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The NO-responsive hemoglobins of *Campylobacter jejuni*: Concerted responses of two globins to NO and evidence *in vitro* for globin regulation by the transcription factor NssR

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ABSTRACT

Campylobacter jejuni possesses NO-responsive and -detoxifying mechanisms to survive NO during transmission and pathogenesis. *C. jejuni* possesses two hemoglobins. The first (Cgb) is a single-domain (non-flavo)hemoglobin encoded by gene *Cj1586* (*cgb*), mutation of which leads to hypersensitivity to *S*-nitrosoglutathione and NO. Transcription of *cgb* is induced by nitrosative stress and confers resistance to NO, presumably via a Cgb-catalyzed dioxygenase or denitrosylase reaction that converts NO and oxygen to nitrate. Expression of Cgb in response to NO is mediated via the positively-acting transcription factor NssR, which regulates expression of a small regulon that includes *cgb* and *ctb* (*Cj0465c*), the latter encoding the truncated hemoglobin, Ctb. The role of Ctb is unclear: it is not directly involved in NO detoxification but is implicated in oxygen delivery or metabolism. Here, we describe attempts to define a function for Ctb by examining the effects of a *ctb* mutation on the NO transcriptome and *cgb* gene expression during normoxia and hypoxia. Mutation of *ctb* does not elicit major compensatory transcriptomic changes but relatively minor changes in genes involved in intermediary metabolism, solute transport and signal transduction. We present and test the hypothesis that, by binding NO or O_2, Ctb dampens the response to NO under hypoxic conditions and limits *cgb* expression, perhaps because Cgb function (i.e. NO detoxification) requires O_2-dependent chemistry. We report the purification of NssR and specific binding to the *ctb* promoter. GSNO does not affect the high affinity of NssR for the *ctb* promoter.

Introduction

Following the recognition in the 1980s that nitric oxide (NO) is the endothelium-derived relaxing factor, and that it participates in the regulation of the nervous and immune systems, it was soon discovered that NO also plays a vital role in the resistance of mammalian hosts to microbial infections. Activated macrophages were shown to form nitrite and nitrate from arginine [1,2] via the formation of NO [3] and to have a powerful cytostatic effect *in vitro* on the fungal pathogen *Cryptococcus neoformans* [4]. Activated macrophages also destroyed the intracellular parasite *Leishmania major in vitro* by an L-arginine-dependent mechanism [5] and mice infected with *L. major* developed exacerbated disease when the lesions were injected with the NOS inhibitor L-NMMA, providing the first compelling evidence for the attenuation by NO of an infectious microorganism *in vivo* [6].

A direct role for NO against intracellular bacteria was soon established, initially with *Mycobacterium bovis* [7]. In murine macrophages, NO has an undisputed role in bacterial clearance [8,9] and mechanisms that are known to confer NO resistance in bacteria, such as flavohemoglobin-catalyzed NO detoxification by *Salmonella Typhimurium* protect the bacterium from NO-mediated killing in human macrophages [10]. Flavohemoglobin (Hmp) catalyzes the reaction of NO with oxygen to give innocuous nitrate via a dioxygenase [11–13] or denitrosylase [14,15] mechanism and *hmp* gene transcription is activated on exposure of bacteria to NO or nitrosating agents [16–18]. Those globins most intensively studied [19] include the two globins of *Campylobacter jejuni* [20,21].

Abbreviations: Cgb, *Campylobacter* hemoglobin; Ctb, *Campylobacter* truncated hemoglobin; GSNO, *S*-nitrosoglutathione; NOC-5, 3-[2-hydroxy-1-(1-methylethyl)-2-nitrosohydrazino]-1-propanamine; NOC-7, 3-[2-hydroxy-1-methyl-2-nitrosohydrazino-*N*-methyl-1-propanamine; NOC-12, [*N*-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino)ethanamine]; NssR, *Campylobacter* nitrosative stress-responsive regulator; trHb, truncated hemoglobin.

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Campylobacter species are the leading cause of gastroenteritis in the developed world [22]. During the course of transmission from avian to human hosts and in the gut, the bacterium encounters NO and other agents of nitrosative stress. In the context of *C. jejuni*, the chief sources of NO are from host NO synthases, especially iNOS (inducible NO synthase), bacterial reduction of dietary nitrate to nitrite and thence to NO, and the reactions of salivary nitrite with stomach acid to generate NO [23]. Acidified nitrite kills *C. jejuni* and NOS (NO synthase) expression is increased in macrophages on exposure to the bacterium [24].

The genome sequence of *C. jejuni* lacks a flavohemoglobin gene [25] but gene *Cj1586* (*cgb*) encodes a single-domain hemoglobin, mutation of which leads to hypersensitivity to GSNO and NO [26]. Expression of Cgb is strongly induced after exposure to nitrosative stress. Cells expressing GSNO-induced levels of Cgb are resistant to NO [26] and Cgb is thought to catalyze a dioxygenase or denitrosylase reaction (like Hmp) that converts NO and molecular oxygen to nitrate [27]. Cgb has recently been structurally and spectroscopically characterized [28] wherein the heme cofactor was shown to be adapted for a dioxygenase or denitrosylase reaction. Expression of Cgb in response to NO is mediated via NssR (Nitrosative stress sensing Regulator, *Cj0466*), a member of the Crp–Fnr superfamily of transcription factors, which acts as a positive regulator of a small regulon that includes both *cgb* and *ctb* (*Cj0465c*), the latter encoding the *C. jejuni* truncated hemoglobin [29]. A *ctb* mutant is not compromised in its tolerance of nitrosative stress-generating agents [30] but Ctb has been implicated in oxygen delivery [30,31] or in catalyzing a peroxidase- or P-450-type of oxygen chemistry [21].

Truncated hemoglobins (trHbs) such as Ctb are small members (~20–40 amino acids shorter) of the hemoglobin superfamily that adopt a 2-over-2 α-helical sandwich motif [32]. Sequence analysis of more than 200 trHbs showed that they can be divided into three groups: I, II and the poorly characterized group III [32]. Recently the trHb III from *C. jejuni*, Ctb, was structurally and kinetically characterized [21,31,33,34]. Resonance Raman studies revealed a complex hydrogen-bond network operating in Ctb that controls oxygen binding and kinetic studies showed that Ctb exhibits extremely high oxygen affinity due to slow dissociation kinetics ($0.0041\ s^{-1}$). Hbs displaying a moderate oxygen affinity act mainly as O_2 carriers or storage proteins [35], whereas those with higher affinities are typically involved in oxygen chemistry [36,37].

In summary, Ctb is a truncated globin whose synthesis is upregulated by NssR on exposure of *C. jejuni* to NO but its function remains unclear. The purpose of this work was to explore the function of Ctb and extend our knowledge of its regulation by NssR. First, we examined the transcriptome of a *ctb* mutant to search for compensatory mechanisms that operate in its absence. Recognizing the microaerobic lifestyle of *C. jejuni*, these studies were conducted at two levels of O_2 provision and lead to a hypothesis of ligand management by Ctb. Second, we tested the hypothesis that the role of Ctb is related to its high oxygen affinity and that Ctb regulates NO availability *in vivo* according to oxygen status. Finally, we report the first purification of the transcription factor NssR and its interaction with the *ctb* promoter.

Methods

Bacterial strains and growth

All strains were derived from the sequenced *C. jejuni* strain NCTC 11168 [25]. The mutant defective in Ctb was RKP1388 (*ctb*:kan^r, identical to RKP1386) [30]. Maintenance and growth of bacteria was as described before [38]. The NO-releasing compounds used were 3-[2-hydroxy-1-(1-methylethyl)-2-nitrosohydrazino]-1-propanamine (NOC-5) and 3-[2-hydroxy-1-methyl-2-nitrosohydrazino-*N*-methyl-1-propanamine (NOC-7), which have calculated half-times of NO release at 42 °C of 10.5 and 3.0 min, respectively [38] and [*N*-ethyl-2-(1-ethyl-2-hydroxy-2-nitrosohydrazino)ethanamine] (NOC-12), which has a half life of 327 min at 22 °C [39]. Stock solutions (0.1 M) of each were prepared in 0.1 M NaOH.

Transcriptomic analysis of wild-type and ctb mutant strains and the response to NOCs and varied aeration

Batch cultures were grown in either 100 ml or 200 ml volumes in 250-ml baffled flasks, thus altering the first order rate of oxygen diffusion, *K*, from gas to liquid [30]. Although our previous transcriptomic analysis used GSNO as a source of nitrosative stress [38], here we explored the response to NO *per se*, since previous studies with *Escherichia coli* have revealed quite distinct transcriptomic profiles in response to GSNO [40] and NO [41]. NOC-5 and NOC-7 (stabilised NO-amine complexes, which decompose on protonation of the oxygen of the NO group) release NO in solution with first-order kinetics [42]. At an optical density (Beckman DU600 spectrophotometer, 600 nm, 1 cm path length) of ~0.3, equimolar NOC-5 and -7 (10 μM each) were added and the cultures incubated in the microaerophilic workstation for a further 10 min. This mixture of NOCs provides a sustained presence of NO over several minutes [43].

Samples (30 ml) were taken from each flask and mixed immediately on ice with 3.56 ml of 100% ethanol and 185 μl of phenol to stabilize RNA. Cells were harvested by centrifugation, and total RNA was purified using a Qiagen RNeasy mini kit. Microarray analysis of three biological replicates of wild-type and the *ctb* mutant stressed with NOC-5 and -7 at high and low aeration rates was carried out using *C. jejuni* NCTC 11168 'Pan' arrays purchased from Ocimum Biosolutions Ltd. (Road 1, Banjara Hills, Hyderabad 500034, India). Microarray analysis was carried out as previously described [38], except that equal quantities (12 μg) of RNA from wild-type and *ctb* mutant cultures stressed with NOC-5 and -7 were primed with 18 μg random primers (Invitrogen) at 65 °C for 10 min followed by 22 °C for 10 min. After priming, 4 μl of 5× RT buffer (Invitrogen), 2 μl dNTP mix (0.5 mM ATP, 0.5 mM TTP, 0.5 mM GTP, 0.2 mM CTP), 2 μl 0.1 M DTT and 200 U of Superscript III reverse transcriptase (Invitrogen) were added before incubation for 3 h at 42 °C.

Real time RT-PCR

This was performed as before [44] except that amplification was done in a Stratagene Mx3500P instrument. Primers are listed in Supplementary material (Table S1).

Detection of the Cgb globin by immunoblotting

Mid-exponential phase cultures of wild-type and the *ctb* mutant were grown under both high and low aeration conditions and stressed with 10 μM NOC-5 and -7 for 10 min or left untreated, then harvested by centrifugation. Preparation of cell-free extracts, Western blotting with anti-Cgb antibody, and protein assays were as described before [38].

Cloning of nssR

The *nssR* gene was amplified from genomic DNA purified from *C. jejuni* using the primers given in Supplementary material (Table S1) and cloned into *Nco*I/*Bam*HI-digested pGEX-KG plasmid as described previously [45]. The plasmid sequence was checked by DNA sequencing. This plasmid was then used to transform