A truncated haemoglobin implicated in oxygen metabolism by the microaerophilic food-borne pathogen *Campylobacter jejuni*

Laura M. Wainwright,¹ Karen T. Elvers,² Simon F. Park² and Robert K. Poole¹

Correspondence

Robert K. Poole

r.poole@sheffield.ac.uk

¹Department of Molecular Biology and Biotechnology, The University of Sheffield, Sheffield S10 2TN, UK

²School of Biomedical and Molecular Sciences, University of Surrey, Guildford GU2 7XH, UK

Of the three groups of haemoglobins identified in micro-organisms (single-domain globins, flavohaemoglobins and truncated globins), the last group is the least well understood. The function of the truncated haemoglobin (Ctb) encoded by Cj0465c in the microaerophilic food-borne bacterial pathogen *Campylobacter jejuni* was investigated by constructing a *ctb* mutant and characterizing its phenotype. The effects of the *ctb* mutation on the kinetics of terminal oxidase function in *C. jejuni* were investigated using oxyleghaemoglobin and oxymyoglobin as sensitive reporters of O₂ consumption. The $V_{\max}$ of *ctb* mutant cells for O₂, calculated using either globin, was greater than that of wild-type cells at extracellular O₂ concentrations up to ~1 µM, suggesting a role for Ctb in moderating O₂ supply for reduction by high-affinity terminal oxidases. However, cells mutated in *ctb* were disadvantaged when grown under conditions of high aeration, as revealed by measurements of growth yields and rates in batch culture. Furthermore, the rate at which *ctb* mutant cells consumed O₂ in an O₂ electrode (10–200 µM O₂) was approximately half the rate displayed by wild-type cells, reflecting a role for Ctb in respiration at physiologically relevant external O₂ concentrations. However, a lack of sensitivity of the mutant to paraquat or H₂O₂ indicated that increased oxidative stress under such conditions was not the cause of these phenotypes. O₂ affinities of cells (K_m values of approximately 40 nM and 1 µM) were unaffected by mutation of either Ctb or the full-length *C. jejuni* globin, Cgb. Although the gene encoding Ctb was found to be upregulated by S-nitrosoglutathione (GSNO) and the NO-donating compound S-nitroso-N-acetylpenicillamine (SNAP), a *ctb* mutant did not display sensitivity to a number of nitrosative stress-generating compounds. The authors conclude that Ctb is involved in moderating O₂ flux within *C. jejuni*.

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INTRODUCTION

Campylobacter jejuni is a Gram-negative spiral-shaped bacterium. It is highly motile and preferentially grows in an atmosphere containing 5–15% O₂ and 3–5% CO₂ (Ketley, 1997) at 42 °C (Hazeleger *et al.*, 1998). This organism is not only a widespread commensal of avian gastrointestinal tracts, but also the predominant bacterial agent of human gastrointestinal illness worldwide. Symptoms of *Campylobacter* infection can include fever, headache, dizziness and myalgia. This is typically followed by

inflammatory diarrhoea. Such infections tend to be self-limiting and of short duration (Skirrow & Blaser, 2000). However, reactive arthritis and temporary paralysis caused by Guillain-Barré syndrome can be late-onset complications (Hadden & Gregson, 2001; Skirrow & Blaser, 2000).

A distinctive feature of *C. jejuni* is that it is a microaerophile, i.e. oxygen is necessary for growth yet also toxic when at atmospheric concentration. The mechanisms behind microaerophily are not understood but there are many hypotheses (Krieg & Hoffman, 1986). These include an increased susceptibility of microaerophiles to reactive oxygen species (ROS) (Hoffman *et al.*, 1979b), an increased sensitivity to inhibition by O₂ of certain respiratory enzymes (Hoffman *et al.*, 1979a), and excessive metabolic generation of ROS (Hoffman *et al.*, 1979b). In light of this, *C. jejuni* may benefit from the presence of an O₂-detoxifying mechanism when exposed to atmospheric O₂ conditions. However, in

Abbreviations: GSNO, S-nitrosoglutathione; Hb, haemoglobin; flavoHb, flavohaemoglobin; trHb, truncated haemoglobin; LHb, leghaemoglobin; Mb, myoglobin; 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; RNI, reactive nitrogen intermediates; ROS, reactive oxygen species; SNAP, S-nitroso-N-acetylpenicillamine.

mammalian or avian hosts, *C. jejuni* is likely to be exposed to lower than optimal O₂ concentrations. In such a situation, the presence of high-affinity O₂ binding and reduction mechanisms would be useful, such as the cytochrome *cbb*₃-type oxidase predicted by the genome sequence (Parkhill *et al.*, 2000). The obligately aerobic bacterium *Vitreoscilla* makes use of a single-domain Hb, Vgb, which is believed to facilitate the transfer of O₂ to cytochrome *bo'* under low-O₂ conditions (Park *et al.*, 2002).

C. jejuni is likely to encounter stresses other than those related to its microaerophilic lifestyle. Among these is nitrosative stress, which can be derived from both macrophages (Stevanin *et al.*, 2002) and, more likely, chemical generation of NO in saliva and the stomach (Duncan *et al.*, 1995; Li *et al.*, 1997). It has also been documented that NO synthesis is increased in patients with infective gastroenteritis (Forte *et al.*, 1999). An established mechanism of protection from NO and related reactive nitrogen intermediates (RNIs) in other bacteria is via the participation of NO-detoxifying haemoglobins (Hbs) (Wu *et al.*, 2003). *C. jejuni* possesses two Hbs, representing two of the three classes now well documented in micro-organisms. The flavohaemoglobins (flavoHbs) possess a C-terminal ferredoxin-NADP⁺ reductase-like domain, whereas the single-domain Hbs are very similar to the N-terminal haem domain of the flavoHbs and to myoglobin. Truncated Hbs (trHbs) are 20–40 residues smaller than the single-domain globins, and possess a considerably altered globin fold (Wu *et al.*, 2003). One of the *C. jejuni* Hbs, Cgb, is of the single-domain type and functions in the detoxification of NO (Elvers *et al.*, 2004). A *cgb* mutant of *C. jejuni* is hypersensitive to the nitrosating agent S-nitrosoglutathione (GSNO), to S-nitroso-N-acetylpenicillamine (SNAP), and to the NO releaser spermine NONOate. Cgb also protects the respiration of *C. jejuni* from inhibition by NO (Elvers *et al.*, 2004).

The second *C. jejuni* Hb is a trHb, i.e. a member of the most enigmatic group of microbial Hbs. trHbs are found in a number of other microbes (Wittenberg *et al.*, 2002) but little is known concerning their functions. *Mycobacterium tuberculosis* and *Mycobacterium bovis* both possess two trHbs, HbO and HbN. HbO is believed to function in the facilitation of O₂ transfer (Pathania *et al.*, 2002) and HbN is thought to detoxify NO (Ouellet *et al.*, 2002). The *C. jejuni* trHb (Cj0465c) is the subject of this paper. Attempts were made to elucidate its function, keeping in mind both the functions of other microbial globins and also the needs of *C. jejuni* as a microaerophilic organism. This study shows that the trHb does not appear to function in the detoxification of NO and is instead involved in the flux of O₂ into and within the cell.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used are listed in Table 1. *C. jejuni* NCTC 11168 was used as the wild-type. The strain of NCTC 11168 used for this study was the version deposited at the National Collection

of Type Cultures, Colindale, UK (obtained directly from this source). This strain is obviously related to the genome-sequenced strain but has a curved shape and is motile. Moreover, it exhibits a clear response to NO and has the same sensitivity to NO (unpublished) as the original wild-type form of this strain termed NCTC 111680 (Gaynor *et al.*, 2004). *Escherichia coli* TOP10 was used for overexpression of Ctb; the expressing strain was named RKP4979. *C. jejuni* strains were grown in Mueller–Hinton medium in a modular atmosphere-controlled system (MACS) VA500 workstation (Don Whitley Scientific) with a constant gas supply of 10% oxygen, 10% carbon dioxide and 80% nitrogen at 42 °C. Agar plates were incubated for 48 h; cells from these plates were inoculated into 50 ml liquid culture in a 100 ml flask and grown for approximately 18 h. The OD₆₀₀ was adjusted to 0.5 and inoculated into the batch culture to 1.33% (v/v). Batch cultures were grown using the desired volume of medium in a 250 ml baffled flask. Liquid cultures were shaken at 115 r.p.m. Mueller–Hinton medium was supplemented with vancomycin (10 µg ml⁻¹) and mutant cultures were also supplemented with kanamycin (50 µg ml⁻¹), chloramphenicol (10 µg ml⁻¹) and tetracycline (10 µg ml⁻¹) where appropriate. *E. coli* strains were grown in Luria–Bertani (LB) medium at 37 °C at 200 r.p.m., and on nutrient agar at the same temperature. The medium was supplemented with ampicillin (50 µg ml⁻¹).

Generation of *C. jejuni* mutants. The construction of the *C. jejuni* *cgb* mutant RKP1336 (also known as CJCGB01) has been described previously (Elvers *et al.*, 2004). The same method was used to generate the trHb mutant. Here the *ctb* gene (Cj0465c) from *C. jejuni* NCTC 11168 was amplified from genomic DNA by PCR using oligonucleotide primers HB1 (5'-GAGCGTTTGTAGACGAG-GGAAAT-3') and HB2 (5'-GGATCCCTCTTCAAAAACAGCATTAG-CAGG-3'), where the underlined sequence is a *Bam*HI site. The resulting 1124 bp fragment was cloned into the pBAD TA-cloning vector to generate pKE26. In this vector, the inserted *ctb* gene contains a unique *Bgl*II restriction site 223 bp from the ATG start. Thus pKE26 was digested with *Bgl*II and a kanamycin-resistance cassette with *Bam*HI ends from pJMK30 (Baillon *et al.*, 1999) (J. Ketley, University of Leicester, UK) was inserted. This generated the suicide plasmid pKE29, which was introduced into *C. jejuni* by electroporation. The homologous recombination that resulted in a double crossover event was verified by PCR and Southern hybridization (data not shown). The resulting mutant was designated RKP1386. To generate a *cgb ctb* double mutant, a second suicide plasmid, pKE34, was constructed. This was identical to pKE29 except that the antibiotic-resistance marker had been replaced with a tetracycline-resistance cassette from pSP105 (Dickinson *et al.*, 1995). This plasmid was introduced into RKP1336, and following verification (see above), a kanamycin- and tetracycline-resistant double mutant strain was selected and designated RKP1389. For both single and double mutants the antibiotic-resistance cassettes are oriented with the same transcriptional polarity as the mutated genes.

Construction of a *ctb*-complemented strain. Complementation of the *ctb* mutation in RKP1386 was achieved by insertion of the wild-type gene into an isolated pseudogene, a technique which has been successful with other mutations (Elvers *et al.*, 2005). PCR was used to amplify *ctb* and its promoter from *C. jejuni* NCTC 11168 genomic DNA using oligonucleotide primers HB3 (5'-GGATCCTT-TAGCCTCTTCGCCTTCAT-3') and HB4 (5'-GGTACCGTGGAGATTGCTCGATGGT-3') containing a *Bam*HI and a *Kpn*I restriction site respectively. The resulting fragment was digested with *Bam*HI and *Kpn*I and then ligated into the corresponding sites of pGEMCWH01 (C. Holmes & P. A. Lund, University of Birmingham, UK, unpublished results), forming plasmid pKE112. Plasmid pGEMCWH01 contains the pseudogene Cj0752 into which a multiple cloning site has been inserted. The chloramphenicol-resistance cassette from pAV103 (kindly donated by J. Ketley, University of

Table 1. Strains and plasmids

Strain or plasmid	Genotype or relevant characteristics	Source or reference
C. jejuni		
NCTC 11168	Parental strain	NCTC
RKP1386	11168 <i>ctb</i> ::Kan ^r	This study
RKP1336	11168 <i>cgb</i> ::Kan ^r	Elvers <i>et al.</i> (2004)
RKP1389	11168 <i>cgb</i> ::Kan ^r , <i>ctb</i> ::Tet ^r	This study
RKP5404	RKP1386 with mutation complemented by insertion of <i>ctb</i> ::Cm ^r into Cj0753	This study
E. coli		
TOP10	<i>araBAD</i> C	Invitrogen
RKP4979	TOP10 + pLW1	This study
DH5 α	F ⁻ ϕ 80dlacZ Δ M15	Life Technologies
Plasmids		
PCR2.1	Cloning vector; Ap ^r Kan ^r	Invitrogen
pBAD-TOPO	Cloning vector, Ap ^r	Invitrogen
PJMK30	<i>Campylobacter coli</i> Kan ^r cassette in pUC19	Baillon <i>et al.</i> (1999)
PSP105	<i>C. coli</i> integrative vector, Tet ^r	Dickinson <i>et al.</i> (1995)
PAV103	Cm ^r cassette cloned into <i>EcoRV</i> site of <i>ahpC</i>	Baillon <i>et al.</i> (1999)
PGEMCWH01	Cj0752 with an internal cloning site in pGEM T-vector	C. Holmes & P. A. Lund (unpublished results)
PKE26	1124 bp PCR product containing <i>ctb</i> in pBAD-TOPO	This study
PKE29	Kan ^r cassette cloned into a unique <i>Bgl</i> II site in <i>ctb</i> in pKE26	This study
PKE2	1187 bp PCR product containing <i>cgb</i> in pBAD-TOPO	Elvers <i>et al.</i> (2004)
PKE18	Kan ^r cassette cloned into a unique PCR-generated <i>Bgl</i> II site in <i>cgb</i> in pKE2	Elvers <i>et al.</i> 2004)
PKE34	Tet ^r cassette cloned into a unique <i>Bgl</i> II site in <i>ctb</i> in pKE26	This study
PKE112	700 bp PCR product containing <i>ctb</i> and its promoter cloned between <i>Bam</i> HI and <i>Kpn</i> I in pGEMCWH01	This study
PKE115	Cm ^r cassette cloned into <i>Kpn</i> I site of pKE12	This study
pBAD/His C	Cloning vector; Ap ^r	Invitrogen
PLW1	380 bp PCR product containing <i>ctb</i> cloned between <i>Nco</i> I and <i>Hind</i> III in pBAD/His C	This study

Leicester) was amplified by PCR using oligonucleotide primers CAT3 (5'-GGTACCTGCTCGGCGGTGTTCCCTTTCCAAG-3') and CAT4 (5'-GGTACCTGCGCCCTTTAGTTCCTAAAGGGT-3') with unique restriction sites for *Kpn*I. This cassette was then inserted into the *Kpn*I site of pKE112 to create construct pKE115. The suicide plasmid pKE115 therefore contains a wild-type copy of *ctb* and, downstream, a selectable chloramphenicol-resistance marker flanked by Cj0752. The plasmid pKE115 was confirmed using PCR and restriction digestion. This plasmid was introduced into competent cells of RKP1386 by electroporation and transformants were selected on Mueller-Hinton agar with chloramphenicol (10 μ g ml⁻¹). The homologous recombination that resulted in a double crossover event was verified by PCR using HB3, HB4 and primers for Cj0752. The resulting strain was designated RKP5404.

Growth curves and viable counts. For growth curves, *C. jejuni* was grown as above. Samples (1 ml) were removed at 2, 4, 7, 10, 12, 24 and 48 h and the apparent absorbance (OD₆₀₀) measured on a Jenway 6100 spectrophotometer. Viable counts were performed using 96-well plates and multiwell pipettes, in triplicate, and each dilution series was plated three times using 10 μ l drops. To measure survival in air, *C. jejuni* were grown for 14 h microaerobically and then transferred to an air incubator, where they were shaken at the same temperature and speed as before for 12 h. Samples (10 μ l) were taken in triplicate every 90 min. *C. jejuni* cells were exposed to NOC-5 and -7, paraquat and H₂O₂ in microtitre plates. Cells were

grown for 8 h before exposure. NOC compounds were used at 5, 20, 50 and 100 μ M and the exposure time was 1 h. H₂O₂ was used at 0.01, 0.1, 0.2 and 0.4 mM, and paraquat at 1, 5, 10, and 100 μ M; the exposure time for both was 30 min. Serial dilutions were performed immediately thereafter.

Disk diffusion assays. The sensitivity of *C. jejuni* NCTC 11168, RKP1386 (*ctb*), RKP1336 (*cgb*) and RKP1389 (*cgb ctb*) to a range of stress-inducing agents, *tert*-butyl hydroperoxide (39 mM), paraquat (117 mM), cumene hydroperoxide (158 mM), hydrogen peroxide (265 mM) and S-nitrosoglutathione (100 mM) was assayed in a plate diffusion assay using the method described by Baillon *et al.* (1999).

Determination of culture oxygen transfer rate constants. Baffled flasks (250 ml) were loaded with 100, 150 and 200 ml distilled H₂O and deoxygenated in the microaerobic cabinet using a few grains of dithionite. The flasks were then shaken at 115 r.p.m. and their reoxygenation within the cabinet followed using a Hanna portable waterproof dissolved oxygen probe (model HI 9142). A plot was made of the percentage deoxygenation against time on a semilog scale, and the rate constant *K* was determined by dividing $\ln 2$ (0.693) by the time at which the water was 50% deoxygenated (Poole, 1976). The oxygen electrode was calibrated by placing in stirred water for the 100% air saturation value; dithionite was added to determine the zero point.

Determination of K_m and V_{max} values of whole-cell respiration. These measurements were made using the deoxygenation of oxyleghaemoglobin (oxyLHb) and oxymyoglobin (oxyMb) as sensitive reporters of oxygen concentration, according to the protocol and calculations detailed by Appleby & Bergersen (1980). LHb was kindly donated by Dr F. J. Bergersen, CSIRO Division of Plant Industry, Canberra, Australia. The LHb was treated with a fivefold excess of sodium dithionite and oxygenated by passage down a 50×5.0 cm column of Sephadex G25 (Amersham) in aerated 50 mM potassium phosphate pH 7.4, 1 mM EDTA. Horse skeletal muscle Mb (Sigma) was oxygenated similarly, using a PD-10 column (Amersham). The oxyLHb or oxyMb was diluted to a concentration of 10–15 μ M in a cuvette with 25 mM potassium phosphate, pH 7.0, plus 1 mM EDTA. The cuvette was sealed and additions of *C. jejuni* cells and formate as respiratory electron donor, 5 mM final concentration, were made. These studies were carried out on a custom-built SDB3.5 dual-wavelength scanning spectrophotometer (University of Pennsylvania School of Medicine Biomedical Instrumentation Group and Current Designs, Philadelphia, PA, USA) (Kalnieks *et al.*, 1998) in time-scanning mode. The ΔA was measured between 576 and 565 nm for oxyLHb, and 582 and 565 nm for oxyMb. The data were transferred to Excel and calculations were performed according to Appleby & Bergersen (1980).

Cloning and expression of *ctb* in *E. coli*. Genomic DNA was isolated from NCTC 11168 using guanidium thiocyanate (Pitcher *et al.*, 1989). The forward (RP268, 5'-AAAATTAACATTTAA-CCATGGCTTATATGAAATTTGAAAC-3') and reverse (RP267, 5'-GAAAAGGTAAAAAAGCTTTGGCAAAAAAATTG-3') primers to amplify *ctb* contained an *NcoI* or *HindIII* site respectively. The 0.38 kb fragment (corresponding to *ctb*) was recovered with a Qiaquick gel extraction kit (Qiagen), cloned between the *NcoI* and *HindIII* sites of pBAD/His (Invitrogen) to make pLW1 and transformed into *E. coli* TOP10 using the method of Inoue *et al.* (1990) to make strain RKP4979. The construct pLW1 was checked by sequencing from just upstream of the promoter through to the end of the insert. For Ctb overexpression, starter cultures in LB were grown overnight. A 1% inoculum was used in expression cultures supplemented with ampicillin, 200 μ M δ -aminolaevulinic acid and 12 μ M $FeCl_3$. The cultures were shaken at 200 r.p.m. until an OD₆₀₀ of ~0.5 had been reached; they were then induced with 0.02% arabinose and grown for a further 4 h. Concentrations of δ -aminolaevulinic acid, $FeCl_3$ and arabinose were selected after preliminary optimization studies.

Ctb purification and expression studies. Polyclonal antiserum against Ctb was raised by immunizing rabbits with pure Ctb. Cells from 4 litres of culture were harvested by centrifugation at 5000 g for 10 min at 4 °C and resuspended in 80 ml 50 mM Tris/HCl (pH 7.0). Cells were disrupted by sonication and centrifuged at 21 000 g for 15 min at 4 °C to remove cell debris. The reddish-brown supernatant was loaded onto a 30 ml DEAE Sepharose Fast Flow (Pharmacia Biotech) column equilibrated with 50 mM Tris/HCl (pH 7.0). The column was washed with 40 ml of the same buffer and Ctb eluted with a NaCl gradient (from 0 to 0.5 M) in 50 mM Tris/HCl (pH 7.0). Fractions to be carried forward for the next step were chosen on the basis of coincidence of the haem and protein peaks in the UV absorption profile. The eluate was concentrated to ~1.4 ml using a Vivaspin 20 concentrator (Vivascience) with a molecular mass cut-off of 5 kDa. This fraction was further purified by gel filtration. A Superdex-200 column (16 $\times$ 60 cm, Amersham Biosciences) was equilibrated with 50 mM Tris/HCl (pH 7.0), 0.2 M NaCl. A 1 ml portion of the previous fraction was applied and eluted in the equilibration buffer at a flow rate of 1 ml min⁻¹. The haem and protein elution profiles were monitored at 412 and 280 nm respectively. Pure Ctb was stored at 4 °C.

Production of antibody was performed by S. Smith in The University of Sheffield Antibody Resource Centre. Protein (250–500 μ g) in a maximum volume of 250 μ l PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) was mixed with an equal volume of Freund's adjuvant. This was passed through a 23G needle until an emulsion formed. Two rabbits were each injected subcutaneously with a maximum of 500 μ l. After 22 days, the injections were repeated but with incomplete Freund's adjuvant; this was performed again another 20 days later. Sample bleeds took place after a further 10 days, followed by Western blotting to check the antisera. After a final booster injection, the rabbits were bled out 10 days later. The antibody was aliquoted and stored at -70 °C; the aliquot in use was stored at 4 °C.

For Western blotting, crude extracts were resolved on SDS-PAGE using 5–20% Tris/HCl Ready Gels (Bio-Rad). The Bio-Rad protein assay was used with BSA standards to ensure that 20 μ g protein was loaded per lane. Proteins were transferred onto a nitrocellulose membrane in a mini-transblot apparatus (Bio-Rad) at 75 mA overnight. Immobilized proteins were probed with primary anti-Ctb antibody and secondary antibody (horseradish-peroxidase-conjugated anti-rabbit IgG, Sigma) and developed using ECL detection reagents (Amersham).

Polarographic measurements of O₂ consumption. A digital Clark-type electrode system (model 10; Rank Brothers) was used. *C. jejuni* cells were grown to early exponential phase and resuspended in 50 mM potassium phosphate (pH 7.3). Supernatant subcellular fractions were prepared by sonicating whole cells and centrifuging out cell debris at 21 000 g for 15 min. Membranes were spun out at 142 000 g for 1 h. The electrode chamber was stirred and used at a working volume of 1.5 ml at 42 °C. The electrode was calibrated with air-saturated 50 mM potassium phosphate (pH 7.3), assuming an oxygen concentration of 220 μ M. A few grains of dithionite were used to zero the electrode. The same buffer was used to perform experiments. Intact cells or supernatant subcellular fractions were added in sufficient amount to give measurable O₂ uptake rates. Formate (5 mM) was used as a reductant for cells and NADH (0.5 mM) for supernatant fractions (all expressed as final concentrations).

RNA extraction. RNA was extracted from *C. jejuni* NCTC 11168 and RKP1386 (*ctb* mutant) using RNeasy and RNaProtect purification and stabilization kits (Qiagen). Both strains were grown in 50 ml Mueller–Hinton medium microaerobically at 37 °C to an OD₆₀₀ of 0.2 and then 5 ml of each strain was directly pipetted into 10 ml RNaProtect (Qiagen). The cells were pelleted by centrifugation and lysed with TE buffer (100 μ l) containing lysozyme (1 mg ml⁻¹; Sigma). The RNA was extracted and contaminating DNA was removed for 1 μ g total RNA by treating with a DNA-free kit (Ambion) according to the manufacturer's instructions.

Reverse transcriptase PCR. First-strand cDNA synthesis was performed using 100 ng total RNA with random hexamers (250 ng, Invitrogen) and Superscript III RT (Invitrogen). PCR was carried out under the conditions described previously (Elvers *et al.*, 2004) with the following oligonucleotides: to amplify *recG* (Cj0464), forward primer 5'-GTGCGGCTTTGATGGTTTAT-3' and reverse primer 5'-TCTTGGAATAGGCGTAGCTGA-3' were used, and for Cj0466, forward primer 5'-ATGAAGGCGAAGAGGCTAAA-3' and reverse primer 5'-TCGAGATAAAGATTGCGCTCTA-3' were used. DNA gyrase subunit A gene (Cj1027c) was used as a 'housekeeping' control gene with forward primer 5'-AGGCACACGCTTAATATA-ACCA-3' and reverse primer gyrA2 5'-GGTGTGATTATGTTGGCA-ATTCAT-3'. The PCR products were analysed by gel electrophoresis on 1.5% agarose.

Fig. 1. *C. jejuni* cells grown in 100 ml Mueller–Hinton medium in 250 ml baffled conical flasks in an atmosphere of 10% O₂ (v/v). The experiment was performed in triplicate and means were plotted. (a) ◆, Wild-type cells; ■, *ctb* mutant cells; ▲, the complemented *ctb* mutant. (b) ◆, *cgb* mutant cells; ■ the double Hb mutant. Error bars indicate SD (in many cases too small to be seen).

RESULTS

Construction of globin mutants in *C. jejuni*

Analysis of the genome sequence of *C. jejuni* NCTC 11168 (Parkhill *et al.*, 2000) reveals coding sequences for two haemoglobin-like proteins. Cgb is a NO-detoxifying single-domain globin that has been characterized previously (Elvers *et al.*, 2005). The second, encoded by Cj0465c, which we designate Ctb (Campylobacter truncated globin), is of the truncated type as described by Wittenberg *et al.* (2002).

To elucidate the role of Ctb, a deficient mutant, RKP1386, was constructed as described in Methods, with the antibiotic-resistance marker orientated with the same transcriptional polarity as *ctb*. To eliminate the possibility that the phenotype of the *ctb* mutant would be masked by Cgb, the second globin, a mutant deficient in both *C. jejuni* globins, RKP1389, was also constructed (see Methods). The kanamycin-resistance cassette used in the construction of the *ctb* mutant contains its own promoter but lacks a transcriptional terminator and, when the resistance cassette is inserted with the same transcriptional polarity as the mutated gene, mutations have been shown to be non-polar on downstream genes (Hickey *et al.*, 2000). Furthermore, the insertion of the kanamycin-resistance cassette did not affect expression of the genes immediately proximal to *ctb* as determined by reverse transcriptase PCR (data not shown) and polar effects are minimal. To test unequivocally that the *ctb* gene is directly linked to the observed phenotypes, we also constructed a strain (RKP5404) in which the *ctb* mutation was complemented by transforming RKP1386 with a plasmid containing *ctb* under the control of its own promoter that then recombined onto the *C. jejuni* chromosome at a second location.

Growth of *C. jejuni* at various aeration rates

The presence of the single-domain globin Vgb in micro-aerobically growing cells offers a growth advantage to *E. coli* (Khosla & Bailey, 1988) and the implication is that Vgb

functions to this end in *Vitreoscilla*. For this reason, growth curves were performed on *C. jejuni* grown at different aeration rates in order to determine if the presence of Ctb conferred a positive effect under conditions of either low or high oxygen provision. The oxygen transfer rate to cultures was varied by using 100, 150 or 200 ml medium in 250 ml baffled flasks since, in a rotary shaker, the O₂ solution rate falls rapidly as the liquid volume is increased (Pirt, 1975). Values of *K* (the O₂ transfer constant) were calculated for each volume in flasks shaken at 115 r.p.m. An O₂ transfer constant of 0.06 min⁻¹ was found for 200 ml H₂O in a 250 ml flask, 0.16 min⁻¹ for 150 ml and 0.43 min⁻¹ for 100 ml. Thus, the volume of medium determines the rate of oxygen transfer into the culture.

For each strain, the growth curves were performed in triplicate on at least two separate occasions. Wild-type *C. jejuni* cells (Fig. 1a) grown in 100 ml medium reached a significantly higher OD₆₀₀ (1.1) than the same strain grown in a higher volume of medium (data not shown). However, when inoculated into 100 ml medium, the *ctb* mutant grew significantly less well than the wild-type, achieving only 0.7 OD units ($P = 6.77 \times 10^{-5}$, assuming an equal variance (two tailed, Fig. 1a). There was no difference in growth rate of the two strains for the majority of the exponential phase (1–10 h), but the mutant slowed in growth as it approached stationary phase. No significant difference was observed between the *ctb* mutant and wild-type cells when grown at lower oxygen transfer rates (i.e. in 150 and 200 ml Mueller–Hinton broth) but yields attained by both strains decreased as *K* decreased (data not shown). The *cgb* mutant and the wild-type strains grew similarly in all three volumes of medium (Fig. 1b for 100 ml Mueller–Hinton; data not shown for 150 and 200 ml). The strain mutated in both globins consistently reached the same final OD₆₀₀ as the wild-type (Fig. 1b for 100 ml Mueller–Hinton; data not shown for 150 and 200 ml). The complemented *ctb* mutant strain did not display the attenuated growth of the mutant strain when grown in 100 ml medium (Fig. 1a). Hence complementation with the *ctb* gene reversed the effect of the mutation.

The *ctb* and other Hb mutants of *C. jejuni* are defective in survival in air

In light of the decreased ability of the *ctb* mutant to grow at high O₂ transfer rates (Fig. 1), the survival in air of the wild-type and various Hb mutant strains was investigated. Cells were grown to late exponential phase and then transferred to an air incubator, where they were shaken as before. Samples were taken for viable counts in triplicate at intervals. Fig. 2 is representative of three experiments and shows that the three mutant strains survived equally poorly in comparison to wild-type cells. The wild-type strain did not lose viability until 10.5 h (not shown). The *t* test, assuming an equal variance (two tailed), showed that the difference in viability between each mutant and NCTC 11168 at 9 h was significant, showing *P* values of 3.2×10^{-9} – 3.1×10^{-7} . The differences in viability between the three mutants were considerably less significant or insignificant (*P* values 0.001 to 0.08).

Ctb affects the oxygen consumption properties of *C. jejuni*

To determine the effect of the presence of Ctb on respiration rates and substrate-stimulated O₂ uptake rates, O₂ consumption was followed in suspensions of intact cells and supernatant subcellular fractions. For the latter, membranes had been centrifuged out, removing the contribution from terminal respiratory oxidases. *C. jejuni* cells in the electrode chamber at 42 °C were exposed to up to 220 µM O₂, considerably higher than is optimal for growth. Cells in early exponential phase were used and each trace repeated at least six times with different batches of cells. Wild-type cells [$30.5 \text{ nmol O}_2 (\text{mg total cell protein})^{-1} \text{ s}^{-1}$, SD 2.5] displayed almost double the oxygen consumption of the *ctb* mutant [$16.8 \text{ nmol O}_2 (\text{mg total cell protein})^{-1} \text{ s}^{-1}$, SD 1.3]. The *t* test, assuming equal variance (two tailed) found the difference between the two strains to be significant

Fig. 2. Survival in air of *C. jejuni* mutants. Cells were grown at 42 °C in an atmosphere of 10% (v/v) O₂ to late exponential phase, and then transferred to an air incubator. Samples (10 µl) were taken every 1.5 h and viable counts performed in triplicate. ♦, Wild-type cells; ■, the *ctb* mutant; ▲, the *cgb* mutant; ●, the *cgb ctb* mutant. The data are normalized to the viability at 0 h for each strain. The data shown are representative of three experiments.

($P = 1.34 \times 10^{-5}$). The *cgb* and double mutant consumed O₂ at a rate similar to that of wild-type cells (data not shown). The oxygen consumption rates of supernatant fractions, presumed to have contributions from unidentified diaphorases and/or oxygenases, as well as globins, were three orders of magnitude lower than those obtained with whole cells when compared on a protein basis. The difference between the two strains was not statistically significant.

Determination of *V*_{max} and *K*_m of *C. jejuni* whole cells for O₂

Since we had obtained evidence for a role for Ctb in promoting aerobic growth and oxygen consumption, we examined the oxygen consumption of cells at low oxygen tensions to determine whether Ctb affected the *K*_m or *V*_{max} of the cell for O₂. In the past this technique has been used to determine the *K*_m values of several high-affinity oxidases for O₂ (Contreras *et al.*, 1999; D'Mello *et al.*, 1994, 1995, 1996; Smith *et al.*, 1990). The technique relies on the spectrophotometric differences between an oxygenated (exogenous) reporter Hb and its deoxy form. Oxygenated globin (LHb or Mb) was placed with buffer in a cuvette to which further O₂ entry was prevented. Additions of reductant and cells were made: initially cells respired using oxygen present in the buffer; when this had been exhausted they started to deoxygenate the Hb. This deoxygenation was followed spectrally; a typical kinetic trace showing oxygen consumption by the cells is represented in Fig. 3. Both oxyLHb and oxyMb were used. LHb has the higher oxygen affinity and is appropriate for determining respiration by the high-affinity (R. Jackson & R. K. Poole, unpublished) cytochrome *cbb*₃-type oxidase. Use of oxyMb is suited to observing

Fig. 3. Deoxygenation of oxyLHb by *ctb* mutant cells. At time zero, the cuvette contained oxyLHb at approximately 15 µM in buffer (25 °C), which had been previously sparged with nitrogen. Formate (5 mM) (A) was added, followed by whole cells (B). Spontaneous deoxygenation of the globin was observed (C), during which phase *K*_m and *V*_{max} values were derived as described in Methods. Sodium dithionite was added to demonstrate complete deoxygenation (D).

Fig. 4. Deoxygenation of LHb by *ctb* and *cgb* mutants. (a, c) Plots of total oxygen (calculated from the loss of oxyLHb) against time for the *ctb* ($R^2=0.99$) and *cgb* ($R^2=0.99$) mutants, respectively. (b, d) Eadie–Hofstee plots (V against V/S) for the *ctb* ($R^2=0.98$) and *cgb* ($R^2=0.96$) mutants. The data shown are derived from a single scan, but are representative of the four scans that were carried out for each combination of strain and globin.

respiration by the low-affinity oxidase. The experiments were carried out using wild-type cells, and the *ctb*, *cgb* and *cgb ctb* mutants. Only data that were within the working range of the two globins were examined (D'Mello *et al.*, 1994). Fig. 4 displays, as examples, plots of total oxygen (that bound to LHb plus that present in solution in the cuvette) against time and Eadie–Hofstee plots for the *ctb* and *cgb* mutants using oxyLHb as a source of O_2 . Both Eadie–Hofstee and Lineweaver–Burk plots were obtained for all strains. However, Eadie–Hofstee plots were taken to be the more reliable linear transformation as the Lineweaver–Burk plot has a tendency to give overly large estimates of $V_{\max}$ and K_m when applied to unweighted data (Dowd & Riggs, 1965). The t test, assuming an equal variance (two tailed), was used to analyse the significance of the data. No significant difference in K_m values was seen between the wild-type and the globin mutants (Table 2) using either oxyLHb or oxyMb

as reporters. However, the $V_{\max}$ of the *ctb* mutant was 2.5-fold greater than the wild-type when assayed using oxyLHb ($P=0.0027$). The difference was not so pronounced with oxyMb, the *ctb* mutant value being 1.6-fold greater than the wild-type ($P=0.00015$). The *cgb* and double mutant values were not significantly different from the wild-type. All scans were repeated at least four times for each combination of strain and oxygenated globin.

Figure 5 shows a compilation of O_2 uptake rates recorded for both strains over the whole range of extracellular O_2 concentrations measurable by the two exogenous globins and the Clark-type O_2 electrode. Averaged values of O_2 concentrations could not be used for the O_2 uptake rates measured from the two globins as the scanning spectrophotometer recorded changes in absorbance at time intervals and there is some small variation between scans. For both

Table 2. Values of K_m and $V_{\max}$ ($\text{nmol mg}^{-1} \text{s}^{-1}$) of *C. jejuni* cells for oxygen calculated from the deoxygenation of oxyLHb and oxyMb

The values shown are means $\pm$ SD of four determinations. The units of K_m are nM for OxyLHb and μM for OxyMb.

Strain	OxyLHb				OxyMb			
	Lineweaver–Burk		Eadie–Hofstee		Lineweaver–Burk		Eadie–Hofstee	
	$V_{\max}$	K_m	$V_{\max}$	K_m	$V_{\max}$	K_m	$V_{\max}$	K_m
NCTC 1118	2.84 ± 1.71	47.7 ± 3.4	2.54 ± 1.36	41.6 ± 2	35.2 ± 3.5	0.88 ± 0.01	36.2 ± 4.2	0.92 ± 0.02
<i>ctb</i>	7.73 ± 0.69	51.6 ± 1.5	6.45 ± 0.63	39.5 ± 1.2	58.5 ± 1.2	0.89 ± 0.03	59.5 ± 3.6	0.91 ± 0.01
<i>cgb</i>	2.44 ± 0.26	46.9 ± 0.9	2.27 ± 0.28	42.9 ± 1.1	42.5 ± 1.3	0.88 ± 0.02	43.6 ± 0.9	0.91 ± 0.03
<i>cgb ctb</i>	4.05 ± 0.18	49.2 ± 0.7	3.58 ± 0.11	40.8 ± 0.48	37.2 ± 3.4	0.91 ± 0.01	37.3 ± 3.3	0.91 ± 0.01

Fig. 5. O_2 consumption by *C. jejuni* cells at all concentrations of O_2 tested. The dashed line and filled symbols refer to O_2 consumption by wild-type cells; the continuous line and open symbols refer to O_2 consumption by the *ctb* mutant. Squares represent O_2 consumption using oxyLHb as a source of O_2 , triangles represent O_2 consumption determined using oxyMb, and diamonds show O_2 consumption in the O_2 electrode. O_2 consumption measurements where an oxygenated globin was the O_2 source were plotted by determining values of V for arbitrary concentrations of O_2 . The rates of O_2 consumption calculated from the O_2 electrode are averaged from a minimum of four traces; the error bars are the standard deviation of the mean. The brackets below the graph represent the optimal O_2 concentration ranges in which the two reporter globins and the electrode function reliably.

strains the use of oxyLHb as an O_2 source appeared to underestimate O_2 consumption at O_2 concentrations greater than $0.1 \mu M$, as shown for *Rhizobium* by Bergersen & Turner (1979). Loss of Ctb stimulated respiration at O_2 tensions below about $1 \mu M$ (see above, Table 2) but resulted in a marked decline in respiration at higher O_2 tensions. We can conclude that Ctb plays a role in moderating respiration in this microaerophile.

Effects of oxidative and nitrosative stress on *C. jejuni* cells

To explore further the phenotypes of the globin mutants, *C. jejuni* wild-type cells, the two single globin mutants and the double mutant were exposed to a number of stressing compounds using either viable counts or disk diffusion assays as a measure of sensitivity. For the viable counts, *C. jejuni* cells were grown microaerobically to mid-exponential phase, then exposed to the NO-donating compounds NOC-5 and NOC-7 for 1 h. NOC-5 and -7 have half-lives of NO release of 93 and 10 min respectively and, when used in combination, provide sustained levels of NO in solution (Cruz-Ramos *et al.*, 2002). The NOC compounds were each used at concentrations of 5, 20, 50 and $100 \mu M$. Fig. 6 shows that the *ctb* mutant displayed similar sensitivity to the wild-type,

whereas the *cgb* mutant possessed markedly increased sensitivity, showing a sharp drop in viability even at the lowest NOC concentration. The *t* test, assuming an equal variance (two tailed), showed that the difference in viability between the *cgb* mutant and NCTC 11168 at $5 \mu M$ NOC was significant ($P=0.006$). In contrast, the difference in viability between the *ctb* mutant and the wild-type was not significant ($P=0.19$). This is consistent with the role of Cgb in NO detoxification (Elvers *et al.*, 2004). Interestingly, the double mutant did not have the sensitivity of the *cgb* mutant, and behaved very similarly to the wild-type and *ctb* mutant strains (data not shown). Cells were also exposed to paraquat (1 – $100 \mu M$) and H_2O_2 (0.01 – 0.4 mM) for 30 min. However, no significant difference was seen between the viabilities of wild-type cells and the three globin mutants (data not shown).

Table 3 shows the results of disk diffusion assays that were performed on solid medium in the presence of (concentrations given are those applied to the disk): GSNO (100 mM), *tert*-butyl hydroperoxide (39 mM), cumene hydroperoxide (158 mM), paraquat (117 mM) and hydrogen peroxide (265 mM). Under these conditions, both the *cgb* and *cgb ctb* mutant were hypersensitive to GSNO, displaying a significantly greater zone of killing compared to the wild-type [$P=0.002$ (*cgb*) and 0.0001 (*cgb ctb*)]. The *ctb* mutant showed a greater zone of killing with paraquat compared to the wild-type; however, this difference was only marginally significant ($P=0.04$). The *ctb* mutant and the *cgb ctb* mutants displayed a small though significant decrease in sensitivity to *tert*-butyl hydroperoxide ($P=0.02$) and paraquat ($P=0.002$) respectively.

Expression of Ctb in response to stress conditions

Western blots were performed on crude extracts from *C. jejuni* wild-type cells that had been exposed to stressful conditions. Ctb could always be easily detected in unstressed cells. This contrasts with Cgb, which is almost undetectable

Fig. 6. Exposure of *C. jejuni* mutants to the NO-donating compounds NOC-5 and NOC-7. Cells were grown to mid-exponential phase and exposed to both NOC compounds for 1 h. Viable counts were performed in triplicate. The data are normalized to the viability in the absence of NOC for each strain. ♦, Wild-type cells; ■, the *ctb* mutant; ▲, the *cgb* mutant; ●, the *cgb ctb* mutant. The data shown are representative of three experiments.

Table 3. Resistance and sensitivity of *C. jejuni* strains to agents of oxidative and nitrosative stress

The sensitivity of *C. jejuni* NCTC 11168 and the *ctb*, *cgb* and *cgb ctb* mutants to a range of stress-inducing agents, *tert*-butyl hydroperoxide (39 mM), paraquat (117 mM), cumene hydroperoxide (158 mM), hydrogen peroxide (265 mM) and *S*-nitrosoglutathione (100 mM) was assayed on disk diffusion plates. Values are expressed as mean diameter of the zone of killing $\pm$ SEM. Asterisks indicate significant *P* values, where $P < 0.05$ (see Results for further information). *P* values are a result of the *t* test, assuming an equal variance (two tail) comparing the wild-type with each mutant.

Stress inducer	Initial concn on disk (mM)	Diameter of disk inhibition zone (mm)			
		NCTC 11168	<i>cgb</i>	<i>ctb</i>	<i>cgb ctb</i>
GSNO	100	9.2 $\pm$ 0.9	18.4 $\pm$ 2.4*	10.7 $\pm$ 1.2	27.3 $\pm$ 0.5*
<i>tert</i> -Butyl hydroperoxide	38.8	51.7 $\pm$ 2.2	48.0 $\pm$ 1.1	44.8 $\pm$ 1.3*	44.8 $\pm$ 2.6
Cumene hydroperoxide	157.8	43.9 $\pm$ 2.1	42.6 $\pm$ 0.7	39.4 $\pm$ 0.8	44.2 $\pm$ 0.6
Paraquat	116.6	37.2 $\pm$ 0.8	36.3 $\pm$ 0.6	39.6 $\pm$ 1.6*	32.8 $\pm$ 0.5*
Hydrogen peroxide	264.6	38.7 $\pm$ 2.5	37.2 $\pm$ 0.5	35.2 $\pm$ 1.6	37.2 $\pm$ 0.4

in cultures uninduced with GSNO (Elvers *et al.*, 2004). Cells were grown in 150 ml medium to mid-exponential phase and exposed to 100 and 200 μ M GSNO (a nitrosating agent), SNAP (an NO-donating compound), paraquat, hydrogen peroxide, cumene hydroperoxide and *tert*-butyl hydroperoxide for 1 and 2 h. Controls were carried out using H₂O and DMSO (the solvent for alkyl hydroperoxides) (Fig. 7a). Upregulation of *ctb* was observed only on treatment by GSNO or SNAP. Some decrease in Ctb synthesis by the oxidative stress-inducing compounds paraquat, H₂O₂, cumene hydroperoxide and *tert*-butyl hydroperoxide, was seen, particularly at 200 μ M. The inducing effect of GSNO and SNAP was dose- and time-dependent: higher levels of Ctb were seen when using 200 μ M as compared with 100 μ M. When using 100 μ M of either GSNO or SNAP, the level of Ctb increased between the 1 h and 2 h time points.

In light of the attenuated growth of the *ctb* mutant at high O₂ transfer rates, Western blots were performed to determine if the growth phenotype was reflected in the expression of Ctb. *C. jejuni* wild-type cells were grown using 75, 100, 150 and 200 ml medium in 250 ml baffled flasks, for 6, 9, 12, 24 and 48 h. No difference in expression between the different oxygen transfer constants was seen at any of these time points. Fig. 7(b) shows the blot taken from cells grown for 9 h. Finally, wild-type cells were grown in 150 ml medium and harvested at 6, 9, 12, 24 and 48 h in order to observe the expression of *ctb* over time (Fig. 7c). The level of Ctb was decreased slightly at 12 h in comparison to the level of protein at 6, 9, 24 and 48 h.

DISCUSSION

The data described in this paper demonstrate that both *Campylobacter* globins are required for survival on transfer to air (Fig. 2). However, a role for Ctb in cells exposed to higher than optimal levels of O₂ is indicated by both the growth curves at various *K* values (Fig. 1), and perhaps the behaviour of whole cells in the O₂ electrode. Nevertheless, Ctb expression appears not to be upregulated at elevated O₂ tension (Fig. 7). In contrast, the *Vitreoscilla* globin Vgb is

expressed optimally at low O₂ tensions (Dikshit *et al.*, 1989) and, when expressed in *E. coli*, improves growth under microaerobic conditions (Khosla & Bailey, 1988), not excess O₂. Thus it was postulated that Vgb is important for growth

Fig. 7. Western blots using Ctb antibody on *C. jejuni* crude extracts. Cells were grown in an atmosphere of 10% O₂ at 42 °C in 250 ml baffled flasks, sonicated, and the cell debris centrifuged out at 21 000 *g* for 15 min. Twenty micrograms of protein was loaded per lane. For the blots displayed in (a), wild-type cells were grown to mid-exponential phase and exposed to DMSO (solvent control, lane 2), GSNO (lane 3), SNAP (lane 4), paraquat (lane 5), H₂O₂ (lane 6), cumene hydroperoxide (lane 7) and butyl hydroperoxide (lane 8). Lane 1 is the control lane (untreated cells). For (b), wild-type cells were grown under conditions of different O₂ availability by shaking for 9 h in 250 ml flasks the following volumes: 75 ml (lane 1), 100 ml (lane 2), 150 ml (lane 3) and 200 ml (lane 4). For (c), wild-type cells were grown in 150 ml Mueller–Hinton broth for 6 h (lane 1), 9 h (lane 2), 12 h (lane 3), 24 h (lane 4) and 48 h (lane 5). The blots shown are representative of at least three experiments.

of the obligate aerobe *Vitreoscilla* in its native environment where it is exposed to hypoxic conditions.

The *ctb* mutant was not markedly sensitive to the majority of the agents of oxidative stress tested, showing only marginal sensitivity to paraquat. This diminishes the likelihood that the attenuated growth and oxygen consumption of this strain under conditions of high oxygen are due to a heightened sensitivity to oxidative stress. Interestingly, although the *ctb* mutant survived in air considerably less well than wild-type cells, both the *cgb* mutant and the double mutant mimicked the drop in its viability. The latter two mutants did not, however, display the reduced growth and oxygen consumption of the *ctb* mutant. A mutant of *E. coli* defective in the flavoHb Hmp has increased sensitivity to paraquat (Membrillo-Hernández *et al.*, 1999) but the basis of this effect is not understood. No such sensitivity has been found for other microbes mutated in trHbs or indeed any other microbial globins (Wu *et al.*, 2003).

The data derived from the deoxygenation of oxygenated globins also signal an O₂-related role for Ctb. These measurements of O₂ uptake are different from those obtained in the O₂ electrode as, in this experiment, *C. jejuni* is consuming O₂ at very low buffered O₂ tensions. The absence of Ctb, or indeed Cgb, did not affect the affinity of either oxidase for O₂. The values for *K_m* remained close to 40 nM and 0.9 µM, which have recently been assigned to the *cco*- and *cyd*-encoded oxidases, respectively (R. Jackson & R. K. Poole, unpublished). However, the *V_{max}* of cells for O₂ was markedly altered by the absence of Ctb. The deoxygenation of oxyLHb by the *ctb* mutant gave a *V_{max}* value 2.5 times greater than that given by wild-type cells. When oxyMb was used as a source of O₂, the *V_{max}* displayed by cells deficient in Ctb was 1.6-fold greater than that displayed by wild-type cells. Fig. 5 shows that the combined use of oxyLHb, oxyMb and the Clark-type oxygen electrode provides an observable range of free O₂ covering more than four orders of magnitude. Bergersen & Turner (1979) have suggested that, at high levels of oxygenation of LHb, respiration rates (of *Rhizobium* spp.) are usually diminished, perhaps because there is an insufficient concentration gradient of oxyLHb for facilitated diffusion of oxygen to permit maximal respiration rates. Indeed, in Fig. 5, the data points obtained for both strains with oxyLHb at free O₂ concentration above 0.1 µM are lower than those obtained with oxyMb; the brackets (Fig. 5) indicate the preferred ranges of O₂ concentration for each globin. These ranges may be combined with the ability of the O₂ electrode to measure O₂ between air saturation (approx. 200 µM) and (at best) the oxygen concentrations appropriate for oxidases with a *K_m* of approximately 1 µM (Lundsgaard *et al.*, 1978). The combined data (Fig. 5) show that the presence of Ctb reduces oxygen consumption below about 1 µM but increases it between 1 µM and air saturation levels. Thus the physiological function of Ctb *in vivo* may be to moderate oxygen consumption. The optimal oxygen concentration for growth of campylobacters in the laboratory lies in the range 3–15 % (by volume in the gas phase) but

to our knowledge the optimal oxygen concentrations in solution under physiological conditions (i.e. in natural environments – avian and mammalian hosts and foods) have not been defined. However, at 25 °C and assuming a Henry's law constant of 1.2 M atm⁻¹ (see <http://www.henrys-law.org>) the oxygen solubilities in water equate to about 40–200 µM (3–15 % in the gas phase). These are close to the electrode-determined oxygen concentrations in Fig. 5, suggesting that, at high external concentrations, Ctb may function to increase oxygen consumption and decrease oxygen concentrations. This function might be regarded as analogous to respiratory protection in *Azotobacter vinelandii*, in which the oxygen-labile nitrogenase is protected by rapid O₂ consumption catalysed by cytochrome *bd* (Kelly *et al.*, 1990). Conversely, at low external oxygen concentrations, Ctb may serve to 'spare' oxygen consumption in this obligately microaerobic bacterium.

Our results relate only to external oxygen concentrations, and the intracellular oxygen levels in campylobacters (or any other bacteria) are not known precisely. However, Unden *et al.* (1995) have predicted, on the basis of oxygen diffusion constants and bacterial cellular dimensions that, under both aerobic (about 220 µM) and microaerobic (2–0.2 µM) conditions, the bacterial intracellular oxygen tension approximates to extracellular levels. Fig. 8 outlines a hypothetical situation *in vivo*. In the wild-type cell (Fig. 8a), Ctb is proposed to act as a chaperone for oxygen, delivering it to a/both terminal oxidase(s) in a manner similar to that proposed for Vgb expressed in *E. coli* (Park *et al.*, 2002) or LHb-facilitated O₂ flux in soybean nodule cells, where the diffusive flux of free O₂ through the cytoplasm is augmented by a flux of oxyLHb (Bergerson, 1996). In a *ctb* mutant cell (Fig. 8b), oxygen accesses the oxidase without globin-mediated facilitation; however, when O₂ is bound by neither the oxidase nor by Ctb, some oxygen may be utilized by Cgb (or a diaphorase) and reduced, perhaps to superoxide. Indeed, in the absence of nitric oxide, Hmp, the *E. coli* flavoHb, will convert oxygen to superoxide (Membrillo-Hernández *et al.*, 1996; Wu *et al.*, 2004). Because of the strong homology between Cgb and the haem domain of Hmp (33 % identity; Elvers *et al.*, 2004) it is possible that Cgb could function in the same way in the absence of NO. A partner reductase (at present not identified) would be necessary to transfer electrons to the Cgb haem for O₂ reduction. There seems no reason to suppose that the respiratory flux of electrons would be different in a globin mutant; hence it is possible that the value of *V_{max}* may be higher in *ctb* mutant cells because fewer electrons are needed to reduce oxygen to superoxide (equation 1) than are needed to reduce it to H₂O (equation 2).

Fig. 8. Hypothetical interactions between Ctb, Cgb and a respiratory oxidase. In (a), the inner (cytoplasmic) membrane of the wild-type cell is envisaged as accommodating a terminal oxidase that reduces oxygen on the cytoplasmic side after diffusion of the ligand through the membrane down a concentration gradient. Ctb reacts with oxygen, delivering it to the oxidase perhaps by a specific interaction with an oxidase subunit. Oxygen is thus protected from reduction by the second globin, Cgb. In (b), the *ctb* mutant reduces oxygen at the oxidase without intervention of Ctb but the oxygen is also accessible to univalent reduction by Cgb. The reactions are not chemically balanced.

Thus, in *ctb* mutant cells, more oxygen can potentially be consumed per electron than in wild-type cells, since the O_2 -buffering/chaperone role of Ctb is lacking.

This hypothesis can be extended to the *cgb* mutant, which would lack the superoxide-yielding reaction envisaged in Fig. 8(b). The data shown in Table 2 indicate that the *cgb* mutant is not significantly different from wild-type in the measured V_{max} value. However, the double (*cgb ctb*) mutant did not display the markedly elevated V_{max} of the *ctb* mutant (Table 2). We suggest that this may be the result of the lack of a Cgb-catalysed incomplete O_2 reduction and an adequate O_2 flux to the oxidase in the absence of the O_2 -binding Ctb. Fumarate reductase (Sellars *et al.*, 2002) may also reduce oxygen to superoxide, as documented in *E. coli* (Imlay, 1995). However, the rates of oxygen binding to, and dissociation from, the two globins have not been measured. Inspection of Fig. 5 shows that this effect of mutation in *ctb* is evident only at extracellular O_2 concentrations below about $1 \mu M$. Even if the intracellular concentration of oxygen equates to that outside (see above), it seems plausible that intracellular Ctb concentration might be sufficient for

such a role since immunoblotting (Fig. 7) shows significant levels of synthesis under all conditions tested. That bacterial globins can significantly facilitate oxygen transport to an oxidase has been suggested by the modelling by Khosla & Bailey (1989) of the effects of periplasmic Vgb when expressed in *E. coli*.

A different explanation must be proposed for the effects of *ctb* mutation at much higher extracellular concentrations (Fig. 5). Here, we argue that Ctb may play an important oxygen-protective physiological role by increasing oxygen uptake rates (see above), perhaps by facilitated diffusion or direct interaction of the oxygen-bound Ctb with a terminal oxidase. Such a mechanism has recently been demonstrated for the interaction of Vgb with subunit I (where oxygen is reduced at the haem-Cu binuclear centre) of the cytochrome *bo'*-type oxidase in *Vitreoscilla* (Park *et al.*, 2002). Such interaction is proposed to result in enhancement of micro-aerobic growth. Within the trHb family, only HbO of *M. tuberculosis* has been previously shown to facilitate oxygen transfer. In *E. coli* membrane vesicles, HbO was able to enhance oxygen consumption activity approximately two-fold and Liu *et al.* (2004) demonstrated a dynamic and weak interaction between *E. coli* cytochrome *bo* subunit I and HbO.

Ctb is present in *C. jejuni* at a readily detectable basal level. Although exposure to RNIs resulted in upregulation of *ctb* (Fig. 7), the *ctb* mutant was not hypersensitive to NOC compounds or GSNO. This is in contrast to the results obtained for the *cgb* mutant, which displayed increased sensitivity to all the agents of nitrosative stress tested. However, while the double globin mutant was sensitive to GSNO, it was not hypersensitive to the NOC compounds (Fig. 6). This is most likely due to the fact that exposure to GSNO was over a period of 24 h, considerably longer than the 1 h to which cells were exposed to NOC compounds. Furthermore, NO-donating compounds (NOCs) and nitrosating agents (GSNO) have different chemistries and hence differing targets and effects within the cell (Cooper, 1999; Li *et al.*, 2001; Nikov *et al.*, 2003). The hypothesis outlined in Fig. 8 is also relevant to conditions of nitrosative stress. If detoxification of NO by Cgb requires O_2 , its availability may limit oxidase-catalysed respiration. Under such conditions, the facilitation of O_2 transfer to the oxidases by Ctb would be beneficial and would be promoted by the increased expression of the trHb. Thus, since the functions of the two *C. jejuni* globins appear to be intimately related they might be expected to be co-regulated. This is indeed the case, since both *cgb* and *ctb* have recently been shown to be positively regulated by NssR, a member of the Crp-Fnr superfamily, in response to nitrosative stress (Elvers *et al.*, 2005).

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