation of Cgb in *C. jejuni* extracts in the absence of NADH did not produce changes in the spectrum after 25 min incubation (Fig. 4.3B, blue solid line), indicating, as in *E. coli* extracts, the requirement of reducing equivalents for the globin reduction.

A partially reduced Cgb sample was prepared by incubation of the globin in NADH-containing cellular extracts for 25 min (427, 546, 570 nm). Addition of PROLI NONOate (5 μM) to this sample (Fig. 4.4A, red dashed line) produced an immediate shift of the spectrum to oxidised haem forms (FeIII and FeIII-NO) (416, 546, 570, 645 nm) (Fig. 4.4A, green dashed line) followed by a reconversion to the partially reduced mixture once more after 10 min (Fig. 4.4A, blue solid line). Similar results were observed after a second addition of PROLI NONOate (10 μM) (Fig. 4.4B), resembling the re-reduction of the globin recorded in *E. coli* extracts under the same conditions. On the other hand, when an oxidised sample (Cgb in *C. jejuni* extracts without NADH) (Fig. 4.4C, gray solid line) was added with PROLI NONOate (10 μM), a FeIII-NO spectrum was recorded (425, 533, 568 nm) (green dashed line) shifting back to the FeIII form almost immediately (30 s) (Fig. 4.4C, blue solid line).

4.2.3 The Cgb haem is not reduced in *E. coli* membrane suspensions

Even though the reduction of Cgb was demonstrated in NADH-containing soluble cellular extracts of *E. coli* and *C. jejuni*, reaching stable reduced forms took several minutes (15 and 30 min respectively). This might be caused by the absence of a more efficient reductase(s) contained in the insoluble cellular fraction. Thus, in order to test whether the globin could be more rapidly reduced in the presence of both soluble and insoluble cellular content, attempts to test the reducibility of Cgb in crude extracts of *C. jejuni* (containing soluble and membrane fractions) were performed. However, the presence of high concentrations of membrane components (such as cytochrome *c*) (Jackson *et al.*, 2007), which are also reduced by NADH and can react with NO, obscured the globin haem signal making it difficult to distinguish between the redox changes (not shown).

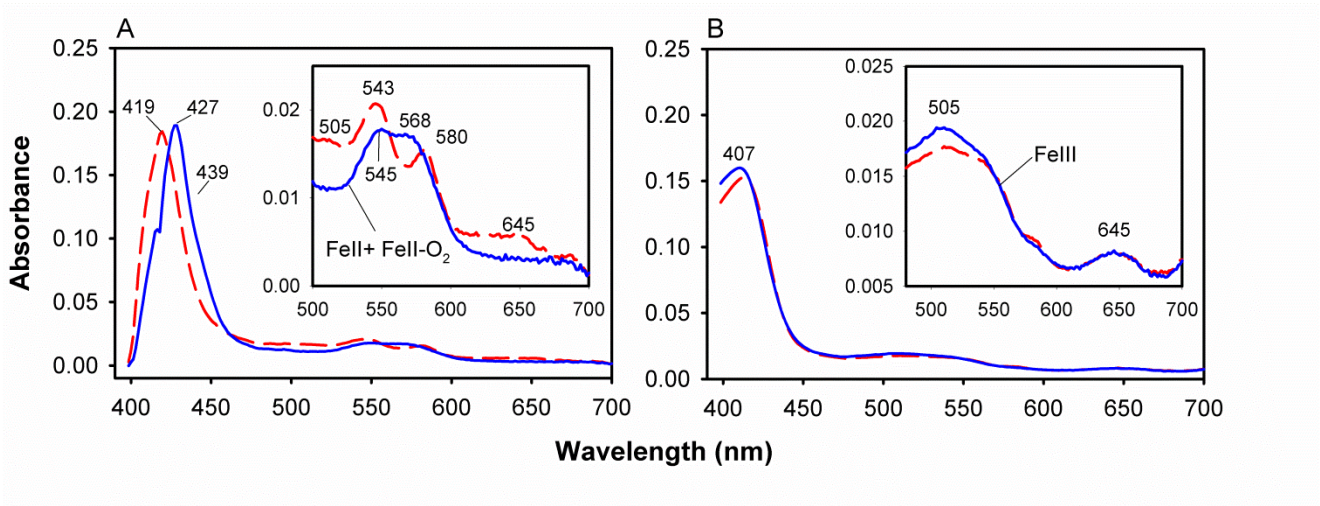


Figure 4.3 Redox state of the Cgb haem cofactor in *C. jejuni* extracts.

Purified protein (5 µM) was mixed with soluble extracts of *C. jejuni cgb ctb* mutant in 50 mM Tris-HCl buffer (pH 7.4), containing NADH (10 mM) at 37 °C (**A)**. Absorption spectra were recorded immediately after addition of the protein (red dashed line) or 30 min (blue solid line) incubation. (**B**) Spectra in soluble extracts without NADH immediately after globin addition (red dashed line) and 25 min later (blue solid line) are shown as a control. Spectra were recorded against a baseline of the soluble extracts in the presence or absence of NADH respectively. The tests were repeated twice with similar results. Modified from Tinajero-Trejo *et al*. (2013).

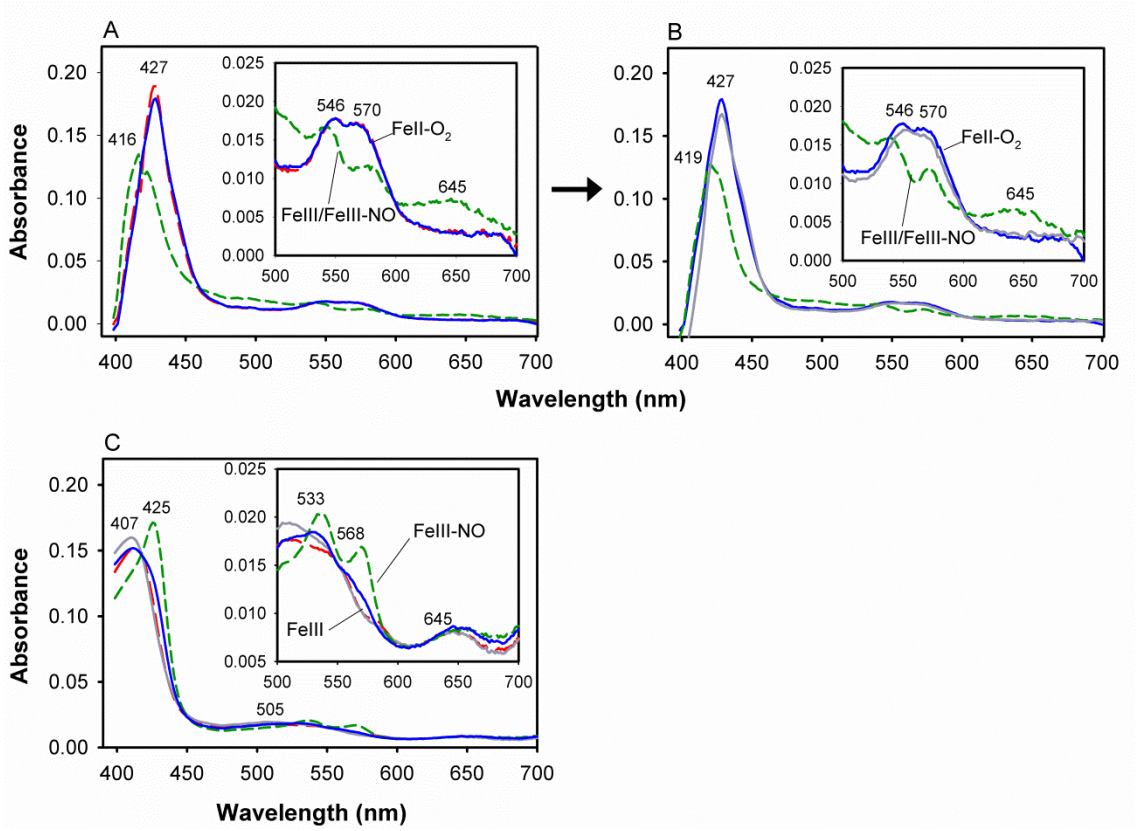


Figure 4.4 Redox state of the Cgb haem cofactor in *C. jejuni* extracts and turnover after oxidation by NO.

Purified Cgb (5 µM) was mixed with soluble extracts of *C. jejuni cgb ctb* mutant in 50 mM Tris-HCl buffer (pH 7.4), containing NADH (10 mM) at 37 °C. Arrow (**A** to **B**) indicates subsequent steps performed with the same sample. (**A**) After Cgb showed a stable FeII-O_2 form (red dashed line), 10 µM PROLI NONOate were added and changes in the absorption recorded immediately (green short dashed line) and after 10 min incubation (blue solid line). (**B**) Additional 10 µM PROLI NONOate was added to the sample (blue solid line) and the changes recorded immediately (green dashed line) and after 2 min incubation (gray solid line). (**C**) Changes in the absorbance of Cgb in soluble extracts without NADH are shown as a control. Spectra were recorded against baseline of the soluble extracts in the presence (**A** and **B**) or absence (**C**) of NADH. The tests were repeated three times with similar results. Modified from Tinajero-Trejo *et al.* (2013).

In a different attempt to test whether insoluble components are involved in the reduction of the globin, membranes were isolated from *E. coli hmp* cells. Since the signal from the *E. coli* membranes was negligible compared to that of Cgb, this system was suitable for testing the redox changes of the globin.

Membrane suspensions in the presence of NADH failed to promote reduction of ferric Cgb (5 μM) after 20 min incubation (513, 505, 645 nm) (Fig. 4.5A, blue solid line) and addition of PROLI NONOate (10 μM) produced a transient FeIII-NO form (427, 536, 570 nm) (Fig. 4.5B, green dashed line) that, after 15 min incubation, shifted back to the native oxidised form (FeIII). These results suggested that the electron donor(s) involved in the reduction of Cgb is soluble. However, the nature of such reductase(s) is still unknown.

4.2.4 NADH promotes reduction of Ctb in cellular extracts of *E. coli*.

Ctb failed to support the growth of the *E. coli hmp* mutant strain under nitrosative stress conditions (Chapter 3, Fig. 3.4). However, the ability of the truncated globin to consume NO in *E. coli* cells in aerobic and anaerobic conditions (Chapter 3, Fig. 3.6D) suggests that Ctb might have a minor function in NO chemistry that would imply changes in the redox state of the haem.

In view of the fact that the native form of the purified Ctb haem is oxidised (FeIII) (410, 512, 542, 582, 640 nm) (Pickford *et al.*, 2008), and consequently unable to bind NO (Shepherd *et al.*, 2011), the haem group must be reduced in the cellular milieu as an initial step in order to allow the NO binding. In agreement with this principle, addition of oxidised Ctb (FeIII) (4.8 μM) to *E. coli hmp* extracts in the presence of NADH (10 mM) (Fig. 4.6A, red dashed line) produced the complete reduction (FeII) of the haem after 30 min incubation at 37 °C (433, 566 nm) (Fig. 4.6A, blue solid line). As observed for Cgb, the reduction was dependent on the presence of both cellular extracts and NADH. Indeed, the addition of the truncated globin to extracts in the absence of cofactor failed to produce changes in the FeIII haem spectrum (Fig. 4.6B), and the presence of NADH in buffer promoted only a partial reduction after 30 min incubation (Fig. 4.6C).

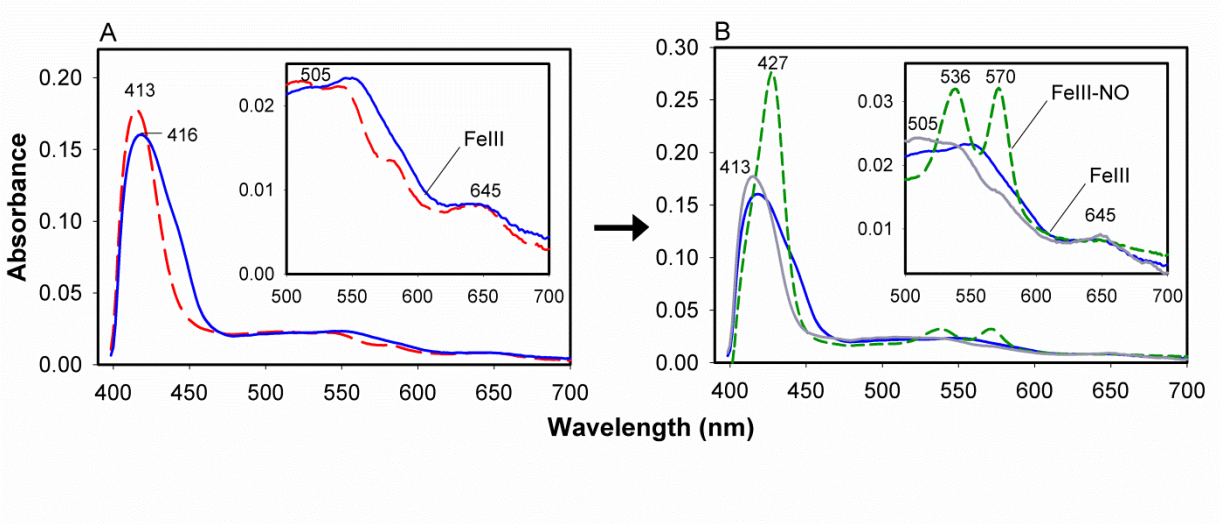


Figure 4.5 Redox state of the Cgb haem cofactor in *E. coli* membrane suspensions and changes after oxidation by NO.

Purified Cgb (5 µM) was mixed with membrane suspensions of *E. coli hmp* mutant in 50 mM Tris-HCl buffer (pH 7.4), containing NADH (10 mM) at 37 °C. Arrow indicates subsequent steps performed with the same sample. (**A**) Absorption spectra were recorded immediately after addition of the protein (red dashed line) and after 20 min incubation (blue solid line). (**B**) PROLI NONOate (10 µM) was added to the sample (blue solid line) and changes in the absorption were recorded immediately (green dashed line) and after 10 min incubation (gray solid line). Membrane suspension containing NADH was used as a baseline. The test was repeated twice with similar results.

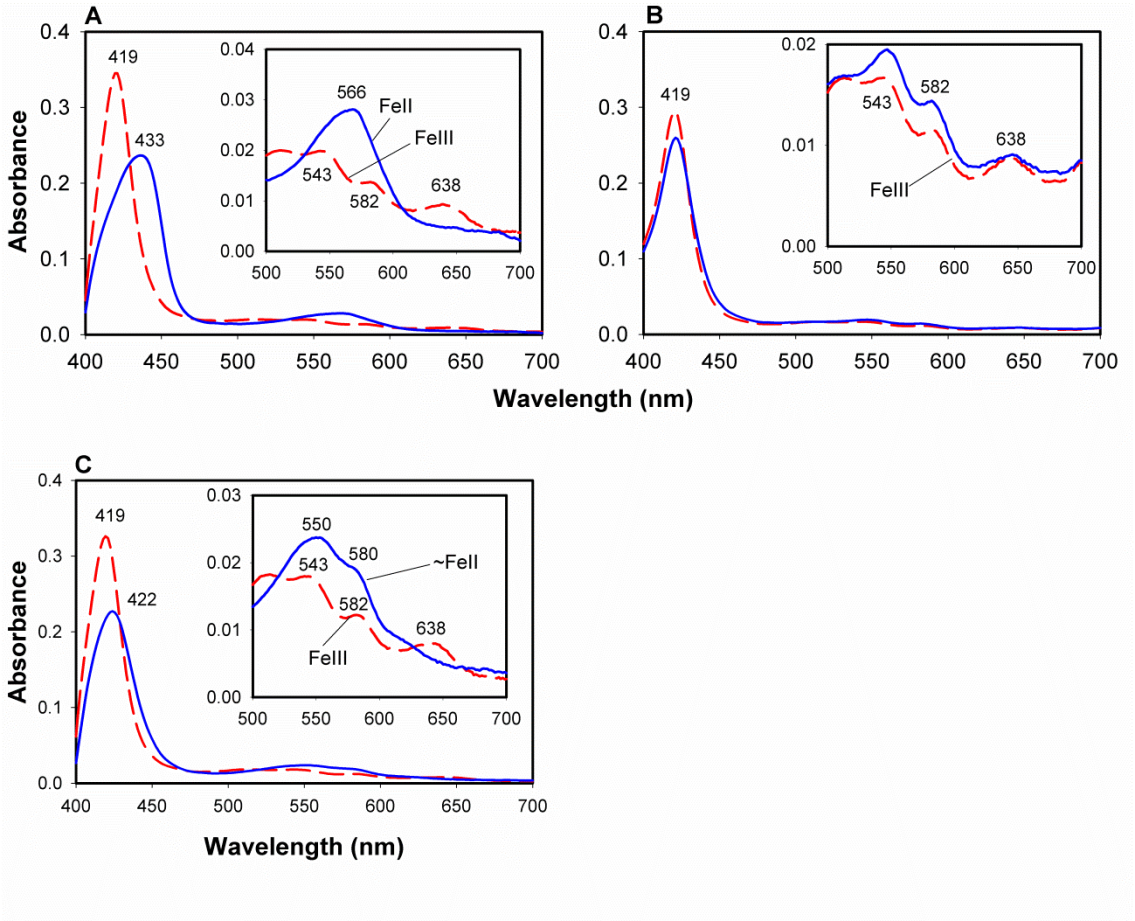


Figure 4.6 Redox state of the Ctb haem cofactor in *E. coli* extracts.

Purified Ctb (4.8 µM) was mixed with soluble extracts of *E. coli hmp* mutant in 50 mM Tris-HCl buffer (pH 7.4), containing NADH (10 mM) at 37 °C. Absorption spectra were recorded immediately after addition of the protein (red dashed lines) and after 30 min incubation (**A**). Changes in absorbance in the presence of soluble extract without NADH (**B**) or in buffer containing NADH (**C**) immediately (red solid lines) and after 20 or 30 min incubation respectively (blue solid lines) are shown as controls. Soluble extracts containing NADH, soluble extracts or buffer added with NADH were used as a baseline (**A**, **B** and **C** respectively). Tests were repeated two times with similar results. Modified from Tinajero-Trejo *et al*. (2013).

The re-reduction of the truncated globin in the heterologous extracts after NO oxidation was investigated. A Ctb reduced sample produced by the incubation of the globin in NADH-containing *E. coli* extracts for 30 min (Fig. 4.7A, red dashed line) was added with an equimolar concentration of PROLI NONOate (4.8 μM). An immediate shift to a fully oxidised spectrum (FeIII) (Fig. 4.7A red dashed line) was followed by reconversion to the reduced state (FeII) after 10 min incubation (Fig. 4.7A, blue solid line). A second addition of PROLI NONOate (10 μM) resulted again in oxidation and then re-reduction of the globin haem (Fig. 4.7B). On the other hand, addition of Ctb to extracts in the absence of NADH showed no changes after addition of NONOate (10 μM) (Fig. 4.7C) in agreement with the inability of oxidised Ctb to bind NO (Shepherd *et al.*, 2011).

4.2.5 NADH promotes reduction of Ctb in cellular extracts of *C. jejuni*.

Reduction of purified Ctb in *C. jejuni* soluble extracts was tested. Addition of native oxidised globin (4.5 μM) to cellular soluble extracts of *C. jejuni* in the presence of NADH (10 mM) (Fig. 4.8A, red dashed line) produced a stable, partially reduced form (FeII/FeII-O_2) after 30 min incubation at 37 °C (425, 548, 571 nm) (Fig. 4.8A, blue solid line). As before, addition of Ctb to extracts in the absence of NADH did not produce redox changes (Fig. 4.8B) pointing out the dependence of reduction on NADH.

Upon reduction of Ctb (4.5 μM) in *C. jejuni* extracts in the presence of NADH (Fig. 4.9A, red dashed line), PROLI NONOate (10 μM) was added to oxidize the sample (Fig. 4.9A, green dashed line). Turnover to the partially reduced form observed after 20 min incubation (Fig. 4.9A, blue solid line) is in agreement with the re-reduction of Ctb observed in *E. coli* extracts.

Finally, in order to test whether a different electron donor could promote the reduction of Ctb in *C. jejuni* extracts, formate (10 mM), one of the most efficient sources of energy for *Campylobacter* (Hoffman and Goodman, 1982), was added instead of NADH. No changes were recorded in the spectrum after 30 min incubation (not shown), suggesting that, at least in the tested conditions, formate fails to mediate the reduction of the globin.

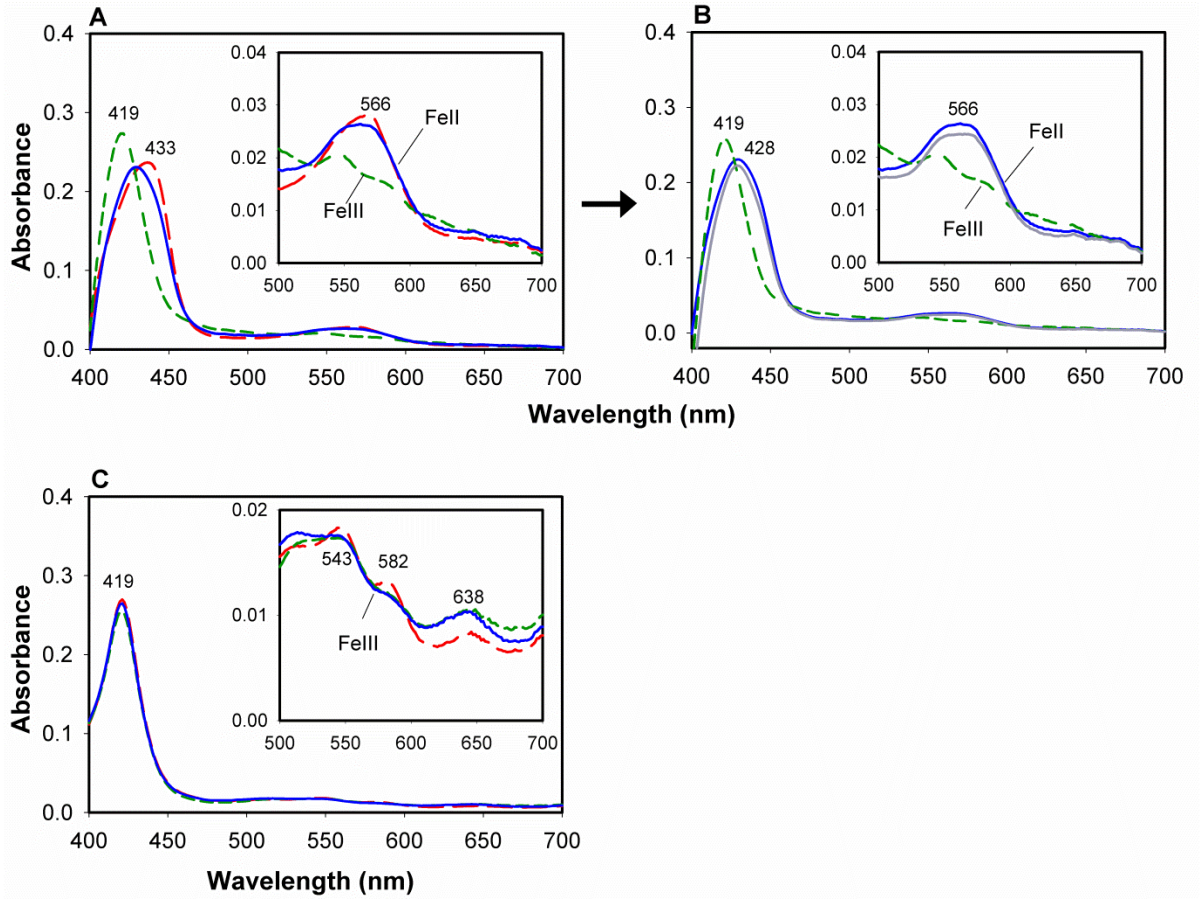


Figure 4.7 Redox state of the Ctb haem cofactor in *E. coli* extracts and turnover after oxidation by NO.

Purified Ctb (4.8 µM) was mixed with soluble extracts of *E. coli hmp* mutant in 50 mM Tris-HCl buffer (pH 7.4), containing NADH (10 mM) at 37 °C. Arrow (**A** to **B**) indicates subsequent steps performed with the same sample. (**A**) After reduction of Cgb (red long dashed line), PROLI NONOate (4.8 µM) was added and changes in the absorption were recorded immediately (green short dashed line) and after 15 min incubation (blue solid line). (**B**) The sample (blue solid line) was added with PROLI NONOate (10 µM) and the changes recorded immediately (green short dashed line) and after 15 min incubation (gray solid line). Spectra were recorded against a NADH-containing soluble extract baseline. (**C**) Changes in the absorbance of Ctb in buffer containing NADH (red dashed line) and after NONOate addition (red and blue lines) are shown as a control. Soluble extracts in the presence (**A** and **B**) or absence (**C**) of NADH were used as the baseline. The tests were repeated twice with similar results. Modified from Tinajero-Trejo *et al*. (2013).

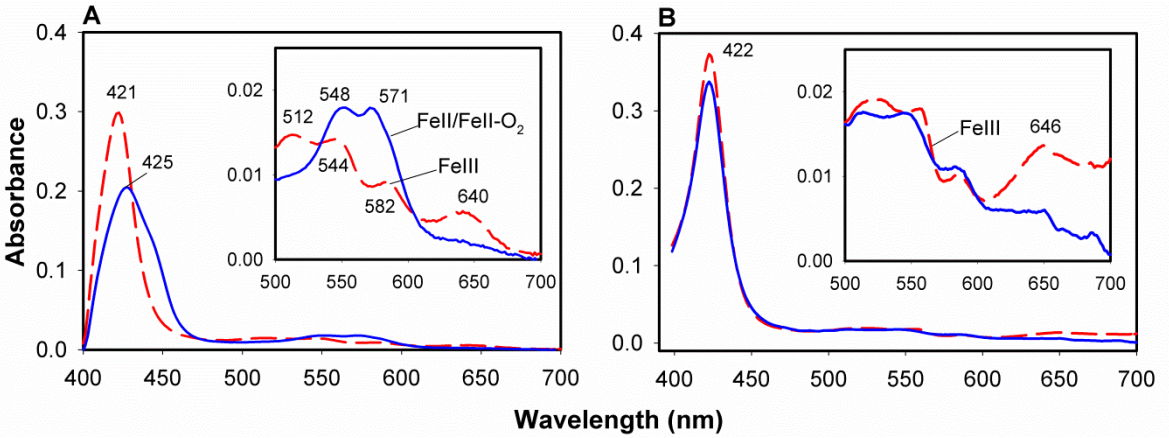


Figure 4.8 Redox state of the Ctb haem cofactor in *C. jejuni* extracts.

Purified Ctb (4.5 µM) was mixed with soluble extracts of the *C. jejuni cgb ctb* mutant in 50 mM Tris-HCl buffer (pH 7.4), containing NADH (10 mM) at 37 °C. (**A)** Absorption spectra were recorded immediately after addition of the protein (red dashed line) and after 30 min (blue solid line) incubation. (**B**) Changes in the absorbance in soluble extracts without NADH immediately after addition of the protein (red dashed line) and after 30 min incubation (blue solid line) are shown as a control. Soluble extracts in the presence (**A**) or absence (**B**) of NADH were used as the baseline. Tests were repeated twice with similar results. Modified from Tinajero-Trejo *et al*. (2013).

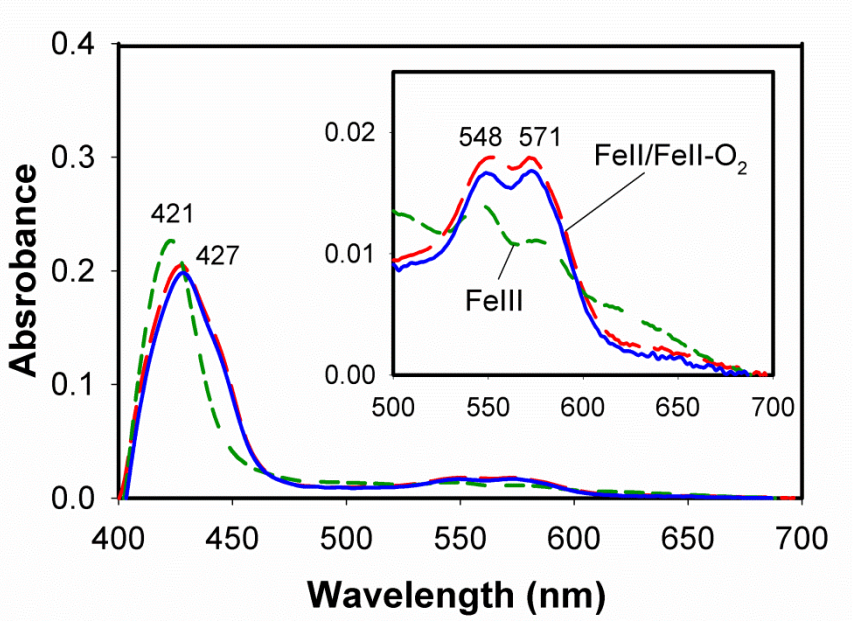


Figure 4.9 Redox state of the Ctb haem cofactor in *C. jejuni* extracts and turnover after oxidation by NO.

Purified Ctb (4.5 µM) was mixed with soluble extracts of *C. jejuni cgb ctb* mutant in 50 mM Tris-HCl buffer (pH 7.4), containing NADH (10 mM) at 37 °C. After reduction of Ctb (red long dashed line), PROLI NONOate (10 µM) was added and changes in the absorption recorded immediately (green short dashed line) and after 20 min incubation (blue solid line). NADH-containing extracts were used as a baseline. The test was repeated twice with similar results. Modified from Tinajero-Trejo *et al*. (2013).

4.3 Discussion

A metmyoglobin (metMb) reductase from bovine heart was first described by Hagler *et al.* in 1979. Reduction of metMb by this reductase was dependent on NADH and ferrocyanide ion. However, the presence of cytochrome b_5 was more efficient than ferrocyanide *in vitro.* Later, the interaction of cytochrome b_5 from microsomal preparations with Mb and Mb reductase was demonstrated electrophoretically and kinetic and ^{1}H NMR data supported the electron transfer from the reductase to myoglobin mediated by cytochrome b_5 in muscle (Livingston *et al.*, 1983). The reduction of metmyoglobin in heart muscle is such an efficient reaction that the oxidised form is not detectable by *in vivo* ^{1}H NMR.

On the other hand, failure in the identification of specific reductases seems to be a common problem, not only for other eukaryotic globins (e.g. neuroglobin and cytoglobin) and prokaryotic non-flavo globin proteins (SDgbs and TrHbs), but also for other haem enzymes. For instance, genes encoding bacterial nitric oxide synthases (bNOS) have been identified in a number of bacterial genomes (e.g. *Deinococcus radiodurans* and *Bacillus subtilis* among many others) (Kunst et al., 1997; White et al., 1999). NO production by bNOS appears to protect bacteria from oxidative stress (Gusarov and Nudler, 2005; Shatalin et al., 2008) and to play a role in resistance to antibacterial agents (Gusarov et al., 2009). Interestingly, these enzymes lack the C-terminal flavodomain that enables the reduction of the haem in mammalian NOS. A reduced haem, consequently able to bind O_2, constitutes a requisite for the production of NO from arginine (Gorren and Mayer, 2002; Wei *et al*., 2003). Thus, as is the case for SDgbs and TrHbs, bNOSs must rely on an independent electron donor protein. The search for such a dedicated redox partner has been unfruitful; the production of NO *in vivo* was demonstrated in *B. subtilis* and *B. anthracis* but efforts to find “the" dedicated redox partner(s) in these microorganisms were unsuccessful (Gusarov et al., 2008). Since over-expression of bNOS from *B. subtilis* in the native host and in the heterologous host *E. coli* (a phylogenetically distant bacterium, lacking *nos* genes) dramatically increased the production of NO, the authors concluded that bNOS function is not dependent on dedicated redox partners.

Is there enough evidence to reach such a conclusion with regards to the reductases of bacterial non-flavo globins? Is it possible that, as it has been proposed for bNOS, the SDgbs and the TrHbs globins rely on promiscuous electron donors more than specific reductases? Mutations in the TrHb2 (PhHbO) and the SDgb (Cgb) encoding-genes from *P. haloplanktis* and *C. jejuni* respectively led to the inference of roles in NO and RNS detoxification (Avila-Ramirez *et al.*, 2013; Elvers *et al.*, 2004; Parrilli *et al.*, 2010; Pittman *et al.*, 2007). This function was later confirmed in both cases by heterologous over-expression in an *E. coli hmp* mutant strain. Both PhHbO and Cgb complemented the NO and GSNO resistance phenotype loss by the absence of the flavohaemoglobin in the heterologous host (Coppola *et al*., 2013 and herein). Besides, the TrHb1 (HbN) from *M. tuberculosis*, which the encoding-gene is up-regulated under nitrosative stress conditions in the native host, also supports the growth of an Hmp-lacking *E. coli* in the presence of NO (Pathania *et al.*, 2002a). Thus, these three structurally different globins, belonging to phylogenetically diverse bacteria, appear to find an efficient reductase(s) partner(s) in *E. coli*. This indeed suggests the presence of general reduction systems common perhaps to any haem protein. Unfortunately, studies of globins for which a function has been demonstrated in both the native host and heterologously are too scarce to confirm this proposal.

Herein, it has been shown that both Cgb and Ctb haems are reduced by soluble extracts of *E. coli* (Fig. 4.1 and 4.6) and *C. jejuni* (Fig. 4.3 and 4.8) in the presence of NADH. These findings suggest the existence of a reductant component(s) in the heterologous host that is able to donate electrons as efficiently as their counterpart(s) in the native host. Furthermore, the turnover of the Cgb and Ctb haems after oxidation by an excess of PROLI NONOate (up to four times more NO compared to the concentration of globin (i. e. 5 μM Cgb and 10 μM NONOate)) (Fig. 4.2, 4.4, 4.7 and 4.9) implies the presence of high levels of the reductase protein(s). Perhaps an unspecific 'diaphorase' such as those described before (Liochev *et al.*, 1994), able to catalyse several cycles of reduction, or a sufficiently reducing environment are responsible.

Cgb, but not Ctb, supported the growth of an *E. coli hmp* mutant under nitrosative stress conditions (Chapter 3, Fig. 3.3). However, the reduction of Cgb and Ctb in cellular milieus (Fig. 4.1A and 4.3A, 4.6A and 4.8A respectively), even after NO oxidation, was comparable (4.2B and 4.4B, 4.7B and 4.9 respectively). Thus, it appears that reduction

is not the limiting factor related to the inability of Ctb to support the growth of *E. coli* in the presence of NO or RNS (Chapter 3, Fig. 3.4).

Hausladen *et al.* (2001) suggested that the Hmp NOD activity implies the formation of a ferric nitrosyl intermediate (Hmp-FeIII-NO) prior to nitrate production. Moreover, in the absence of O_2, a much slower reaction also dependent on the formation of the FeIII-NO intermediate yields N_2O as a result of dimerization and dehydration of nitroxyl (HNO) (Hausladen *et al.*, 1998; Kim *et al.*, 1999). The demonstration of the ability of the Cgb haem to bind NO in the reduced (Cgb-FeII-NO) and the oxidised (Cgb-FeIII-NO) forms led to the proposal of a NOD or denitrosylase activity as the detoxification mechanism mediated by this globin (Shepherd *et al.*, 2011). We hypothesise that differences in the fate of the NO molecule bound to the ferrous form of one or the other globin may explain the functional differences. Since reduced Ctb is able to bind NO (Ctb-FeII-NO) (Wainwright *et al.*, 2006) but the oxidised form fails to do so (Shepherd *et al.*, 2011) (Fig. 4.6B, 4.7C and 4.8B), a detoxification reaction (e.g. NOD, denitrosylase or NO reductase activity) seems unlikely. However, this suggestion fails to explain the NO consumption shown in Ctb-expressing *E. coli* cells (Chapter 3, Fig. 3.6D). More experiments are required in order to better understand these apparent discrepancies.

Even though the NADH-dependent reduction of the *C. jejuni* globins was demonstrated in cellular soluble extracts, the periods of incubation needed for the stabilisation of the reduced forms were, perhaps, too long to be physiologically relevant (e.g. 10 min for Cgb and 20 min for Ctb in *C. jejuni* extracts) (Fig. 4.3A and 4.6A respectively). However, this might be related to the artificial system used in this study. Indeed, it is clear that heterologous globins synthesised in *E. coli* are natively reduced (see section 4.1). Another possibility is that insoluble components, absent in our system, participate in a more efficient globin reduction (e.g. the electron flux from the respiratory chain). If this is the case, spectroscopic changes in cellular extracts containing both soluble and membrane fractions may produce a more efficient reduction. Indeed, a preliminary study of the reduction of Cgb in *C. jejuni* crude extracts using formate as the electron donor showed features of reduction (Smith, 2010). However, as reported here, the signal of cytochrome *c* made it difficult to distinguish clearly the globin spectrum. On the

other hand, failure of membrane suspensions containing NADH to reduce Cgb in *E. coli* (Fig. 4.5A) argues against this hypothesis.

Considering the experimental challenges faced when using extracts to test the role of membrane components in the reducibility of the *C. jejuni* globins, a more suitable model might be, for example, the use of a respiration defective strain to test the ability of Cgb to detoxify NO *in vivo* in the absence of a functional respiratory chain. Such a possibility is explored in the following Chapter.

4.4 Conclusions

The single domain haemoglobin Cgb and the truncated globin Ctb from *C. jejuni* are reduced in soluble extracts of *E. coli* and *C. jejuni* in the presence of NADH. Transient oxidation of the globin haems by NO, followed by the reconversion to the reduce forms are dependent of both cellular soluble extracts and NADH.

Chapter 5. Looking for the reductase partners of Cgb in *E. coli* and *C. jejuni*

5.1 Introduction

Frey *et al.* (2000) showed that the expression of Cgb in an *E. coli* wild type strain improved the growth under nitrosative stress conditions. However, NO uptake of soluble cellular fractions obtained from a Cgb-expressing strain, failed to show differences when compared to the wild type. According to the authors, this discrepancy could be explained by the absence of a membrane-bound protein involved in the reduction of Cgb, as has been previously described for myoglobin (Livingston *et al.*, 1985). In the present work, the NO uptake and the protection of respiration against NO inhibition has been demonstrated in Cgb-expressing cells of an *E. coli hmp* mutant strain (Chapter 3, Fig. 3.6). Even though the NADH-dependent reduction of the Cgb haem after oxidation by NO was demonstrated spectrophotometrically in soluble fractions of *E. coli* and a *C. jejuni*, technical difficulties hampered the investigation of whether, in the presence of membrane and soluble fractions (crude extracts), a more efficient turnover might take place (see Chapter 4).

The ability of bacterial haemoglobins to associate with membranes has been previously described. Mycobacterial hemoglobin HbO (Pathania *et al.*, 2002b) and the Vgb globin from *Vitreoscilla* (Ramandeep *et al.*, 2001) bind to bacterial membranes and have been implicated in oxygen transfer (Dikshit *et al.*, 1992; Park *et al.*, 2002; Pathania *et al.*, 2002b). Vgb interacts with subunit I of cytochrome *bo'* ubiquinol oxidases (Park *et al.*, 2002), supposedly playing a role as an O_2 shuttle. For this reason, the *Vitreoscilla* globin is widely used for biotechnological applications (reviewed in Frey *et al.*, 2011).

Comparison between the Cgb and the Hmp structures suggests that the former may not interact with reductases as is true for the reductase domain of flavohaemoglobins. The conserved residue Lys-84, responsible for the formation of a salt bridge between the domains in the flavohaemoglobins (Ermler *et al.*, 1995a), is absent in the *C. jejuni* globin. As Cgb and Vgb are homologues (42% amino acid identity), the former may interact with membranes and take electrons from the respiratory chain for the reduction of its haem during NO detoxification.

5.1.1 The respiratory chain of *E. coli*

E. coli possesses a variety of energy-producing pathways. This characteristic makes it a suitable model for genetic studies of respiratory electron transfer and ATP synthesis (Cox and Downie, 1979; Poole and Williams, 1987). Mutants lacking aerobic respiratory metabolism are able to grow fermentatively or anaerobically with adequate electron acceptors. Indeed, *E. coli* possesses many NADH dehydrogenases (Kim *et al.*, 2008; Patridge and Ferry, 2006; Thorn *et al.*, 1995), lactate and succinate dehydrogenases and a number of terminal reductases (Gennis and Stewart, 1996). Three different quinones (ubiquinone, menaquinone and demethylmenaquinone) and three terminal oxidases (cytochrome *bo'*, cytochrome *bd-I* and cytochrome *bd-II*) are synthesised in *E. coli* (Poole and Cook, 2000). Thus, electrons can be transferred to O_2, the main terminal acceptor, or donated to alternative acceptors (e.g. dimethylsulfoxide, nitrate or fumarate).

NADH constitutes the major carrier of electrons from glycolysis and the Krebs cycle, while $FADH_2$ carries electrons transfered specifically from the oxidation of succinate by succinate dehydrogenase (Cecchini, 2003). It has been assumed that, in these processes, electrons are transferred to ubiquinone while the other quinones are mainly involved in transferring electrons from anaerobic metabolism (Wissenbach *et al.*, 1990). The ubiquinone biosynthetic pathway has been studied in detail in *E. coli* (Meganathan, 2001), and mutants defective in ubiquinone synthesis have been isolated (Gibson and Cox, 1973) due to their capacity to grow on glucose by fermentation (Wu *et al.*, 1993), but not on non-fermentable carbon sources such as malate or succinate.

Deletion of the *ubiCA* operon in *E. coli* leads to strains defective in chorismate lyase and 4-hydroxybenzoate octaprenyl transferase. These enzymes catalyse the first and second steps in the ubiquinone synthesis pathway and are rate limiting in ubiquinone synthesis (Soballe and Poole, 1998). Indeed, deletions in *ubi* genes render extremely poorly growing strains (Sharma *et al.*, 2012; Wu *et al.*, 1993; Wu *et al.*, 1992).

5.1.2 The flavorubredoxin reductase (NorW) of *E. coli*

The main mechanism for anaerobic NO detoxification in *E. coli* is catalysed by the flavorubredoxin (NorV). This oxygen-sensitive protein contains an NO reactive di-iron centre able to reduce NO to N_2O (Gomes *et al.*, 2002) in a mechanism that depends on the presence of a reductase partner: the FAD-containing NADH:flavorubredoxin oxidoreductase (NorW) (Gardner *et al.*, 2003; Gardner *et al.*, 2002; Gomes *et al.*, 2002; Gomes *et al.*, 2000). The induction of the *norVW* operon is regulated by the haem-containing transcription factor (NorR) under nitrosative stress conditions, either in the presence or absence of O_2 (Flatley *et al.*, 2005; Pullan *et al.*, 2007; Spiro, 2006).

It has been suggested that NorW might be involved in the reduction of neuroglobin *in vivo* when the globin is heterologously expressed in *E. coli*. Indeed, NorW has been successfully utilised to reduce mouse neuroglobin *in vitro* in a reaction dependent on NADH (Giuffre *et al.*, 2008).

5.1.3 The lactate dehydrogenase (Cj1585c) of *C. jejuni*

In the *C. jejuni* genome, the *cj1585c* gene is positioned upstream of *cgb*, yet divergently transcribed. Cj1585c is an FAD-containing oxidoreductase using L-lactate as the electron donor (Thomas *et al.*, 2010). It has been previously suggested that such a protein may play a similar role to the reductase partner of Cgb during NO detoxification. Indeed, *cj1585c* is up-regulated in response to NO under oxygen-limited conditions. However, induction of the members of the NssR regulon (including *cgb*) under nitrosative stress conditions occurs in microaerobiosis, but not in oxygen limitation (Avila-Ramirez *et al.*, 2013) (see Chapter 1, section 1.8). Since the Cgb detoxification reaction appears to be O_2-dependent in *Campylobacter* (Avila-Ramirez *et al.*, 2013; Elvers *et al.*, 2005; Elvers *et al.*, 2004; Monk *et al.*, 2008; Wainwright *et al.*, 2005), a role for Cj1585c is unlikely. However, disk diffusion assays in MH broth in the presence or absence of L-lactate revealed that a *cj1585c* mutant was significantly more sensitive than the wild type to SNP or spermine NONOate; however, this sensitivity was independent of the presence of L-lactate (Thomas, 2009). It is possible that basal levels of Cj1585c, expressed microaerobically, donate the electrons for globin reduction under nitrosative stress conditions.

The objective of the present chapter is to assess the role of the respiratory chain and the flavorubredoxin reductase (NorW) as the source of electrons for the reduction of the Cgb haem during NO detoxification in *E. coli*. Additionally, the function of the lactate dehydrogenase (Cj1585c) as the cognate reductase of Cgb in *C. jejuni* is evaluated.

5.2 Results

5.2.1 The ability of Cgb to detoxify NO in *E. coli* is independent of the respiratory chain.

Since the *Vitreoscilla* hemoglobin Vgb interacts with the cytochrome *bo'* terminal oxidase of *E. coli* (Park *et al.*, 2002), it was hypothesized that electron flux could supply reductant power to the *C. jejuni* globin Cgb during NO detoxification in aerobic conditions. In order to investigate this, an NO-sensitive *E. coli* strain (*hmp* mutant) unable to produce ubiquinone, due to the replacement of the intergenic region of the *ubiCA* operon with a kanamycin resistance gene, was constructed (see section 2.3.5). Characterization of the mutant included testing the absence of ubiquinone (Angie Vreugdenhil, personal communication) and the ability of the *hmp ubiCA* mutant to grow in rich medium under aerobic and anaerobic conditions, but not in medium containing succinate as the only carbon source (not shown). Additionally, the respiration rates of membranes isolated from the *hmp* mutant and the *hmp ubiCA* mutant were compared (Table 5.1). The O_2 uptake rate promoted by the addition of succinate (10 mM) as the electron source was 7.5-fold slower in the membranes from the triple mutant than those from the *hmp* mutant. However, upon addition of coenzyme Q1 (20 μM) and succinate simultaneously, the rates were similar. Thus, the suitability of the system to test Cgb function in the absence of an efficient electron flux from the aerobic respiratory chain was confirmed.

The growth profiles of the *hmp ubiCA* mutant expressing Cgb, Hmp or carrying the empty vector in 2 X TY medium were all comparable. However, *ubiCA* mutant (*hmp*$^+$) used as a control during the test showed a diminished ability to grow in this condition (Fig. 5.1A). Even though there is not an obvious explanation for this behaviour, the same has been previously observed in our lab for *ubiG* and *ubiA* single mutants compared to *hmp ubiG* and *hmp ubiA* double mutants (not shown), suggesting that the deletion of *hmp* partially alleviates the growth defect that is characteristic of the ubiquinone-lacking strains.

Addition of 1 mM DETA NONOate to cultures at the beginning of the exponential phase (30 Klett units) failed to inhibit the growth of the Cgb-expressing *hmp ubiCA*

Table 5.1 Respiration rates of membrane suspensions from an *E. coli hmp ubiCA* mutant strain.

	Oxygen consumption* (nmol O_2 min^{-1} mg $protein^{-1}$)	
Strain	Succinate	Succinate + Q1
MG1655 *hmp*	50.54	52.97
MG1655 *hmp ubiCA*	6.68	52.61

*polarographic measurements were performed upon addition of succinate (10 mM) or succinate plus coenzyme Q1 (8 µM).

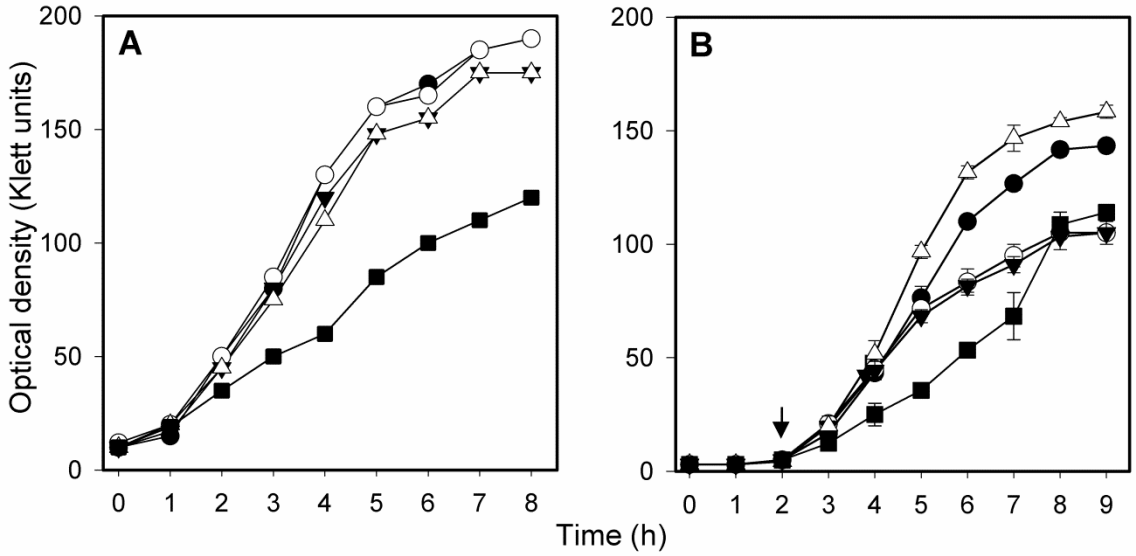


Figure 5.1 Growth profile and resistance to nitrosative stress of a respiration-defective *E. coli hmp* mutant strain expressing Cgb.

(**A**) Aerobic cultures of an *hmp ubiCA* mutant strain expressing Hmp (pPL341) (●) or Cgb (pMT1) (Δ) were grown in 2 X TY broth supplemented with arabinose and incubated for 9 h at 37 ºC, 240 rpm. (**B**) When cultures reached 30 Klett units (arrow), DETA-NONOate (1 mM) was added. Cultures of *E. coli ubiCA* mutant (■), *hmp ubiCA* carrying the empty vector (○), or pMT1 but in the absence of arabinose (▼) were included as controls. Bars represent standard error from three independent experiments. Modified from Tinajero-Trejo *et al*. (2013).

mutant strain, but produced detrimental effects to the strains harbouring the empty vector or pMT1 in the absence of the inducer arabinose (Fig. 5.1B). Interestingly, the complemented strain with pPL341 grew slightly less than the strain expressing Cgb. These differences might be the consequence of a negative effect caused by production of the superoxide ion linked to the over-expression of Hmp (Membrillo-Hernandez *et al.*, 1996; Wu *et al.*, 2004), rather than a positive effect due to the expression of Cgb.

The ability of cell suspensions of *hmp ubiCA* mutants to consume NO when expressing Cgb, Ctb or Hmp was tested polarographically in fully oxygenated buffer. The accumulated NO upon subsequent additions of 1 μM PROLI-NONOate was efficiently consumed by cells expressing either Cgb or Ctb. Conversely, cells carrying the empty vector accumulated a high concentration of the toxic compound (Fig. 5.2). The electrode failed to show any NO accumulation when Hmp-expressing cells were tested.

The ability of Cgb to support the growth of the *E. coli hmp ubiCA* mutant, and the efficient consumption of NO by both Cgb- and Ctb-expressing cells, support the lack of dependence of Cgb and Ctb reduction on aerobic electron flux beyond the UQ pool in *E. coli*.

5.2.2 The NADH:flavorubredoxin oxidoreductase plays a minor role as the cognate reductase of Cgb in *E. coli.*

The function of Cgb as a NO detoxification mechanism able to sustain the aerobic growth of an Hmp-lacking *E. coli* strain in the presence of NO and RNS was demonstrated (Chapter 3, Fig. 3.3). However, expression of the globin failed to support the anaerobic growth of an *hmp norR* mutant under nitrosative stress conditions (Chapter 3, Fig. 3.7). Since, in the latter system, the expression of NorW was prevented by the absence of the NorR regulator (Gardner *et al.*, 2003), it was suggested that NorW may have a role as the heterologous reductase partner of Cgb *in vivo*. Indeed, the lack of NorW might explain the inability of the globin to detoxify NO anaerobically in *E. coli*.

In order to test whether the absence of NorW would affect the detoxification capacity of Cgb, aerobic cultures of the *hmp norR* mutant expressing Cgb were grown overnight in the presence of arabinose and with increasing concentrations of DETA NONOate. A concentration of 1 mM DETA NONOate produced a significant decrease in the OD

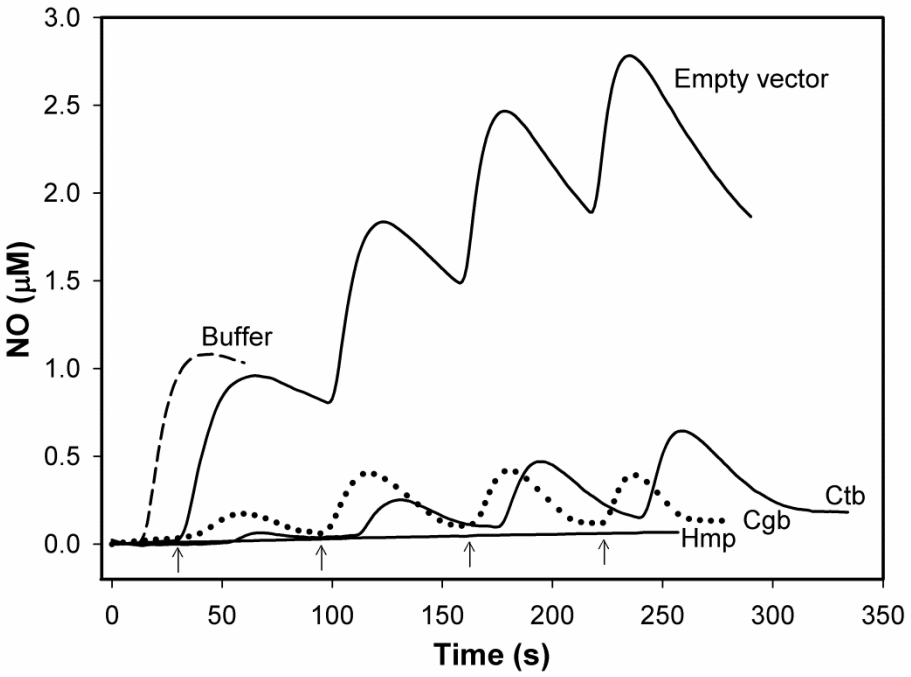


Figure 5.2 NO consumption of *E. coli hmp ubiCA* cell suspensions expressing Cgb or Ctb.

Cells were resuspended in Tris-HCl buffer (pH 7.4), and incubated aerobically in a closed chamber at 37°C; NO consumption was polarographically recorded following four subsequent additions of 1 μM PROLI NONOate. For Cgb- and Ctb-expressing cells, each addition was performed after the NO trace reached the electrode base line. Signals from cells carrying the empty vector or expressing Hmp are shown as controls. Arrows indicate PROLI-NONOate additions to the Hmp-expressing cells; for other traces, the additions immediately precede each rise in the electrode response. The signal produced from the addition of 1 μM PROLI NONOate to buffer is shown for comparison. The assay was repeated twice with similar results. Taken from Tinajero-Trejo *et al*. (2013).

while 2 mM completely inhibited the growth compared to the control in the absence of NONOate. On the other hand, the presence of DETA NONOate failed to cause growth inhibition of the complemented strain (pPL341) or the *norR* single mutant (*hmp*$^{+}$) strain, but impaired the growth of the double mutant carrying pMT1 in the absence of arabinose and of cells carrying the empty vector (Fig. 5.3A). In agreement with these results, growth of cultures after addition of 1 mM DETA NONOate showed a considerable decrease of the Cgb-expressing *hmp norR* while the Hmp-expressing cells were not affected (Fig. 5.3B).

To corroborate the results above, an *hmp norW* mutant strain was constructed. The double mutant was transformed with pMT1, pPL341 and the empty vector (pBAD/HisA) and the growth profile in the presence of 1 mM DETA NONOate tested. In agreement with the result showed in Fig. 5.3B, the growth of the Cgb-expressing *hmp norW* was slightly inhibited compared to the control (Fig. 5.4). However, comparison of the periods needed for the reduction of Cgb haem in extracts of *E. coli hmp* (Chapter 4, Fig. 4.2) and *E. coli hmp norR* (Fig. 5.5) followed spectrophotometrically after oxidation with NO did not show differences. Thus, the data above indicate a minor role for NorW as the Cgb electron donor in *E. coli*.

5.2.3 The ability of Cgb to detoxify NO in *C. jejuni* is independent of the lactate dehydrogenase (Cj1585c).

In order to investigate whether Cj1585c has a function as the cognate reductase of Cgb in *C. jejuni*, the effect of the deletion of the *cj1585c* gene in the resistance of the microorganism to nitrosative stress was tested by following the growth under microaerobic conditions. Addition of 500 μM DETA NONOate (Fig. 5.6A) or 400 μM GSNO (Fig. 5.6B) failed to produce inhibition of the *cj1585c* mutant or the isogenic strain. As expected, the *cgb* mutant was severely inhibited in both conditions. This result strongly suggests that Cj1585c is not involved in the nitrosative stress resistance mechanism mediated by Cgb.

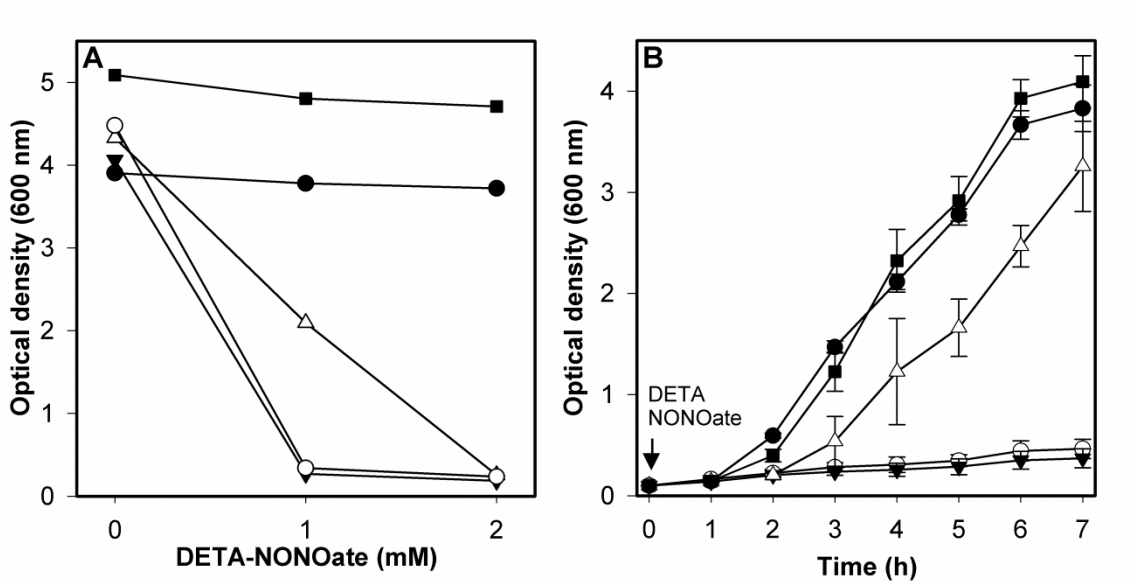


Figure 5.3 Susceptibility tests and growth curves of an *E. coli hmp norR* mutant expressing Cgb under nitrosative stress conditions.

Aerobic cultures of the *hmp norR* mutant expressing Hmp (pPL341) (●) or Cgb (pMT1) (Δ) were grown in LB supplemented with arabinose. The indicated concentrations of DETA NONOate were added and cultures incubated for 24 h at 37 ºC, 240rpm; OD was recorded at 600 nm (**A**). In (**B**), 1 mM DETA-NONOate was added (arrow) and the OD recorded every hour. *E. coli norR* (■), *hmp norR* carrying the empty vector (○) and pMT1 in the absence of arabinose (▼) were included as controls. Bars represent the standard deviation of three independent experiments. Taken from Tinajero-Trejo *et al*. (2013).

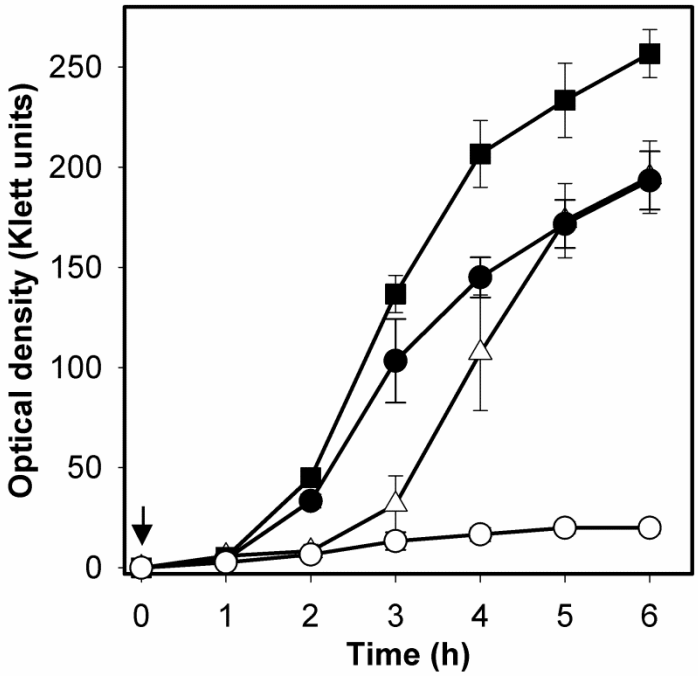


Figure 5.4 Growth curve of an *E. coli hmp norW* mutant strain expressing Cgb under nitrosative stress conditions.

Aerobic cultures of the *hmp norW* mutant carrying Hmp (pPL341) (●) or Cgb (pMT1) (Δ) were grown in LB supplemented with arabinose. DETA NONOate (1 mM) was added (arrow) and cultures incubated at 37 ºC, 240rpm; OD was recorded every hour. *E. coli norW* (■) and *hmp norW* carrying the empty vector (○) were included as controls. Bars represent the standard error of three independent experiments. Taken from Tinajero-Trejo *et al*. (2013).

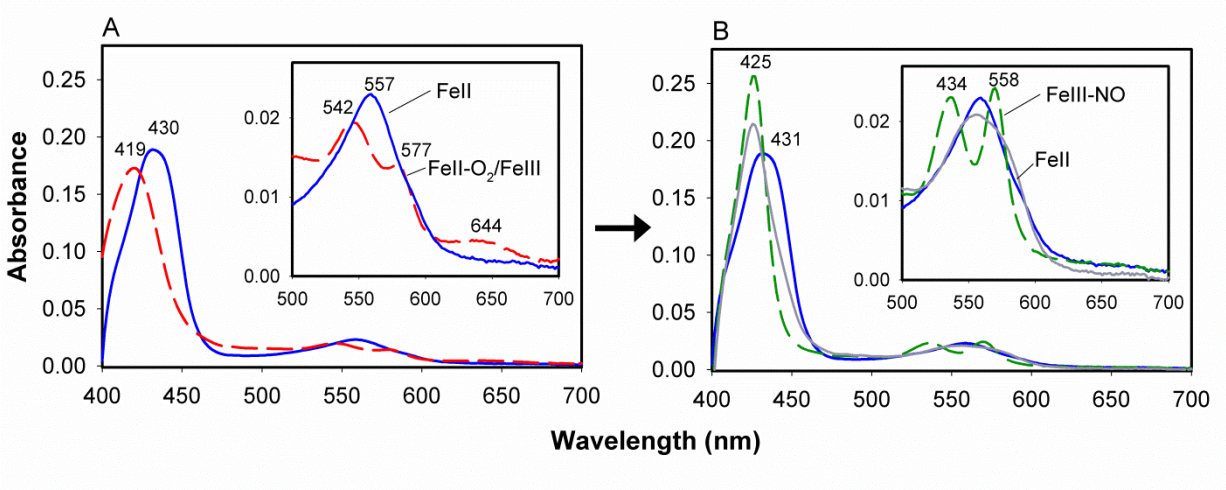


Figure 5.5 Redox state of the Cgb haem cofactor in *E. coli hmp norR* extracts and turnover after oxidation by NO.

Purified Cgb (5 µM) was mixed with soluble extracts of *E. coli hmp norR* mutant in 50 mM Tris-HCl buffer (pH 7.4), containing NADH (10 mM) at 37 °C. Arrow (**A** to **B**) indicates subsequent steps performed with the same sample. (**A**) Absorption spectra were recorded immediately after addition of the protein (red dashed lines) and after 20 min incubation (blue solid line). (**B**) PROLI NONOate (10 µM) was added to reduced Cgb (blue solid line) and changes in the absorption were recorded immediately (green dashed line) and after 10 min incubation (gray solid line).

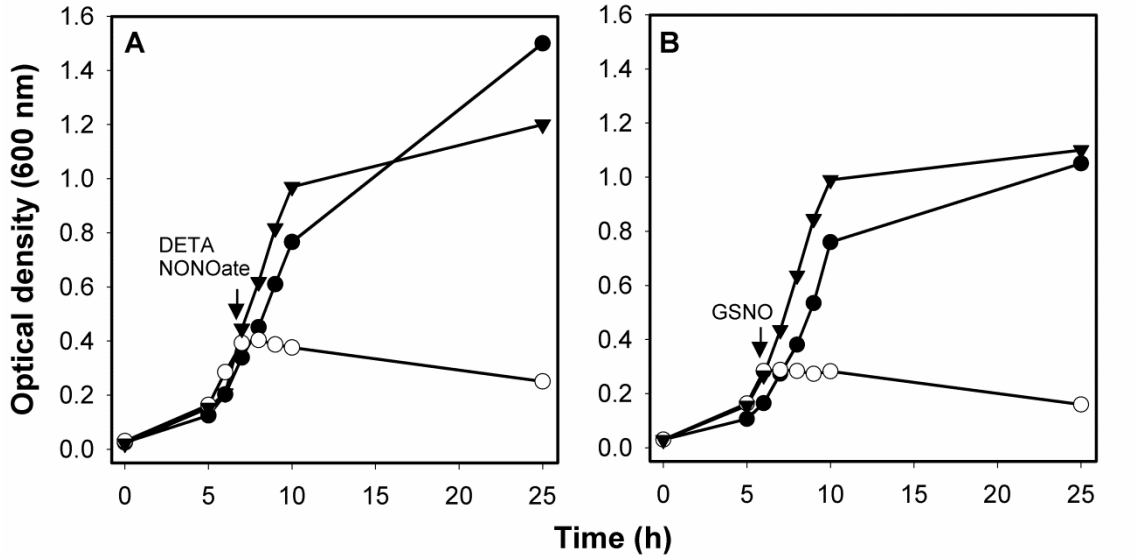


Figure 5.6 Growth curves of a *C. jejuni cj1585c* mutant strain under nitrosative stress conditions.

Microaerobic cultures of the *cj1585c* mutant (▼) in MH broth weres added (arrow) with (**A**) 500 µM DETA NONOate or (**B**) 400 µM GSNO. Cultures were incubated at 42 ºC, 240rpm; OD was recorded every hour. *C. jejuni* wild type (●) or *cgb* mutant (○) were included as controls.

5.3 Discussion

Heterologous expression of globins as an alternative method by which to explore functional activities when genetic manipulation in the native host is not suitable represents a valuable tool. However, differences between the heterologous and the actual host should be taken into consideration before making assumptions about globin function(s). This is especially significant for the cases of SDgbs and TrHbs, where reduction of the haem cofactor does not depend on an intra-protein reaction, as is the case of the FHbs where a reductase domain fused to the haem domain provides the electrons for the reduction (Hernandez-Urzua *et al.*, 2003). Consequently, the non-flavo globins putatively require interaction with an associated electron donor. It is possible that the reducing power comes from a reductase that is present in the heterologous host but not in the native organism. Indeed, Vgb has been expressed in numerous prokaryotic and eukaryotic organisms (reviewed by Frey *et al*., 2011 and Stark *et al*., 2011) and a range of functions have been attributed to these (e.g. NO and RNS detoxification). However, there are no data supporting such functions in *Vitreoscilla* itself.

On the other hand, the evidence of the nitrosative stress sensitivity caused by the deletion of the *cgb* gene in *C. jejuni* (Avila-Ramirez *et al.*, 2013; Elvers *et al.*, 2004), together with the ability of Cgb to detoxify NO and RNS in *E. coli* (Chapter 3), indicate that the single-domain globin plays a crucial role in the defence against nitrosative damage in the native host. This also implies the presence of efficient reductase partners for the globin in both the native and the heterologous host.

A recent study has shown that the respiration of an *E. coli* strain containing only ubiquinone has a decreased rate (67%) compared to the wild type, while a strain producing demethylmenaquinone exclusively loses 92% of its respiratory capacity (Sharma *et al.*, 2012). This indicates a minor, yet significant role for demethylmenaquinone under aerobic conditions (Sharma *et al.*, 2012; Soballe and Poole, 1998). In agreement, in the present study, the deletion of the *ubiCA* operon in the genome of the *hmp* mutant strain produced an 87% decrease of the respiration rate compared to the *hmp* mutant (*ubiCA*$^{+}$) measured in membrane fractions (Table 5.1). The full recovery of the rate after addition of coenzyme Q1 (a ubiquinone homologue) suggests that disruption of the electron flux from ubiquinone to the terminal oxidases is

the cause of the O_2 uptake impairment and agrees with the inability of the *hmp ubi* mutant cells to grow in succinate (not shown).

Leakage of electrons from the respiratory chain, forming superoxide, occurs mainly via menaquinone (Korshunov and Imlay, 2006). However, the aim of this work was to explore whether electrons from the aerobic respiratory flux were transferred to Cgb. Since menaquinone is only a minor species in aerobic respiration (Sharma *et al.*, 2012), it was not expected to play an important role in the growth of the aerobic cultures of this study. Moreover, cytochrome *bo'* terminal oxidase oxidizes predominantly ubiquinone (Kita *et al.*, 1986) and Vgb associates with this complex in *E. coli.* Thus, *ubi* mutant (not an *ubi men* mutant) appeared to be suitable for the objective**;** electron flux from the membrane-bound respiratory chain could supply reducing power if globins were to associate with the membrane. A similar capability of Cgb-expressing *hmp ubiCA* and *hmp* mutant cells to grow in the presence of NO (compare Fig. 3.3C and 5.1B), and to consume NO (compare Fig. 3.6C and 5.2), strongly suggests that the source(s) of electrons for the reduction of the Cgb haem is different from the flux of the respiratory chain.

On the other hand, the flavorubredoxin reductase NorW appears to play a minor, yet effective role in the *in vivo* NO detoxification performed by Cgb in *E. coli* (Fig. 5.3 and 5.4), which might have important implications. For instance, the use of purified NorW as the reductase partner of Cgb (and perhaps Ctb), as been used for neuroglobin (Giuffre *et al.*, 2008), would allow *in vitro* tests. Indeed, exploration of the molecular mechanism(s) involved in the NO detoxification (e.g. confirmation of the suggested NOD and NO reductase activities) is hampered by the absence of an electron donor.

It has been previously reported that the up-regulation of Cj1585 under conditions of nitrosative stress is restricted to very low O_2 concentrations (oxygen transfer constant, 0.06 min^{-1}) while the induction of Cgb occurs in microaerobiosis (0.43 min^{-1}) (Avila-Ramirez *et al.*, 2013). Herein, it is shown that deletion of the *cj1585c* gene failed to produce negative effects in the growth of *C. jejuni* in the presence of NO and GSNO whereas these agents completely inhibited the growth of a *cgb* mutant. Thus, it is concluded that the lactate dehydrogenase does not have a function as the cognate reductase of Cgb in the native host.

Attempts to uncover the source of electrons associated to the reconversion of the haem group of the *C. jejuni* globins, either in the heterologous host *E. coli* or in the actual host, have been unfruitful. However, additional strategies may be considered. The most sensible approach to identify specific or unspecific reductase partners for Cgb should come from a study in the native host. Thus, random mutagenesis in *C. jejuni* (Grant *et al.*, 2005) followed by screening for NO sensitivity, might render mutants affected in genes different from *cgb*. Mapping of the insertion sites could provide candidates for the reductase(s) of Cgb. Furthermore, identification of homologues of the *C. jejuni* cognate reductase(s) in *E. coli* would be possible by bioinformatics-based searching in order to identify heterologous reductases. Once the reductase(s) is/are identified, purification and *in vitro* assays of the interaction between Cgb and the reductase(s) might provide valuable information about the molecular mechanism involved in the NO and RNS detoxification. Failure to isolate NO-sensitive mutants or the finding that these mutants are *cgb*, *nrf* or *nssR*, might suggest the absence of a specific globin reductase. Besides, the isolation of potential reductases from *C. jejuni and E. coli* cell extracts using column-immobilised Cgb as 'bait' has been previously considered. However, attempts in our laboratory to do so have not been successful (J. L. Pickford and R. K. Poole, unpublished).

5.4 Conclusions

The demonstration of the ability of the single-domain globin Cgb to support the growth and consume NO in *E. coli* lacking ubiquinone indicates that the reduction of the globin haem is independent of the electron flux from the respiratory chain. The flavorubredoxin reductase NorW from *E. coli* plays a minor role as the reductase partner of Cgb *in vivo* while the NO and GSNO detoxification mediated by Cgb in *C. jejuni* is independent of the lactate dehydrogenase Cj1585c.

Chapter 6. NO evolution from *E. coli* expressing Cgb and Ctb and a preliminary study of Ctb as a nitrite reductase.

6.1 Introduction

Denitrifying bacteria generate NO endogenously as an obligate intermediate in the reduction of nitrate (NO_3^-) to N_2 (Watmough *et al.*, 1999; Zumft, 1997). NO is also produced by non-denitrifying bacteria including a number of enterobacteria (Corker and Poole, 2003; Ji and Hollocher, 1988). Indeed, nitrite and nitrate metabolism in *Salmonella* and *E. coli* produces NO as a side-product (Corker and Poole, 2003; Gilberthorpe and Poole, 2008; Spiro, 2006). The nitrate reductase NarG has been identified as the major, but not the sole, source of NO by the reduction to nitrite (Gilberthorpe and Poole, 2008; Vine *et al.*, 2011), leading to the expression of NO detoxification systems via the NO-sensing transcriptional factor NsrR (Filenko *et al.*, 2007a; Gilberthorpe *et al.*, 2007). Reactive nitrogen species (e.g. NO and N_2O) are produced during growth with electron donors such as nitrite and nitrate (Corker and Poole, 2003; Weiss, 2006) eliciting, as a consequence, the expression of Hmp in *E. coli* (Poole *et al.*, 1996).

It has been shown that expression of Cgb is induced by both nitrite and nitrate (Pittman *et al.*, 2007); thus, an analogous mechanism to the one described in *E. coli* for the transcriptional activation of the globin is proposed in *Campylobacter*; however, there is no direct evidence of the production of NO or its reactive derivatives in this bacterium. In *C. jejuni* lacking the nitrate reductase NapA, Cgb expression occurs in the presence of nitrite but not nitrate, suggesting that the source of nitrosative stress is the reduction of nitrite (involving perhaps NO production). Since the levels of Cgb expression by nitrite and nitrate in the wild type and a *nrfA* mutant strain are comparable, the role of NrfA as the generator of nitrosative stress (NO production from nitrite) in *C. jejuni* seems unlikely (Pittman *et al.*, 2007). Thus, the elucidation of the putative source(s) of NO during nitrite reduction in this microorganism requires study.

Production of NO by eukaryotic globins such as haemoglobin and myoglobin has been documented (Minneci *et al.*, 2008). A dual function has been proposed for Mb: under normoxia, the globin functions as a NO scavenger, whereas decreasing oxygen

concentrations in myocardial tissue promotes generation of NO from nitrite, leading to down-regulation of cardiac energetics and function (Rassaf *et al.*, 2007). Indeed, NO produced from the reduction of nitrite by deoxy-Mb inhibits mitochondrial respiration (Shiva *et al.*, 2007). Besides, the function of the human neuroglobin and PhHbO, the TrHb2 from *P. haloplanktis*, as nitrite reductases has been recently demonstrated *in vitro* (Li *et al.*, 2012; Russo *et al.*, 2013; Tiso *et al.*, 2011). However, whether or not this putative function has physiological implications remains unknown.

The *ctb* gene is up-regulated in response to nitrosative stress via NssR but it appears not to play a direct role in protection against NO toxicity resistance. Thus, an interesting idea is proposed: Ctb functions as a nitrite reductase producing NO, which in turn is converted to nitrate by Cgb. However, this is a hypothetical mechanism, and must be experimentally verified. Thus, the objective of the present chapter is to produce preliminary data as a base to explore the function of the truncated globin Ctb as a nitrite reductase *in vivo* and *in vitro*.

6.2 Results

6.2.1 Anaerobic NO evolution by *E. coli hmp* mutant cells is increased by the expression of Ctb and decreased by the expression of Cgb.

The ability of the *C. jejuni* globins to uptake NO in anaerobic conditions has been shown by polarographic measurements (Chapter 3, Fig. 3.6). However, expression of either Cgb or Ctb failed to support the anaerobic growth of *E. coli* under nitrosative stress conditions. Generation of NO upon addition of nitrite to *E. coli* wild type cells cultured anoxically with nitrate as terminal electron acceptor has been reported (Corker and Poole, 2003). Thus, the use of such a feature to test whether the expression of the *C. jejuni* globins were able to modify the levels of the endogenously-produced NO by the heterologous host (i.e. decrease it by consumption or increase it by production) was suggested. However, the inability of an *hmp* mutant to produce NO reported in the same study represented an important limitation in view of the fact that the presence of the flavohaemoglobin in the system was undesirable for the purposes of the experiment. Surprisingly, when anaerobic NO evolution was tested polarographically in cell suspensions of *E. coli* wild type and the *hmp* mutant, following the protocol described by Corker and Poole, but from cultures grown in aerobic standard conditions, significant NO production was recorded not only from the wild type but from the *hmp* mutant sample upon addition of nitrite (see below). This finding provided a suitable model for the determination of the anaerobic consumption of intracellularly-produced NO by Cgb and Ctb *in vivo*.

Respiration of Cgb- and Ctb-expressing *E. coli hmp* mutant cells was promoted by the addition of glycerol (15 mM) and O_2 consumption was recorded polarographically in a closed O_2 electrode chamber fitted with an NO electrode. Once the O_2 was depleted (the O_2 trace reached zero), $NaNO_2$ (25 mM) was injected into the chamber and NO accumulation followed until the trace showed a plateau. Finally, an excess of the NO scavenger carboxi-PTIO (50 μM) confirmed the presence of NO (Fig. 6.1). Unlike the *hmp* mutant cells transformed with the empty vector, NO accumulation by cells complemented with pPL341 was very poor (compare Fig. 6.1A and B), presumably due

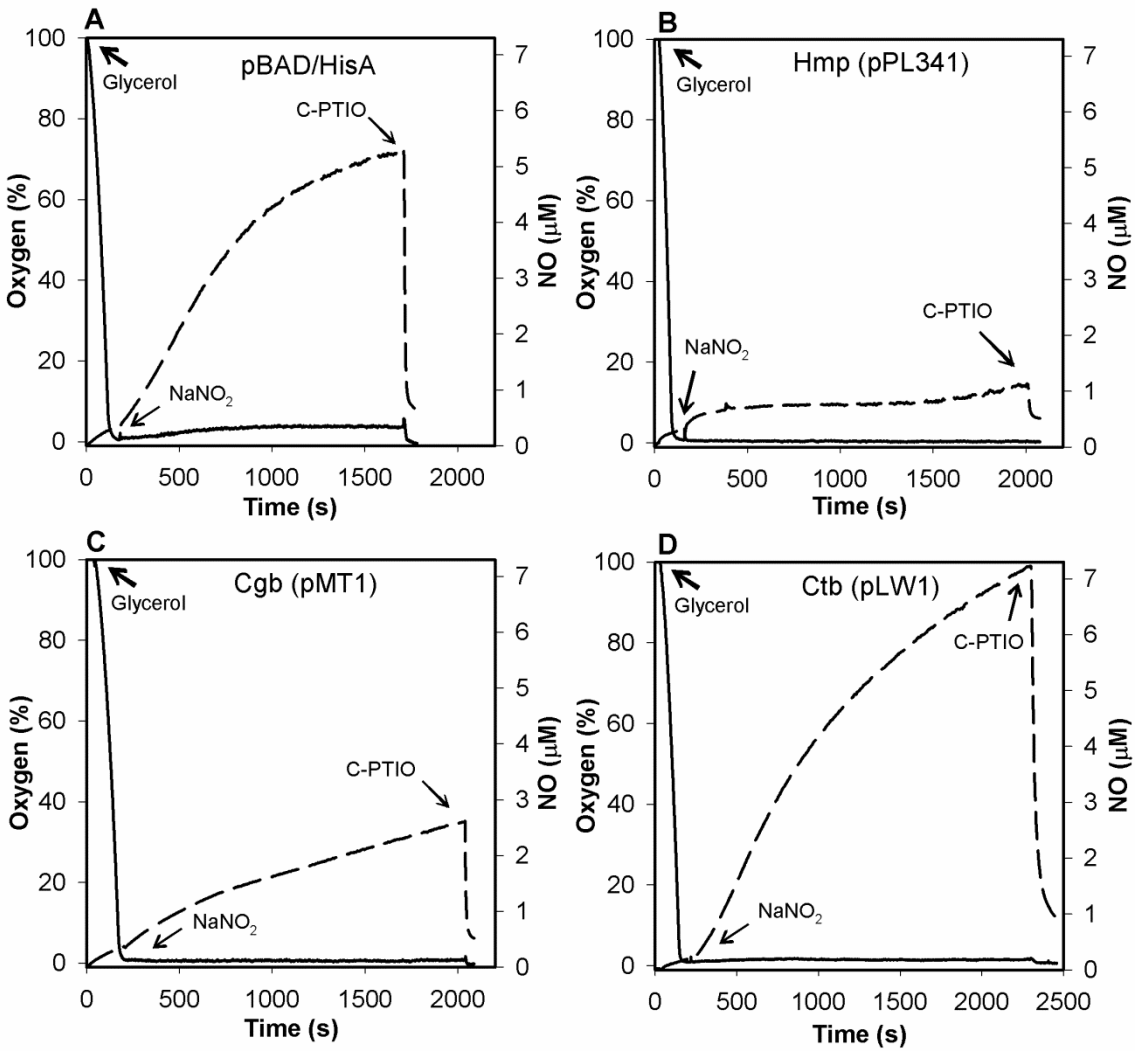


Figure 6.1 Endogenous production of NO by *E. coli* expressing Cgb or Ctb.

Cultures of an *E. coli hmp* mutant transformed with the empty vector (pBAD/HisA) (**A**) or expressing Hmp (pPL341) (**B**), Cgb (pMT1) (**C**) or Ctb (pLW1) (**D**) were grown overnight in LB supplemented with arabinose at 37 ºC, 240 rpm. Cells were resuspended in 50 mM Tris-HCl buffer (pH 7.4) and O_2 depleted by cellular respiration upon addition of glycerol (15 mM). Sodium nitrite (25 mM) was added after the oxygen trace reached zero (solid line) and the NO production (dashed line) was recorded. NO and O_2 levels were followed polarographically. The NO-reactive compound carboxy-PTIO (C-PTIO) was added at 50 µM final concentration. Similar results were obtained in at least five independent experiments performed in duplicate.

to the NO consumption and detoxification by the NO-reductase activity of Hmp (Mills *et al.*, 2001). In the same way, cells over-expressing Cgb showed lower levels of NO than the control carrying the empty vector (compare Fig. 6.1C with 6.1A), supporting the ability of this globin to reduce NO in the absence of O_2. On the other hand, cells expressing Ctb did not prevent the accumulation of NO (Fig. 6.1D).

NO production rates of cell samples expressing the *C. jejuni* globins or carrying the empty vector were calculated from the linear phase of the NO traces in Figure 6.1 and normalized by the total cellular protein content. A significantly lower rate for the Cgb-expressing cells (~5-fold) compared to the cells carrying the empty vector was found. However, cells expressing the truncated haemoglobin Ctb produced NO ~1.5-fold faster than the control carrying the empty vector (Fig. 6.2) and ~1.3-fold faster than the wild type (MG1655) even though in the last case, the difference was not statistically significant (Fig. 6.2). These findings suggested that Ctb might be able to reduce nitrite in anaerobic conditions, resulting in a higher accumulation of the toxic compound NO.

6.2.2 Ctb reacts with nitrite and produces NO in the presence of sodium dithionite.

Salhany (2008) studied the wavelength-dependent kinetics of nitrosyl haemoglobin formation from the reaction of the globin with nitrite in the presence of the O_2 scavenger sodium dithionite. Since variations in the concentration of dithionite did not modify the reaction rate, it was concluded that dithionite fails to react with nitrite to produce NO. Thus, changes in the absorbance of dithionite-reduced globins (FeII) towards the NO-FeII form in the presence of nitrite can be associated with nitrite reduction activity: NO production implies the oxidation of the globin haem (FeIII), which in turn is re-reduced by the excess of dithionite. Finally, the newly reduced globin (FeII) binds the NO produced being FeII-NO the sole final product (Helbo *et al.*, 2012; Russo *et al.*, 2013; Salhany, 2008; Tiso *et al.*, 2012) as follows:

$$FeII + NO_2^- + H^+ \longrightarrow FeIII + NO + OH^- \qquad \text{(Eq. 3)}$$

$$NO + FeII \longrightarrow FeII\text{-}NO \qquad \text{(Eq. 4)}$$

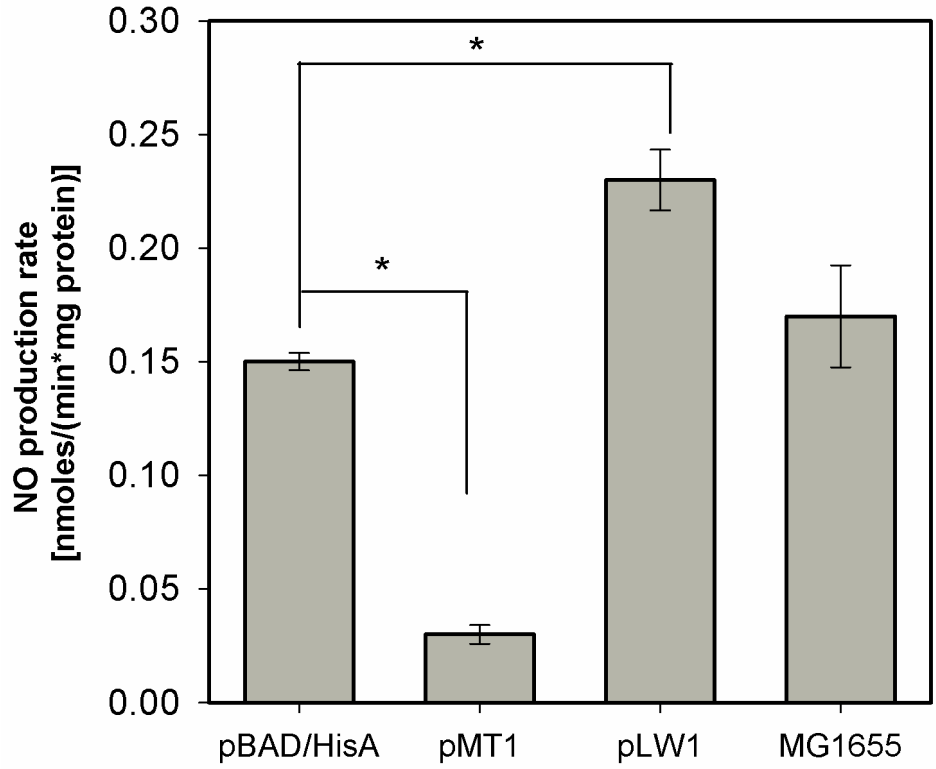


Figure 6.2 NO production rates of *E. coli* expressing Cgb or Ctb.

Anaerobic NO production rates from nitrite by *E. coli hmp* mutant cells expressing Cgb (pMT1), Ctb (pLW1) or carrying the empty vector (pBAD/HisA) were calculated from the linear phase of the NO electrode traces from the experiments described in Fig. 6.1. Rates from the wild type (MG1655) were calculated as a control. Bars represent ± the standard error of at least five independent experiments performed in duplicate.

*$P < 0.001$.

In order to further investigate whether Ctb was able to produce NO from nitrite, purified globin (3.7 μM) in degassed buffer (50 mM Tris-HCl 50 mM, pH 7.4) was added with a range of sodium dithionite concentrations (50 μM-10 mM) and the reduction was followed spectroscopically. Concentrations below 10 mM failed to reduce efficiently the haem globin (not shown) while 10 mM dithionite produced a stable ferrous spectrum (FeII 432, 566 nm) (for reference, see Table 4.1) after 20 min incubation at room temperature (Fig. 6.3A). The need for such a high concentration of dithionite to reduce Ctb is in agreement with the exceptionally high O_2 affinity reported for this globin (Lu *et al.*, 2007a). Addition of 0.5 mM sodium nitrite but not sodium nitrate to dithionite-reduced Ctb samples produced a FeII-NO-like spectrum (423, 543, 566 nm) after 20 min incubation at room temperature (Fig. 6.3B), while addition of 0.5 mM nitrate did not produce spectroscopic changes (Fig. 6.3C). As a control, 10 μM PROLI NONOate was added to a dithionite-reduced Ctb sample; an immediate shift to the FeII-NO spectrum was observed (Fig. 6.3D). Since the resulting spectra after addition of NONOate and nitrite were similar (compare Fig. 3B and D), NO production by Ctb from nitrite appears to be occurring.

When spectroscopic changes after addition of nitrite (0.5 mM) to dithionite-reduced Ctb (10 μM) were followed over time (Fig. 6.4A), an isosbestic point was clearly observed at 426 nm. Values from the difference in absorbance (434 nm minus 426 nm and 420 minus 426 nm) were graphed showing apparent first order kinetics (Fig. 6.4B). However, the spectrum of the dithionite-reduced globin before the addition of nitrite failed to cross the isosbestic point, suggesting the presence of a third species in the reaction mixture (FeII-O_2 perhaps).

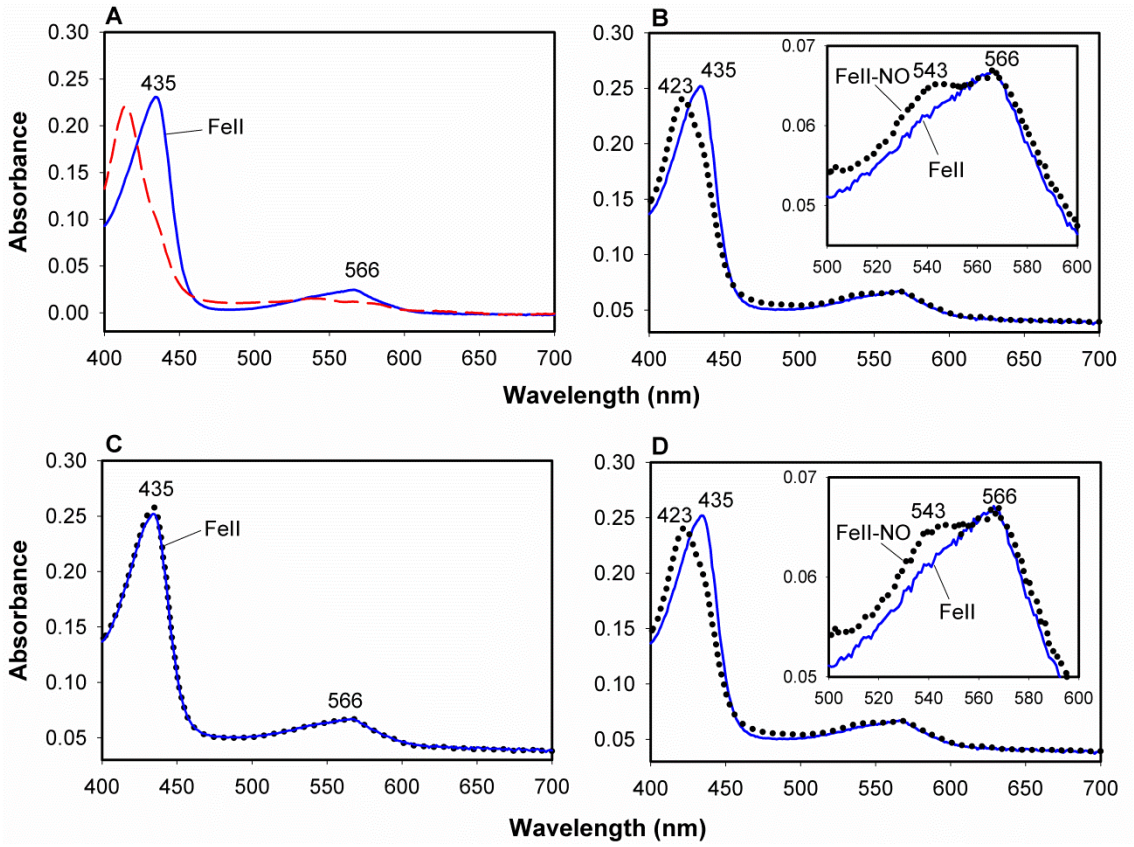


Figure 6.3 Spectroscopic changes of dithionite-reduced Ctb in the presence of nitrite.

Native Ctb (3.7 µM) in degassed buffer (Tris-HCl 50 mM, pH 7.4) was reduced by addition of 10 mM sodium dithionite (**A**, dashed and solid lines respectively). Dithionite-reduced Ctb (solid lines) was supplemented with 0.5 mM sodium nitrite (**B**) or sodium nitrate (**C**) and changes in the spectra were recorded after 20 min incubation at room temperature (dotted lines). Spectroscopic changes of dithionite-reduced Ctb (solid line) upon addition of 22 µM PROLI-NONOate (dotted line) were recorded as a control (**D**). Absolute spectra were recorded against a buffer baseline.

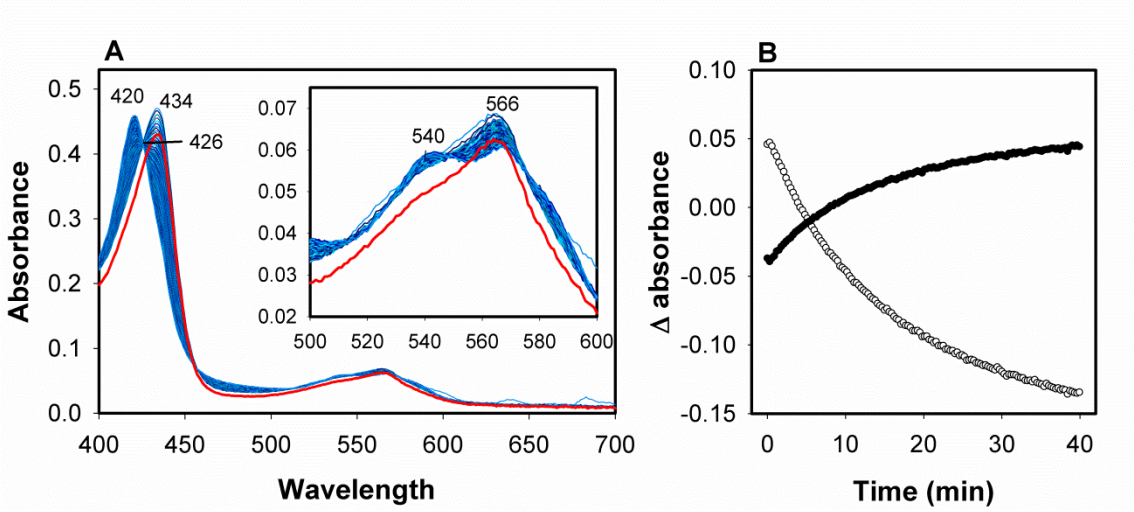


Figure 6.4 Kinetics of the NO-reduced Ctb formation from nitrite in presence of sodium dithionite.

(**A**) Native Ctb (10 µM) in degassed buffer (Tris-HCl 50 mM, pH 7.4) was reduced by addition of 10 mM sodium dithionite (red line). Changes in absorbance after addition of 0.5 mM nitrite were recorded every 0.3 min for 40 min at room temperature (blue lines). (**B**) Difference in absorbance at 434 nm minus 426 nm (open circles) and 420 nm minus 426 nm (closed circles) were plotted.

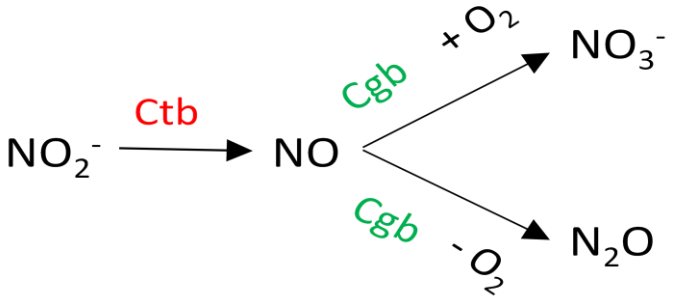
6.3 Discussion

Production of NO by prokaryotic organisms associated with the presence of NOS has been reported. Certainly, bNOS are encoded in many genomes of Gram-positive species (Gusarov *et al.*, 2009). However, the physiological significance of NO bacterial production, a bactericidal compound, is far from being well understood. It has been suggested that NO offers protection against oxidative stress. Thus, endogenous production of NO defends bacteria against the oxidative burst produced by macrophages during infection (Gusarov and Nudler, 2005; Shatalin *et al.*, 2008). Furthermore, Gusarov *et al*. (2009) showed that NO produced by the *Bacillus subtilis* bNOS protects the bacterium against a number of antibiotics. It was suggested that such protection may be a consequence of chemical modification of the antibiotic by NO (e.g. nitrosation of aromatic amino groups on acriflavine) or due to the reduction of the oxidative stress caused by antibiotics (Dwyer *et al.*, 2009; Kohanski *et al.*, 2007). However, new evidence against the production of ROS as the mechanism linked to antibiotic action question the latter hypothesis (Keren *et al.*, 2013; Liu and Imlay, 2013).

An interesting example of an interplay between NO consumption and production is the globin-containing organism *Giardia lamblia* (Rafferty et al., 2010), a flagellated protozoan parasite of the small intestine. A functional FHb is encoded by the genome of this parasite. The recombinant flavohaemoglobin metabolizes NO efficiently in the absence of O_2 (Mastronicola et al., 2010) and probably defends the parasite against NO toxicity. Interestingly, NOS activity and NO production by this organism, together with the presence of a *nos*-like sequence in the genome, have been reported (Harris *et al.*, 2006). However, the physiological meaning behind the NO production/consumption of *G. lamblia* has not been unravelled.

The *C. jejuni* genome does not contain *nos* genes. However, NO is produced during nitrite reduction in *Campylobacter* in a Nrf-independent manner (Pittman *et al.*, 2007). Herein, preliminary data *in vivo* (Fig. 6.2) and *in vitro* (Fig. 6.3) provide new evidence suggesting that Ctb is a nitrite reductase, in common with an increasing number of globins including myoglobin, neuroglobin and cytoglobin. Furthermore, the putative ability of the truncated globin to produce NO is in agreement with the reports showing that loss of viability associated with nitrosative stress and growth sensitivity to GSNO

in a *cgb* mutant are significantly reduced by the mutation of the *ctb* gene (Avila-Ramirez *et al.*, 2013; Wainwright *et al.*, 2005). Thus, the following sequence of reactions is suggested:



(Eq. 5)

Nitrite is produced in the oral cavity from bacterial reduction of salivary nitrate. Thus, nitrite in saliva reaches concentrations as high as 230 μM (Mirvish *et al.*, 2000) and constitutes the major source of gastric nitrite (Eisenbrand *et al.*, 1980). During infection, it is likely that *Campylobacter* encounters this toxic compound as the first source of nitrosative stress and, consequently, as an elicitor of defence responses against RNS, including NO, that are chemically produced in the acidic gastric environment. Indeed, it has been shown that Cgb is expressed in response to nitrite in *C. jejuni* (Pittman *et al.*, 2007). However, there is no evidence of nitrite detoxification by this globin and whether nitrite is able to directly interact with the transcription factor NssR is also unknown. An attractive hypothesis is that the truncated globin Ctb, constitutively expressed at low levels (Wainwright *et al.*, 2005), produces NO from nitrite at early stages of the infection, triggering the up-regulation of *cgb* (via NssR) that in turn protects the bacterium against NO and *S*-nitrosothiols produced in the digestive system as a part of the host defence response. However, the data presented herein are preliminary and require more experimental support.

Investigational approaches aimed to confirm the role of Ctb as a nitrite reductase might include the heterologous expression of the truncated globin in *E. coli* lacking not only the *hmp* and *nor* genes but also the main proteins implicated in NO production (e.g. *nir*, *narG*, *nrf*, etc.) (Prof. Jeff Cole, personal communication). In this way, endogenous anaerobic evolution of NO from nitrite may be attributed only to the presence of the heterologous globin. Furthermore, endogenous production of NO in *C. jejuni* cells

might be measurable polarographically; therefore, comparison among the isogenic strain and strains mutated in the globin genes could render useful data. For instance, NO evolution by cells after addition of nitrite should be higher in a *cgb* mutant than in the isogenic strain since the former lacks the NO detoxification provided by Cgb. If NO production was related to Ctb, NO accumulation in a Cgb-lacking strain should be prevented by the deletion of the truncated globin gene. Similarly, addition of high concentrations of nitrite should have an increased inhibitory effect on the respiration rate of the *cgb* mutant due to the putative production of NO by Ctb. However, the respiration rate should be less affected in cells lacking both Cgb and Ctb.

Pittman *et al.* (2007) showed that, under oxygen-limitation, nitrite concentrations above 2 mM are toxic to *C. jejuni*. However, cultures are able to grow with nitrate at concentrations as high as 20 mM. Since these authors showed that cultures of *C. jejuni* growing with nitrate produced stoichometric concentration of nitrite, it would be interesting to explore the effect of Cgb-lacking cultures growing in such conditions. Those cultures may be inhibited due to the inability of the strain to detoxify the produced NO. However, if the NO production is Ctb-dependent, cultures of a double mutant (*cgb ctb*) should be able to cope with the nitrosative stress conditions.

The high O_2 affinity reported for Ctb (Lu *et al.*, 2007a) makes it difficult to follow the kinetics of NO production from nitrite *in vitro*. Indeed, the amount of sodium dithionite needed to reduce the *C. jejuni* truncated globin exceeded the amount required for reduction of other globins in similar experiments (e.g. 10 mM for Ctb reduction (Fig. 6.4) and less than 100 μM for carp Mb (Helbo *et al.*, 2012)). Furthermore, even though the samples were contained in sealed cuvettes and bubbled with N_2 to keep them O_2-free, the spectra of the dithionite-reduced Ctb before and after addition of nitrite failed to show an isosbestic point, suggesting the presence of oxy-ferrous Ctb species contaminating the reaction mixture. Thus, preparation of the samples in an anaerobic glovebox would facilitate the determination of the reaction kinetics and kinetics values either in the presence or absence of dithionite.

Chapter 7. Ctb as a sink to test CO release from CO-releasing molecules (CO-RMs) *in vivo*.

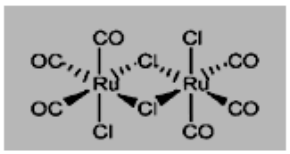
7.1 Introduction

CO is a poisonous odourless molecule able to avidly bind reduced (FeII) haemoglobin (Hb) resulting in the formation of carboxy-haemoglobin (CO-Hb). This binding hampers the formation of oxy-haemoglobin (O_2-Hb) and, consequently, inhibits respiration (Keilin, 1966). However, CO has important physiological functions in mammalian systems involving signalling and regulation. Indeed, CO is endogenously produced by inducible haem oxygenase-1 and constitutive haem oxygenase-II. These enzymes catalyse the rate-limiting step in the haem degradation pathway, producing biliverdin IXa (BV), CO and free iron (FeII). Endogenously produced CO is a gasotransmitter involved in the modulation of a number of key cellular functions acting as an anti-inflammatory, anti-apoptotic and cytoprotective molecule (Mann, 2010; Marks *et al.*, 1991; Motterlini and Otterbein, 2010; Ryter *et al.*, 2006). Interestingly not only animals but also plants and some pathogenic microorganisms produce CO via HO enzymes (Boczkowski *et al.*, 2006; Shekhawat and Verma, 2010).

CO-releasing molecules (CO-RMs) are mainly metal carbonyl compounds used for the delivery of CO to biological systems in controlled amounts (Motterlini *et al.*, 2002). The use of CO-RMs has allowed substantial advances in biological studies without the difficulties and risks associated with the use of CO gas in the laboratory. A variety of different CO-RMs are now available (e.g. with ruthenium, manganese, iron and boron centres), showing different rates, kinetics and conditions for CO release (Desmard *et al.*, 2012; Mann, 2010; Schatzschneider, 2011). For instance, the [$Ru(CO)_3Cl$ (glycinate)] compound (CORM-3) (Fig. 7.1) (Clark *et al.*, 2003) has been successfully exploited in models of vascular dysfunction, inflammation and ischemic injury (Alcaraz *et al.*, 2008; Motterlini *et al.*, 2005).

The ability of CO to bind transition metal compounds such as haem groups and iron-sulfur clusters (Boczkowski *et al.*, 2006; Roberts *et al.*, 2004) led to the suggestion that this compound might have antibacterial effects by targeting, for instance, terminal oxidases. Since Nobre *et al*. (2007) demonstrated the lethal effects of CO delivered via

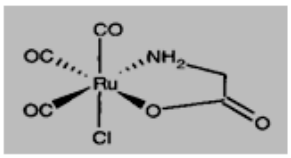
CORM-2



$[Ru(CO)_3Cl_2]_2$

Motterlini *et al.*, 2002

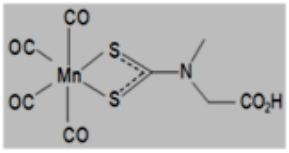
CORM-3



$Ru[CO]_3Cl(glycinate)$

Clark *et al.*, 2002

CORM-401



$Mn(CO)_4(S_2CNMeCH_2CO_2H)$

Crook *et al.*, 2011

Figure 7.1 Structure of CO-releasing molecules frequently used for biological studies.

CORM-2 is a DMSO soluble compound releasing 0.7 mol CO/mol CO-RM, CORM-3 is a water-soluble compound releasing 1 CO/mol CO-RM and CORM-401 is soluble in phosphate buffer saline releasing 1 CO spontaneously by a reversible, dissociative process.

organometallic CO-RMs against pathogenic bacteria, a number of papers studying the antibacterial properties and transcriptomic effects of CO-RMs have been produced (reviewed by Wilson *et al*., 2012). Indeed, CO-RMs, as antibacterial agents, may have great potential against bacterial infection (Motterlini and Otterbein, 2010).

The carboxy-myoglobin assay (CO-Mb) is a spectrophotometric test routinely used for the determination of CO release form CO-RMs (Davidge *et al.,* 2009b; Motterlini *et al.*, 2002). This assay measures the conversion of deoxy-Mb to CO-Mb by following changes in absorbance either in the α, β (Atkin *et al.*, 2011) or Soret regions of the visible spectrum (Davidge *et al.,* 2009b). However, the test requires the use of the O_2-scavenger sodium dithionite, which promotes the reduction (deoxygenation) of the globin. McLean *et al*. (2012) demonstrated that the release of CO from ruthenium-based and 401 CO-RMs is triggered by the presence of dithionite (and other sulfite compounds) promoting the formation of CO-Mb. These findings helped to clarify the previously described discrepancies among the CO release rates of CORM-2 and CORM-3 determined by the Mb assay and those determined by other methods such as polarographic measurements of CO (Desmard *et al.*, 2012).

In a study aimed at investigating inhibition of the NOD (or denitrosylase) activity of the *E. coli* flavohaemoglobin Hmp by CO, it was shown that CORM-3 inhibits the Hmp-based resistance to NO *in vivo* (Tinajero-Trejo *et al*., manuscript in preparation). However, CO gas but not CORM-3, inhibited the Hmp NOD activity *in vitro*, in agreement with the inability of this compound to release CO in the absence of dithionite. Spectroscopic measurements showed that CORM-3 produced CO-Hmp in a reaction where dithionite was used as a reductant but failed to do so when the flavohaemoglobin was reduced with NADH (a natural Hmp electron donor) (Anjum *et al.*, 1998). Interestingly, formation of CO-Hmp by CORM-3 was observed in soluble cellular extracts of *E. coli* but it was limited in membrane suspensions, arguing in favour of the presence of soluble compounds in the bacterial cytoplasm that promote CO release (e.g. sulfite-containing molecules).

CO release from CO-RMs to bacterial terminal oxidases in whole cells and membrane suspensions in dithionite-reduced samples has been demonstrated (Davidge *et al.,* 2009b; Jesse *et al.*, 2013). However, in order to determine the "natural" rates and

kinetics of CO release from these compounds in, for instance, living cells, dithionite-free approaches must be developed. Thus, it was suggested that intracellular Hmp might be exploited to investigate details of CO released from CORM-3 by following the formation of CO-Hmp in whole cells. However, it is well-known that over-expression of this flavohaemoglobin in *E. coli* produces oxidative stress by generation of superoxide via a one-electron reduction of O_2 in the absence of NO producing detrimental effects on growth (Membrillo-Hernandez *et al.*, 1996; Wu *et al.*, 2004). Therefore, an alternative globin must be sought.

High levels of intracellular globin are required to more easily observe spectral changes associated with CO binding when whole cells are used. In the present work, it was demonstrated that the transformation of *E. coli* with pLW1 renders up to 7 µM intracellular Ctb upon addition of arabinose without causing negative effects on growth (Chapter 2, Figs. 3.2 and 3.5). Therefore, Ctb appears to be a suitable candidate for intracellular CO-release experiments from CO-RMs in the absence of "artificial" compounds. Thus, the objective of the current chapter is to develop a Ctb-based technique aimed at allowing measurements of CO release rates and kinetics from CO-RMs to be determined *in vivo* in the absence of the reductant sodium dithionite.

7.2 Results

7.2.1 *E. coli* over-expressing Ctb is a suitable model to record spectroscopic changes of the globin *in vivo.*

E. coli hmp mutant cells transformed with pLW1 or carrying the empty vector were cultured with arabinose in aerobic conditions and cell suspensions prepared and standardized to the same OD at 600 nm. Spectroscopic measurements were carried out in a SDB-4 spectrophotometer at room temperature (Kalnenieks *et al.*, 1998). When the absolute spectra of the samples were obtained against a buffer baseline, a clear difference in absorbance was observed; a much stronger signal from the Ctb-expressing cells than from the control sample was obtained (Fig. 7.2A). Such a signal corresponded to the oxy-ferrous Ctb form (FeII-O_2) (417, 543, 579 nm) (see Table 4.1 for reference), in agreement with a previous report showing the spectrum of intracellular Ctb in *E. coli* (Wainwright *et al.*, 2005). Furthermore, when the absorbance values of cells carrying the empty vector, which correspond to the signal produced by the native haem proteins, were subtracted from the values of the Ctb-expressing sample, an even clearer spectrum was observed (Fig. 7.2B), indicating that the level of the globin signal was suitable for clearly recording intracellular spectroscopic changes.

7.2.2 Intracellular dithionite-reduced but not native Ctb binds CO from CO gas and CORM-3.

In order to test whether it was possible to observe the intracellular formation of the carboxy-Ctb compound (CO-Ctb) spectrometrically, Ctb-expressing cells were reduced by the addition of sodium dithionite followed by bubbling with CO gas. Difference spectra (CO-reduced minus reduced) clearly showed the expected peak and trough signal in the Soret region (Wood, 1984) that indicates the presence of the CO-Ctb redox form (Fig. 7.3, red solid line). Again, a similar but much weaker signal was observed from cells carrying the empty vector (Fig. 7.3, blue dashed line), indicating that the spectrum of the Ctb-containing sample was mostly due to the presence of the globin with only a small contribution from other haem-containing proteins.

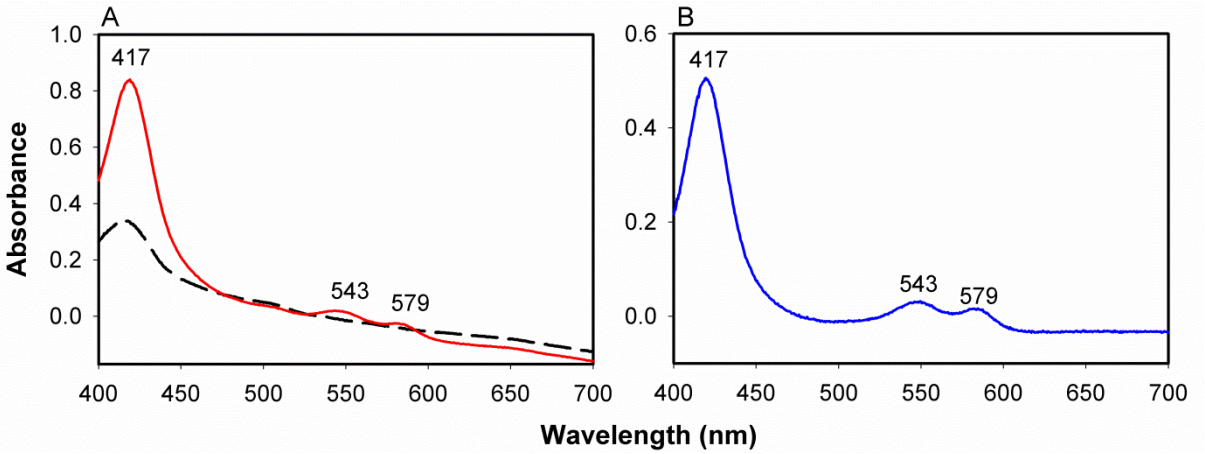


Figure 7.2 Absorbance spectra of intracellular Ctb.

Overnight cultures of an *E. coli hmp* mutant carrying pLW1 were grown in LB supplemented with arabinose. (**A**) Cells expressing Ctb (red solid line) or transformed with the empty vector (black dashed line) were resuspended in a small amount of Tris-HCl 50 mM (pH 7.5) and the absolute spectra measured against a buffer baseline. (**B**) Ctb spectrum obtained by using the absorbance values from the spectrum of cell suspensions carrying the empty vector as the baseline.

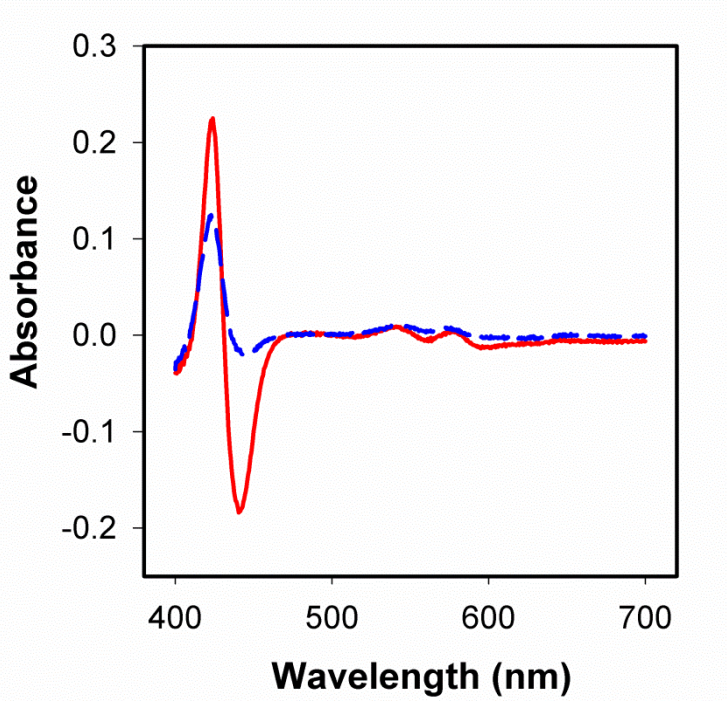


Figure 7.3 Intracellular formation of CO-bound Ctb in the presence of sodium dithionite and CO gas.

Native *E. coli* cell suspensions over-expressing Ctb (red solid line) or carrying the empty vector (blue dashed line) were reduced with a few grains of sodium dithionite followed by bubbling with CO gas for 2 min. The difference spectrum (CO-reduced minus reduced) was plotted.

Comparison of the difference spectra of Ctb-expressing cells in the presence and absence of dithionite clearly showed the need for full globin reduction as a condition for the formation of the CO-Ctb complex (Fig. 7.4). Indeed, bubbling of the sample with CO gas rendered a very small difference signal in the absence of dithionite (Fig. 7.4A, blue-dashed line) compared to that obtained in the presence of the O_2 scavenger (Fig. 7.4A, red solid line). Moreover, addition of CORM-3 (300 µM) elicited the formation of CO-Ctb in cells reduced with the artificial reductant, producing a strong and defined signal (Fig. 7.4B, red solid line), comparable to that obtained with CO gas; however, no signal was observed in the absence of dithionite (Fig. 7.4B, blue dashed line).

7.2.3 Reduction of intracellular Ctb can be achieved by depletion of O_2 via cellular respiration.

The results presented in the previous section strongly suggest that Ctb might be useful as a sink to measure intracellular CO-release from CO-RMs; however, reduction of the globin prior to the addition of the CO-releasing compound was imperative and the use of dithionite, explained before, unsuitable.

In an attempt to produce the de-oxygenation of the intracellular FeII-O_2-Ctb pool in a dithionite-free way, glucose (13.8 mM) was added to globin-expressing cells to promote respiration as a means of scavenging the O_2 from the sample. Consumption of O_2 was followed polarographically in a closed O_2 electrode chamber with constant stirring. Less than 2 min after addition of glucose the electrode trace reached zero, indicating the depletion of O_2 in the chamber (not shown). However, since during the spectroscopic experiments the samples are not maintained in anaerobic conditions, it was important to determine whether, upon exposure to air, cellular respiration was sufficient to maintain an anoxic environment. Thus, after O_2 depletion, the lid of the chamber was removed, allowing air diffusion into the stirred sample and the O_2 levels recorded for a further 1 hour. Re-oxygenation of the chamber occurred after 45 min incubation (not shown), indicating a sustainable O_2 consumption via only respiratory metabolism.

To test whether or not the anoxic conditions in the sample elicited by cellular respiration were sufficient for de-oxygenation of intracellular globin, glucose was added to cell suspensions of the Ctb-expressing cells and that carrying the empty vector to promote

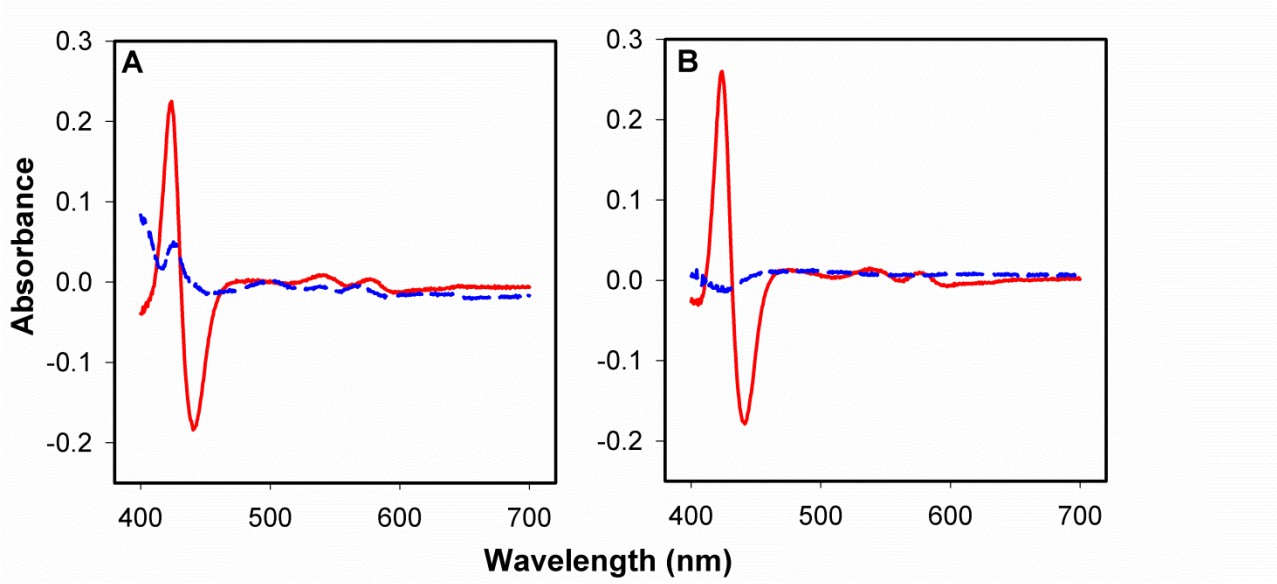


Figure 7.4 Intracellular formation of CO-bound Ctb in the presence of sodium dithionite and CORM-3.

Ctb expressing cells were reduced with sodium dithionite and then bubbled with CO gas for two min (**A**) or added with 300 µM CORM-3 (**B**) (red solid lines). The difference spectra (reduced-CO minus reduced) were plotted against the difference spectra of Ctb expressing cells bubbled with CO gas (**A**) or added with 300 µM CORM-3 (**B**) in the absence of dithionite (CO-oxy-ferrous minus oxy-ferrous) (blue dashed line).

respiration, and the samples were incubated for 5-10 min at room temperature. The absolute spectrum of Ctb was obtained by subtracting the absorbance values of the glucose-reduced cells carrying the empty vector from the values of the glucose-reduced Ctb-expressing sample. In this way, a characteristic ferrous (FeII) Ctb form was observed with an absorption maximum in the Soret region at 431 nm and a single broad band in the α, β region (~560 nm) (Fig. 7.5A). Moreover, when the glucose-reduced sample was bubbled with CO gas for 2 min, and the absolute spectrum obtained by using CO-glucose-reduced cells carrying the empty vector as a baseline, a clear Ctb CO-reduced spectrum was observed (422, 537, 567 nm) (Fig. 7.5B) (see Table 4.1 for reference). Finally, the difference spectrum of Ctb-expressing cells (CO-glucose-reduced minus glucose-reduced) produced a signal with intensity comparable to that obtained in the samples containing dithionite (compare Fig. 7.5C with Fig. 7.3 red solid lines). These results indicated that removal of O_2 by cellular respiration could be successfully exploited to obtain CO-Ctb in whole cells in the absence of the artificial reductant dithionite.

7.2.4 A preliminary experiment suggests that intracellular Ctb binds CO from CORM-3 in glucose-reduced samples with defined kinetics.

In order to test the CO release from CORM-3 in the dithionite-free system, *E. coli* cells over-expressing Ctb were reduced with glucose, as described in the previous section, and CORM-3 (300 μM) was added. The time-course of CO release from CORM-3 was followed looking at the difference spectra (CO-glucose-reduced minus glucose-reduced) and plotted together with the difference spectrum of a glucose-reduced sample bubbled with CO gas for 2 min (Fig. 7.6A, blue solid lines and red dashed line respectively). When the difference in the absorption maxima (peak minus trough) was plotted for every time point, a first order kinetic plot was observed with a half-life of ~2 min.

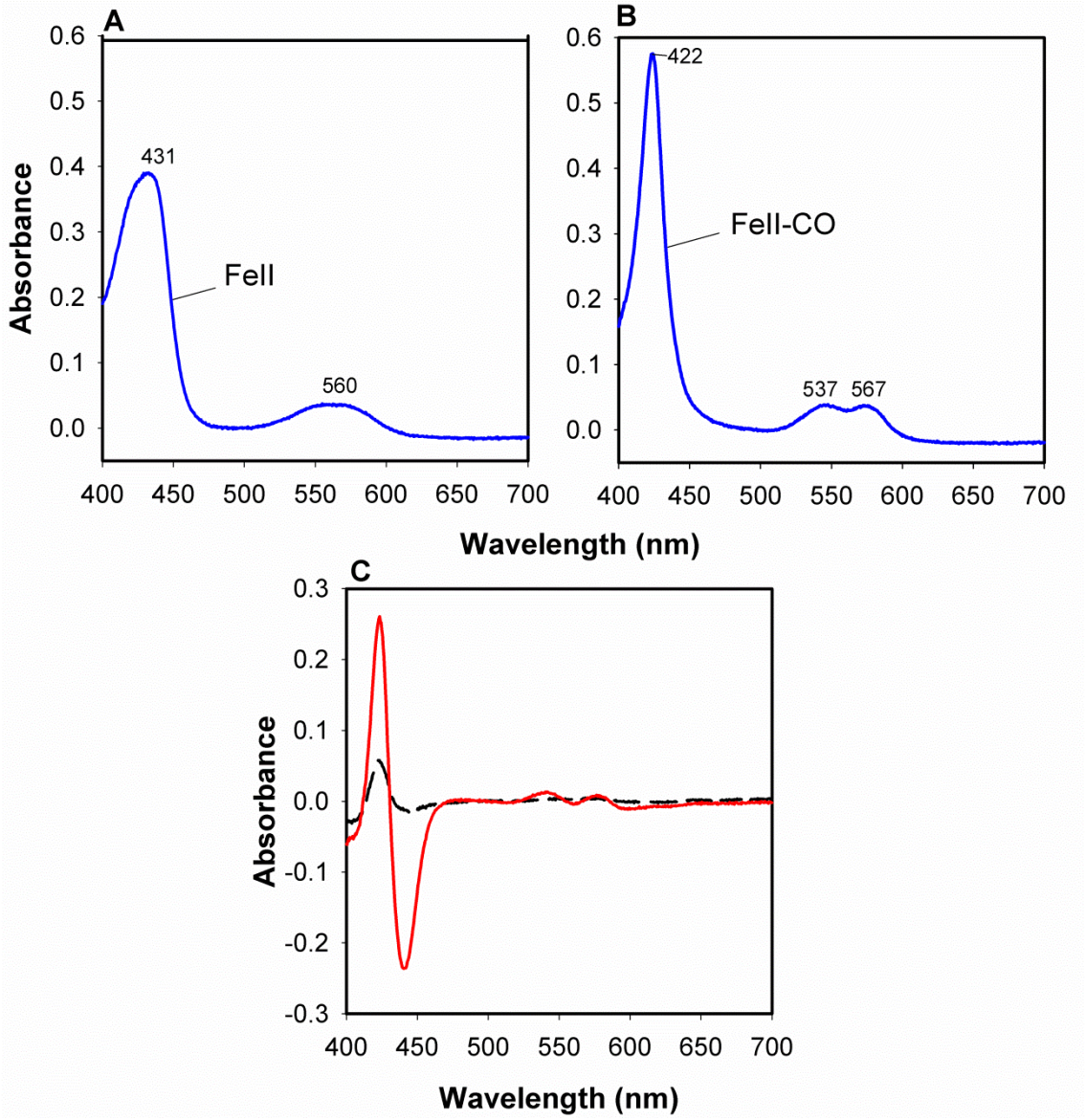


Figure 7.5 Intracellular formation of CO-bound Ctb in the presence of glucose and CO gas.

E. coli cell suspensions over-expressing Ctb or transformed with the empty vector were pre-treated with glucose (15 mM) for 7 min followed by bubbling with CO gas for 2 min. Absolute spectra of Ctb glucose-reduced (**A**) or Ctb CO-glucose-reduced (**B**) were obtained by using the absorbance values from the glucose-reduced or CO-glucose-reduced cells carrying the empty vector as the baseline respectively. (**C**) Difference spectra (CO-glucose-reduced minus glucose-reduced) were plotted for the Ctb-expressing cells (red solid line) and the cells carrying the empty vector (black dashed line).

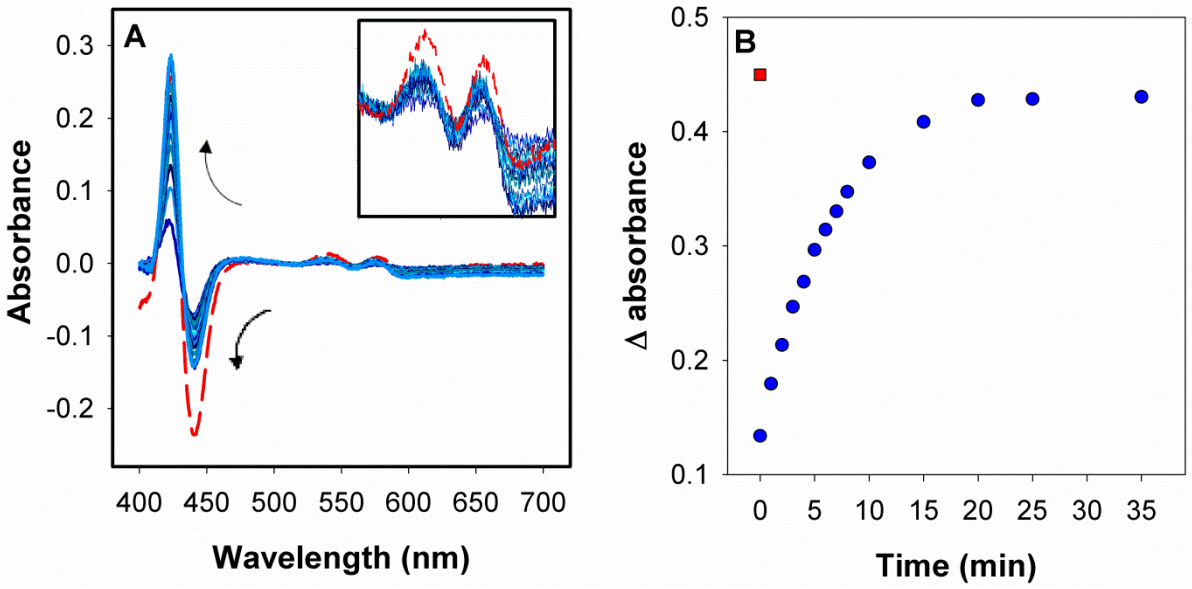


Figure 7.6 Intracellular formation of CO-bound Ctb in the presence of glucose and CORM-3 and CO release kinetics.

(A) *E. coli* cell suspensions over-expressing Ctb were pre-treated with glucose (15 mM) for 7 min and 300 µM CORM-3 was added. Changes in the spectra were followed every minute for 10 min and then at 15, 20, 25 and 35 min. Difference spectra (CO reduced minus reduced) were plotted (blue lines). Arrows indicate the direction of the changes. Difference spectrum of pre-treated cells with glucose followed by bubbling with CO gas for 2 min is shown as a control (red dashed line). (**B**) The differences in the absorption maxima (peak minus trough) were plotted for every time point (●) and were plotted together with the single point corresponding to the CO gas control (■).

7.3 Discussion

As mentioned previously, the truncated globin Ctb has a very high affinity for O_2 (Lu *et al.*, 2007a). Indeed, in order to fully reduce 3.7 µM of purified Ctb, at least 10 mM sodium dithionite had to be added (Chapter 6, Fig. 6.3A) and intracellular globin expressed in *E. coli* is observed in the oxygenated form (oxy-FeII) (Wainwright *et al*., 2006 and Fig. 7.2B). This is interesting since it was demonstrated that oxy-FeII Ctb does not bind either CO gas or CO from CORM-3 (Fig. 7.4); however, growing cells are indeed inhibited by CORM-3 (Davidge *et al.*, 2009a; Davidge *et al.*, 2009b; Desmard *et al.*, 2009) and the ability of Hmp to detoxify NO in culture is also decreased in the presence of the CO-releaser (Tinajero-Trejo *et al*., manuscript in preparation). Thus, it appears that metabolically active cells are able to maintain a sufficiently reduced inner environment, allowing the CO binding to, for instance, ferrous-haem proteins (such as haemoglobins) and terminal oxidases even in aerobic growth conditions. Indeed, respiring *E. coli* cells exposed to air in the open electrode consumed O_2 so efficiently that the electrode failed to detect it for at least 45 min in the presence of glucose (see section 7.2.3). Thus, it is clear that the reductant power supplied by respiration can entirely replace the use of dithionite to reduce cells for studying CO-release from CO-RMs (Fig. 7.7). However, it is important to consider whether the presence of glucose may also interact with CO-RMs. Experimental evidence generated in our lab argues against it. Certainly, 30 mM glucose failed to promote CO releasing from CORM-3 tested spectroscopically by following CO-Hmp formation from NADH-reduced Hmp *in vitro* (Tinajero-Trejo *et al.,* manuscript in preparation).

The development of new strategies to combat antibiotic-resistant pathogens is a worldwide priority (Lewis, 2013). CO has proved to possess important antibacterial effects such as increasing *E. coli* phagocytosis (Otterbein *et al.*, 2005) and protecting against lethality during bacterial sepsis (Chung *et al.*, 2008). Thus, the use of CO-RMs may be part of a new era in the treatment of bacterial infection where these compounds may function as adjuvants of the classical or new generation antibiotics. One of the most attractive features of CO-RMs is the fact that they appear to be more effective than CO gas. Indeed, bacterial growing cells are inhibited by CORM-3 but not by the same concentration of dissolved CO gas, suggesting that the CO-RM is taken up by the cells

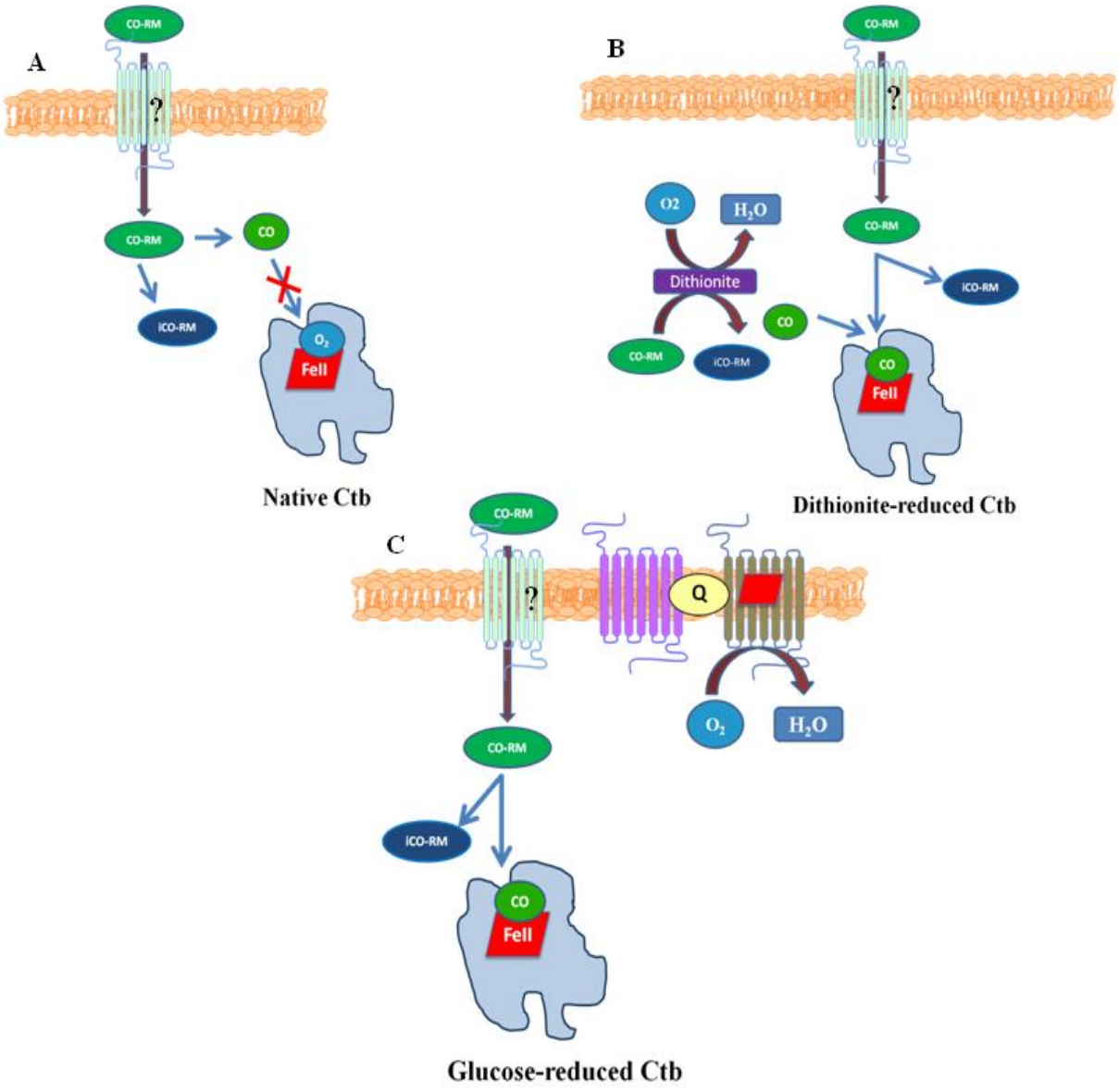


Figure 7.7 Simplified schematic diagram of intracellular CO release from CO-RMs and CO binding to Ctb in the presence and absence of sodium dithionite.

The CO-releasing molecule (CO-RM) is transported to the cell interior (possibly through a transport system) where it reacts with the soluble cellular content rendering the inactive form of CO-RM (iCO-RM) and CO. (**A**) CO released from CO-RM is unable to bind the oxy-FeII haem (red square) of Ctb. (**B**) Sodium dithionite scavenges intracellular O_2 promoting de-oxygenation of the Ctb haem and allowing CO binding to the FeII haem of Ctb; however, dithionite also reacts with CO-RM releasing CO. (**C**) Consumption of O_2 by respiration facilitates the de-oxygenation of the Ctb haem allowing CO binding to the FeII haem of Ctb.

delivering the CO *in situ* (Davidge *et al.*, 2009b; Jesse *et al.*, 2013; Wilson *et al.*, 2013); which has been called "The Trojan horse mechanism" (Wilson *et al.*, 2013). However, the data concerning the modes of action of CO and CO-RMs in biological systems are still very limited, making it difficult to pursue further aims.

7.4 Conclusions

The presence of intracellular CO can be determined spectrophotometrically *in vivo* in *E. coli* cells over-expressing the heterologous truncated globin Ctb. Promotion of respiration by addition of glucose efficiently reduces the intracellular pool of globin, avoiding the use of the artificial reductant sodium dithionite. Thus, Ctb can be used as a sink to measure CO release from CO-RMs. This technique might represent a valuable tool to determine CO release rates and kinetics form a range of CO-RMs. At the moment, this system is being successfully used to determine CO-release and kinetics of CORM-3 and the Mn-based CO-releaser CORM-401 (Lauren Wareham personal communication).

Chapter 8. General discussion

The microaerobic capability of *C. jejuni* to survive under conditions of nitrosative stress has been linked to the presence of the SDgb Cgb (Avila-Ramirez *et al.*, 2013; Elvers *et al.*, 2005; Monk *et al.*, 2008; Pittman *et al.*, 2007; Wainwright *et al.*, 2005). This globin, together with the TrHb3 Ctb, a protein putatively involved in O_2 chemistry (Wainwright *et al.*, 2005), belong to a small regulon positively controlled by the transcription factor (NssR) under nitrosative stress conditions (Elvers *et al.*, 2005; Monk *et al.*, 2008). In O_2-limited conditions, a different but still inducible system provides protection against NO toxicity (Avila-Ramirez *et al.*, 2013). The nature of this mechanism is still unclear.

Investigation of the molecular mechanism(s) of the *C. jejuni* globins *in vitro* is limited by the absence of the reductase domain that, in the flavohaemoglobins, reduces the haem that is oxidized during the conversion of NO and O_2 to nitrate (Gardner *et al.*, 1998a; Hausladen *et al.*, 1998; Hernandez-Urzua *et al.*, 2003). Besides, as mentioned, direct confirmation of Cgb function as a NO and RNS detoxification system and the further investigation of Ctb activity *in vivo* is hampered in *Campylobacter* by complications to express proteins in plasmids. Therefore, the current study mainly pursued two objectives: first, to corroborate the ability of Cgb to protect against nitrosative stress and second, to explore possible functions of Ctb. A natural and convenient approach for functional characterisation is the heterologous expression of globins. Indeed, a number of bacterial globins have been studied in a microorganism different from the native one (Table 1.3). Thus, both *cgb* and *ctb* genes, cloned in commercial vectors under control of arabinose-inducible promoters, were expressed in a NO-sensitive *E. coli* strain (*E. coli hmp* mutant) and a number of experimental approaches including growth tests in the presence of nitrosative and oxidative compounds and polarographic measurements of NO consumption were carried out. Furthermore, exploration of the reduction of Cgb and Ctb haems in cellular milieus after oxidation by NO and a search for "the cognate reductases" of these globins in *E. coli* and *C. jejuni* were also performed.

Confirmation of the Cgb function as an efficient detoxification system, able to complement the NO and RNS resistance phenotype in the Hmp-lacking *E. coli*, constitutes perhaps the most valuable contribution of the current study. Indeed, the

ability of Cgb to support the growth of the *E. coli hmp* mutant strain in the presence of NO and GSNO (Fig. 3.3) clearly agree with the proposed role of this globin as an efficient resistance system in *C. jejuni*. On the other hand, expression of Ctb failed to provide protection under similar conditions, in agreement with the suggestion that Ctb has a function different from NO and RNS tolerance (Fig. 3.4). Interestingly, polarographic measurements showed that not only Cgb-expressing cells but also those expressing Ctb were able to consume NO either in the presence of O_2, providing protection to cellular respiration, or in anoxic conditions (Fig. 3.6).

The O_2-independent consumption of NO by the *C. jejuni* haemoglobins is puzzling and raises several questions related to the molecular mechanisms linked to Cgb and, indeed, to Ctb. Do these globins reduce NO in the absence of O_2? The flavohaemoglobin Hmp acts as a NO reductase, albeit of low activity, in anaerobiosis (Mills *et al.*, 2001). Thus, this is an interesting and certainly feasible suggestion. If this is the case, does this activity have physiological relevance considering that the NssR regulon is not expressed under O_2-limited conditions (Avila-Ramirez *et al.*, 2013)? Is this activity as efficient as the putative denitrosylase function proposed for Cgb (Shepherd *et al.*, 2011)?

Hausladen *et al*. (2001) established the denitrosylase activity of the flavohaemoglobin Hmp that consists of the production of nitrate from haem-bound NO (NO^-) with O_2. Measurements of NADH consumption and haem-ligand turnover under physiologically relevant concentrations of O_2 (between 9 and 125 µM) and NO (between 10 and 50 µM), revealed that Hmp primarily binds NO forming a nitrosyl–haem complex that in turn reacts with O_2 to form nitrate. Why does a microaerophilic organism such as *C. jejuni* rely on an O_2-dependent system to detoxify NO and RNS? The O_2 and NO concentrations encountered by *C. jejuni in vivo* remain elusive. However, it is suggested that certain segments of the alimentary canal are fully anaerobic whereas others can have microaerobic concentrations. It has been recently demonstrated that even aerobic *E. coli* cultures are found in O_2-limited conditions during exponential phase (less than 5% of dissolved O_2 (< 10 µM)) (Potzkei *et al.*, 2012), whereas antimicrobial concentrations of NO that pathogenic bacteria experience can reach up to 0.28 µM (reviewed in Hausladen *et al*., 2012). How is O_2-dependent NO detoxification efficiently performed in environments where O_2 presence seems to be so variable? A very complex and intricate interplay between expression and perhaps protein stability

may be the answer. It is possible that, in the presence of O_2, nitrosative stress triggers the expression of Cgb (via NssR). This globin might be sufficiently stable to remain present during transient periods of O_2 absence, performing the detoxification activity (denitrosylase/reductase) without the need for a constant NssR-dependent *cgb* induction. As an approach to determining protein stability, the presence of Cgb in cultures after exposure to pulses of NO or RNS could be followed by Western blotting

The inability of a Cgb-expressing *E. coli hmp norR* mutant to grow anaerobically (Fig. 3.7) suggests that there are important differences in the level of protection conferred by this globin in the presence or absence of O_2. Experimental approaches aimed at determining the products of the chemical reaction(s) mediated by the *C. jejuni* globins in the presence of NO may cast light on the matter. Measurements of nitrate production from NO and O_2 *in vitro* and/or *in vivo* can be approached by colorimetric techniques and determination of N_2O in anaerobic cultures by Fourier Transform Infrared Spectroscopy. On the other hand, experiments aimed at the determination of the denitrosylase activity of Cgb are a major challenge, limited by the absence of a cognate reductase that allows turnover measurements.

More and more evidence seems to indicate the presence of promiscuous, rather than specific, reductases able to donate electrons to native and heterologous globins and other haem proteins (Coppola *et al.*, 2013; Dewilde *et al.*, 2001; Gusarov *et al.*, 2008; Trandafir *et al.*, 2004; Van Doorslaer *et al.*, 2003). The *C. jejuni* globins appear not to be the exception. Specific candidates for the Cgb cognate reductase include the flavorubredoxin reductase NorW in *E. coli* (Figs. 5.3-5.5) or the lactate dehydrogenase Cj1585c in *C jejuni* (Fig. 5.6) but there is no indication of such a function. Furthermore, the role of the *E. coli* electron flux of the respiratory chain as the source of reduction was also ruled out (Fig. 5.1 and 5.2). However, herein it was demonstrated that regardless of the source of the cellular soluble extracts (*E. coli* or *C. jejuni*) both Cgb and Ctb were efficiently reduced after oxidation by NO (Chapter 4). The dependence of the reduction on the presence of NADH suggested that the "mysterious" reductase(s) might function in a similar way as the reductase domain of the flavohaemoglobin Hmp that also requires the presence of NADH or NADPH (Anjum *et al.*, 1998). However, experimental evidence to support this must be generated.

Bacterial haemoglobins from pathogenic bacteria are unlikely to be candidates for pharmacological purposes due to their homology to human Hb and Mb. However, identification of the cognate reductase(s) of such globins (e.g. SDHbs and TrHbs from *C. jejuni* and *M. tubercuolosis*), might be valuable since it would represent an opportunity for exploring those reductases as targets for pharmacological intervention. For instance, specific compounds able to inhibit the interaction between the globin and the reductase partner may be extremely useful for understanding and perhaps manipulating signalling networks in the globin-containing bacteria.

Extensive efforts have been made in order to reveal the physiological function(s) of the truncated haemoglobin Ctb (Bolli *et al.*, 2008; Lu *et al.*, 2007a; Nardini *et al.*, 2006; Wainwright *et al.*, 2006). However, this enigmatic protein is still far from being understood. Even though Ctb has been involved in oxygen chemistry, it appears to be up-regulated only under conditions of nitrosative stress (Wainwright *et al.*, 2005) and otherwise constitutively expressed at low levels. Are the constitutive levels of the globin sufficient to allow a significant function in regulation of intracellular oxygen tensions? Why is Cgb tightly repressed in the absence of nitrosative stress while Ctb is not? Moreover, are increased levels of the truncated globin required to play the same, or a different role under nitrosative stress conditions?

The demonstration of a significant increase in NO evolution from nitrite by the expression of Ctb in *E. coli* (Fig. 6.2) suggests a nitrite reductase activity linked to the truncated globin. Here, it was suggested that NO production by Ctb from nitrite might be a primary mechanism aimed at triggering the NssR-dependent expression of Cgb that in turn detoxifies NO. Thus, elucidation of the molecular mechanism leading to the activation of NssR in the presence of nitrosative stress (NO, GSNO, nitrite, etc.) represents a challenging and crucial next step in the functional characterisation of the NO-mediated globin responses. A requirement for iron acquisition and oxygen has been demonstrated for the NssR NO-mediated response (Monk *et al.*, 2008). However, the molecular mechanism for activation of NssR remains elusive. Smith *et al.* (2011) showed that purified NssR has a high affinity for the *ctb* promoter by using gel shift analyses, yet GSNO and the NO donor NOC-12 had little effect on DNA binding. However, three possible explanations have been suggested: (i) the observed DNA binding of the apoprotein is due to the lack of an NssR cofactor and, consequently an

artefact, (ii) NssR actually binds the DNA and post-translational modifications of the apoprotein promote transcriptional activation or (iii) a downstream intermediate of NO metabolism activates the transcription. Approaches such as structural modelling, investigation of the NssR metal-containing cofactors and post-translational modifications of the regulator in the presence of candidate metabolites should be considered to test such hypotheses.

Initial structural modelling approaches have been performed using the online RaptorX server (Kallberg *et al.*, 2012; Peng and Xu, 2011). Structural homology with transcriptional regulators that bind cyclic nucleotides, haem, and α-ketoglutarate were found (Dr. Mark Shepherd, personal communication); of special interest are Catabolite Activator Protein (CAP) from *Thermus thermophilus*, CooA from *Carboxydothermus hydrogenoformans* (binds haem), and NtcA from *Synechococcus elongatus* (a global nitrogen regulator). Since the iron requirement for NssR activity *in vivo* has been demonstrated (Monk *et al.*, 2008), the most likely candidate to occupy the ligand-binding cleft is haem. Indeed, a histidine residue commonly found in haem ligands is predicted in the vicinity of the putative haem-binding site (Dr. Mark Shepherd, personal communication). If haem is found to bind to NssR, gel shift assays performed with haem-loaded NssR may produce perturbations of the NssR:DNA interaction in the presence of NO. Furthermore, the requirement of NssR for haem can be tested *in vivo* using a mutant of *C. jejuni* that is deficient in haem synthesis (Δ*hemA*); NO-induced expression of Cgb can be followed by Western blotting and growth sensitivity to NO monitored in culture. Besides, *S*-nitrosation of cysteine residues, the most common protein modification during nitrosative stress, and nitration of tyrosine residues by peroxynitrite, produced *in vivo* by the reaction of NO and superoxide (Hughes, 1999), as possible post-translational modification of NssR could be approached by treating purified NssR with GSNO and NO to cause nitrosylation and peroxynitrite to produce nitration. Increasing mass can be measured by a Bruker micrOTOF-Q Electrospray Mass Spectrometry (Dr. Mark Shepherd, personal communication).

Experimental validation of Ctb as a nitrite reductase able to activate the expression of Cgb via NO production, would represent an important contribution to the understanding of bacterial haemoglobin functions. Certainly, it would constitute the first example of haemoglobins working in an integrated response. A combination of mutagenesis,

molecular dynamics simulation and ligand-binding measurements could be exploited to investigate the molecular bases of the Ctb nitrite reductase activity. Additionally, transcriptomic analysis of nitrite and nitrate metabolism in *C. jejuni* might provide a wider picture of the changes elicited in such conditions. Indeed, if NO is generated during nitrite metabolism in *C. jejuni*, it is plausible that the responses to cultures fed with nitrite will trigger not only the expression of genes related to the presence of this ion but also those observed in the NO regulon. A tightly controlled study in continuous culture could provide reliable data on the effects of nitrite (predicted to generate NO *in vivo*) and NO *per se* (added as a fast NO-releasing NONOate) that would allow comparisons, as has been done previously in Prof. Poole's laboratory for assessing the full distinct effects of NO, GSNO and peroxynitrite in *E. coli* (Pullan *et al.*, 2007).

Even though bacterial globins have been widely studied in recent years, the information obtained does not always reveal the physiological significance of such proteins in their hosts. For instance, although for several microbial globins the crystal structure has been solved (Table 1.2), in many cases, there is not a hint of the functions. Nonetheless, *C. jejuni* has proved to possess important advantages for the investigation of globin function. Indeed, this pathogenic bacterium deals with NO and RNS during infection and pathogenesis. This means that elucidation of the resistance mechanisms against nitrosative stress may have profound implications for fundamental and medical science. Besides, *C. jejuni* is a genetically tractable organism with which it has been possible to produce mutants allowing inference of functions (e.g. the role of Cgb in NO and RNS resistance) and regulation (the transcription factor NssR). These data, together with (i) an extensive exploration of the structure and biochemical characterisation of Cgb and Ctb *in vitro* and (ii) the confirmation of the Cgb function and exploration of the Ctb activities by heterologous expression in *E. coli* have positioned the *C. jejuni* globins among the most studied bacterial globins (Table 8.1).

Table 8.1 Functions and regulation of the haemoglobins Cgb and Ctb from *C. jejuni*.

Family, subfamily and globin name	Crystal Structure	Function(s) inferred by mutation	Regulation		Heterologous expression		References
			Regulator(s) involved	Environmental factors and/or compounds involved in up-regulation	Host	Function(s) in the host	
M SDHb Cgb	Solved	Protection of growth against NO and GSNO toxicity in microaerophilic conditions. Protection of respiration from NO inhibition. Consumption of NO under aerobic and anaerobic conditions.	NssR	GSNO, NOC-18, SNP, spermine NONOate, nitrate and nitrite	*E. coli wild type.* *E. coli hmp*	Protection of growth against NO and GSNO toxicity. Protection of respiration from NO inhibition. Consumption of NO under aerobic and anaerobic conditions.	(Avila-Ramirez *et al.*, 2013; Bollinger *et al.*, 2001; Elvers *et al.*, 2005; Elvers *et al.*, 2004; Frey *et al.*, 2002; Monk *et al.*, 2008; Pittman *et al.*, 2007; Shepherd *et al.*, 2010; Wainwright *et al.*, 2005 and this study).
TrHb TrHb3 Ctb	Solved	Oxygen metabolism	NssR	Constitutively expressed at low levels. Nitrosative stress.	*E. coli hmp*	Consumption of NO under aerobic and anaerobic conditions. Production of NO from nitrite under anaerobic conditions.	(Avila-Ramirez *et al.*, 2013; Elvers *et al.*, 2005; Monk *et al.*, 2008; Nardini *et al.*, 2006; Smith *et al.*, 2011; Wainwright *et al.*, 2005 and this study).

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A question

2 messages

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