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Published

2008

Journal Title

Journal of Bacteriology

DOI

10.1128/JB.00100-08

The *Campylobacter jejuni* thiol peroxidases Tpx and Bcp both contribute to aerotolerance and peroxide-mediated stress resistance but have distinct substrate specificities

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Running title: Roles of Tpx and Bcp in *Campylobacter jejuni*

Abstract

The microaerophilic food-borne pathogen *Campylobacter jejuni* experiences variable oxygen concentrations during its life-cycle, especially during transitions between the external environment and the avian or mammalian gut. Single knockout mutations in either one of two related thiol peroxidase genes, *tpx* and *bcp*, resulted in normal microaerobic growth (10% v/v oxygen) but poorer growth than the wild-type under high aeration conditions (21 % v/v oxygen). However, a *tpx/bcp* double mutant had a severe microaerobic growth defect and did not grow at high aeration in shake flasks. Although the single mutant strains were no more sensitive than the wild-wild-type in disc diffusion assays with hydrogen peroxide, organic peroxides, superoxide or nitrosative stress agents, in all cases the double mutant was hypersensitive. Quantitative cell viability and cellular lipid peroxidation assays indicated some increased sensitivity of the single *tpx* and *bcp* mutants to peroxide stress. Protein carbonylation studies revealed that the *tpx/bcp* double mutant had a higher degree of oxygen- and peroxide-induced oxidative protein damage than either of the single mutants. An analysis of the peroxidase activity of the purified recombinant enzymes showed that, surprisingly, Tpx only reduced hydrogen peroxide as substrate, whereas Bcp also reduced organic peroxides. Immunoblotting of wild-type cell-free extracts with Tpx or Bcp specific antibodies showed increased abundance of both proteins under high aeration compared to microaerobic growth conditions. Taken together, the results suggest that Tpx and Bcp are partially redundant antioxidant enzymes that play an important role in protection of *C. jejuni* against oxygen-induced oxidative stress.

Introduction

Campylobacter jejuni, a Gram-negative bacterium with a microaerobic growth requirement, is a major food-borne pathogen in both the developing and developed world (40; 26). *C. jejuni* is a commensal in chickens and contaminated chicken meat is a major source of infection (18). The genome sequence of *C. jejuni* strain NCTC 11168 was published in 2000 (27), and several other strains have been recently sequenced including strains 81116 (28) and 81-176 (15). However, despite the prevalence of *C. jejuni* mediated illness, the physiology of the bacterium is relatively poorly understood.

Oxidative stress is a major problem for any organism that uses oxygen as a terminal electron acceptor, as incomplete reduction of oxygen to water can yield reactive oxygen species (ROS) such as the superoxide anion (O_2^-) hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^\bullet$) (39). Build-up of these highly reactive species can lead to damage to proteins, nucleic acids and membranes. ROS are also produced by the immune system to kill invading microbes. Therefore, an ability to combat these compounds is key to the survival of bacterial pathogens in the environment and the host (39; 16). Bacteria have evolved a wide variety of mechanisms to combat ROS mediated stress. These include superoxide dismutase (29), catalase (39; 7), cytochrome *c* peroxidases (1), and alkyl hydroperoxide-reductase (AhpC) (2). AhpC belongs to a large family of enzymes known as the peroxiredoxins, which are present in both eukaryotes and prokaryotes (45; 31) with AhpC being the most widely studied. In bacteria, AhpC confers resistance to a broad range of oxidative stress agents including hydrogen peroxide, organic peroxides and lipid peroxides (14; 42; 10; 35) as well as stress caused by reactive nitrogen species in the form of peroxynitrite (3). *C jejuni* contains an AhpC homologue, which in strain

81116 has been shown in previous work to be important for aerotolerance and organic peroxide stress resistance but which, from mutant phenotype data, does not appear to contribute to hydrogen peroxide resistance (2).

Two other peroxiredoxins are widespread in bacteria. These are thiol peroxidase (Tpx) and the "bacterioferritin co-migratory protein" (Bcp). Tpx and Bcp appear to be able to use a wide variety of peroxides as substrates *in vitro*, such as hydrogen peroxide, organic peroxides, and lipid peroxides (6; 43; 32). Tpx has been reported to be periplasmic in *E. coli* (4), but has been shown to use the cytoplasmic thioredoxin system as an electron donor (46), so the location of the protein remains unclear. An *E. coli* mutant lacking Tpx activity is more sensitive to various oxidative stresses (5), and studies with a *Helicobacter pylori tpx* mutant suggested that Tpx plays a significant role in defence against both superoxide and peroxide mediated stress, as well as high oxygen concentrations (9; 25). The *H. pylori tpx* mutant also showed a deficiency in mouse colonisation when compared to wild type, implicating Tpx as an important virulence factor in this bacterium (25). Mutants lacking Bcp in *E. coli* are also more sensitive to stress induced by hydrogen peroxide and organic peroxides (17). *H. pylori bcp* mutants were somewhat more sensitive to peroxide stress when compared to the wild type (9; 43). In general, however, Bcp has been less well studied than Tpx.

C. jejuni contains both Tpx and Bcp homologues, which in strain NCTC 11168 are encoded by *Cj0779* and *Cj0271* respectively. In this study we have characterised the roles of these enzymes through phenotypic analysis of single and double mutants in the *tpx* and *bcp* genes, and by over-expression and purification of both enzymes in order to determine their preferred substrates. The results show that Tpx and Bcp are partially

redundant but together play important roles in resistance to a number of oxidative and nitrosative stress agents, as well as to molecular oxygen. Peroxidase assays with the purified proteins showed that Tpx is a dedicated hydrogen peroxide reductase, whereas Bcp is able to act on a wider variety of peroxide substrates. Finally, evidence from cellular fractionation studies suggests that both Tpx and Bcp are cytoplasmic enzymes in *C. jejuni.*

Materials and Methods

Bacterial strains, media, and culture conditions. *Campylobacter jejuni* strain NCTC 11168 was routinely grown at 37 ºC in a microaerobic atmosphere (10% [v/v] O_2, 5% [v/v] CO_2, 85% [v/v] N_2) in a MACS-VA500 growth cabinet (Don Whitley Scientific, Shipley, UK) on Columbia agar (Oxoid, Basingstoke, UK) containing 5% (v/v) lysed horse blood and amphotericin B and vancomycin (10 μg ml^{-1} each). Kanamycin and chloramphenicol were added to plates at final concentrations of 50 and 30 μg ml^{-1} respectively to select for *C. jejuni tpx* and *bcp* mutants where relevant. Microaerobic liquid cultures of *C. jejuni* were grown in 25-100 ml batches of Brain Heart Infusion broth supplemented with 5% (v/v) foetal bovine serum (BHI-FBS) contained in 250 ml shake flasks in the above microaerobic atmosphere and orbitally shaken at 100 rpm. For fully aerobic growth of *C. jejuni*, microaerobically grown overnight cultures were used to inoculate 50 ml BHI-FBS media contained in 250 ml baffled conical flasks . These cultures were grown in normal atmosopheric oxygen conditions at 37 ºC with 300 rpm shaking in order to aerate the cells as fully as possible.

Escherichia coli was routinely grown at 37 ºC with shaking at 200 rpm in Luria-Burtani (LB) supplemented with appropriate antibiotics. Growth of *C. jejuni* and *E. coli* was monitored by measuring the optical density (OD) at 600 nm using a Pharmacia Biotech Ultrospec 2000 spectrophotometer.

DNA isolation and manipulation. Plasmid DNA was isolated from *E. coli* using the QIAquick miniprep spin kit (QIAGEN). *C. jejuni* chromosomal DNA was isolated using the Wizard® Genomic DNA purification kit (Promega). Standard techniques were employed for the cloning, transformation, preparation, and restriction analysis of plasmid DNA extracted from *E. coli* (34).

Construction of *C. jejuni tpx* and *bcp* mutants. The *tpx* gene was PCR amplified in two fragments (Tpx1, encompassing part of the upstream *peb2* gene and the 5' end of *tpx*, and Tpx2, encompassing the 3' end of *tpx* and part of the downstream *napA* gene) to create a unique internal deletion flanked by *Hpa*I sites, using the following primers (*Hpa*I sites underlined in bold): Tpx 1F: (5'- GGACTTTATGAAGATATGGC-3'), Tpx 1R: (5'-ACAG**GTTAAC**CATACTTACTACAATCACTTC-3'), Tpx 2F (5'-ACAG**GT TAAC**GCTATGGGAAGATTTTGCAGT-3'),

Tpx 2R (5'-GATCTTAGCATTAAAATAACC-3'). Both fragments, each 800 bp, were independently cloned into the *Eco*RI site of plasmid pCR2.1 and transformed into *E.coli* TOPO-TA (Invitrogen, Netherlands). Transformants were recovered by selection plating onto ampicillin (50 μg ml^{-1}) and clones identified with inserts of the correct size.

The two *tpx* fragments were removed from the pCR2.1 plasmids by digestion with *Eco*RI and *Hpa*I, then ligated and cloned into the pUC based vector pMTL20. The *tpx* gene already with an internal deletion was disrupted by the insertion of a chloramphenicol resistance gene isolated from isolated from plasmid pAV35 (41) into the unique *Hpa*I site of pMTL20 to produce p-*tpx*-CAT.

For construction of a *bcp* mutant strain, primers *bcp*-F (5'-ACG TGA ATT CAA TTT TAT CTG CTG AGA TCA T-3') and *bcp*-R (5'-ACG TGA ATT CTC ACA TTC TTT TTG TAA AGC-3') were used to amplify a 1400bp fragment of the *C. jejuni* NCTC 11168 genome containing the full length *bcp* gene plus 5' and 3' flanking regions by PCR, and the gene cloned into cloning vector pGEM 3Zf (-) to produce plasmid pGEM-Bcp. The *ahpA*III kanamycin resistance cassette from *C. coli* (44; 41) was cloned into a unique *Cla*I restriction site in the centre of the *bcp* gene to produce plasmid pGEM-*bcp*-KAN. Transformation of *C. jejuni* NCTC 11168 with plasmids p-*tpx*-CAT and pGEM-*bcp*-KAN was carried out by electroporation and transformants were selected on Colombia blood agar plates supplemented with chloramphenicol at a final concentration of 30 μg ml^{-1} or kanamycin at a final concentration of 50 μg ml^{-1}. Colonies were re-streaked onto Colombia blood agar plates and correct insertion of the antibiotic resistance cassettes into the target genes was verified by extraction of chromosomal DNA by MicroLYSIS (Web Scientific Ltd, Crewe, UK) according to manufacturer's instructions. PCR using gene-specific primers for *tpx* (forward primer - T-*tpx*-F - 5'-CAC CAT GAG TAT AGT AAA TTT TAA AGG AAA-3'; reverse primer - T-*tpx*-R - 5'-ATG GCA ACC ACA ACC ACC G -3') and *bcp* (forward primer - T-*bcp*-F - 5'-CAC CAT GAG TTT AAA TAT AGG AGA TAA GG-3'; reverse primer - T-*bcp*-R - 5'-AAG ACT TTC

AAG CAC TTT TAA AG-3') confirmed allelic exchange by double cross-over, as demonstrated by an increase in PCR product of approximately 0.8 or 1.4 kb for the chloramphenicol or kanamycin cassette insertions respectively. A *tpx/bcp* double mutant was constructed by electroporation of the *C. jejuni* 11168 *tpx* mutant with pGEM-*bcp*-KAN and selecting on Columbia blood agar plates containing both chloramphenicol and kanamycin.

Disc diffusion assays. 50 ml cultures of *C. jejuni* NCTC 11168 and isogenic mutant strains were grown microaerobically at 37 ºC to early stationary phase and the OD_{600} values adjusted to 1.0 with BHI. 20 ml of each culture was added separately to 400 ml cooled Mueller Hinton agar (Oxoid, UK) containing amphotericin and vancomycin at final concentrations of 10 μg ml^{-1}. Following pouring of plates, a sterile 8mm paper disc made from Whatman No.1 paper (Whatman, Brentford, UK) was placed in the centre of the plate and 5 μl of the agent being tested was added to the disc. The agents and concentrations used are as follows: 500 mM H_2O_2; 10 % (v/v) cumene hydroperoxide; 400 mM *tert* Butyl-hydroperoxide; 50 mM methyl viologen; 200 mM sodium nitroprusside. Plates were incubated microaerobically at 37 ºC for 3 days and the diameter of the zones of inhibition created around the discs measured.

Viability assays. To determine the effect of peroxides on viability, 50 ml cultures of *C. jejuni* NCTC 11168 and isogenic mutant strains were grown microaerobically at 37 ºC to early stationary phase and the OD_{600} values adjusted to 1.0 with BHI. The agents being tested were added to final concentrations as follows; H_2O_2 (1 or 2 mM) cumene

hydroperoxide (0.1 mM), *tert* butyl-hydroperoxide (0.1 mM). At time points 0, 60, 120, 180 and 240 min, 20 µl aliquots were removed in triplicate and diluted from 10^{-1} to 10^{-8} in 200 µl volumes. 5 µl aliquots of each dilution were plated in triplicate onto blood agar plates and incubated at 37 ºC for 3 days, after which time the colonies were counted. For aerobic cell viability assays, 50 ml cultures of the relevant strains in 250 ml flasks were grown overnight microaerobically in the growth cabinet as described previously, then transferred to standard atmopsheric incubation conditions at 37 ºC with 300 rpm shaking to aerate the cells as fully as possible.

Detection of protein carbonylation. Cell-free extracts (CFEs) were prepared from wild type *C. jejuni* NCTC 11168 and the three isogenic mutant strains by growing cells microaerobically in 50 ml BHI-FBS with standard antibiotics for 16h until cells reached early stationary phase. For CFEs of stressed cells, stress agents (H_2O_2 at 1 mM or cumene hydroperoxide at 0.1 mM final concentrations) were added and cells grown for a further 2 hours. For aerobic stress, stationary phase cells were transferred to an aerobic environment and shaken at 300 rpm at 37 ºC for 2 hours. Cells were pelleted and resuspended in 1/10 original volume of 10 mM Tris-HCl pH7.5, and disrupted by sonication. Cell debris and unbroken cells were then removed by centrifugation at 8000 x g for 15 mins at 4 ºC. The method for estimation of protein carbonyl content was based on dinitrophenylhydrazine (DNPH) derivitisation, as described by Shacter *et al* (36). This method is highly specific for protein carbonyls (19, 20, 36). Samples were added to an equal volume of 12% (w/v) SDS to give a final concentration of 6% (w/v) SDS. To this solution an equal volume of 10 mM dinitrophenylhydrazine (DNPH) in 10% (v/v)

trifluoroacetic acid was added and incubated at room temperature for 15 minutes to allow derivatization to form protein carbonyl-DNP groups. The sample was prepared for loading onto SDS-PAGE gels by adding an equal volume of 2 x sample buffer. 15% SDS-PAGE gels were run at 150mV until the dye front had reached the bottom of the gel, and gels were electroblotted onto PVDF membrane. DNP groups were detected immunologically using primary anti-DNP antibody raised in rabbit (Sigma). Anti-rabbit-HRP antibody was used as the secondary antibody (Sigma). Primary and secondary antibodies were both used at a dilution of 1:2000. Detection was carried out using an enhanced chemi-luminesence kit (Amersham).

Lipid peroxidation assays. Detection of total cellular lipid peroxides was carried out using the FOX II reagent, which provides a sensitive colorimetric assay for peroxides when measured spectrophotometrically at 560nm. The method used was adapted from that described by Master *et al*, (22). The FOX II reagent contained 90% methanol, 25 mM H_2SO_4, 250 μM ferrous sulphate and 100 μM xylene orange. 50 ml cultures of *C. jejuni* NCTC 11168 and isogenic mutant strains were grown microaerobically at 37 ºC to early stationary phase. For stressing the cells, 1 mM hydrogen peroxide was added to stationary phase cultures and these grown microaerobically for a further 2 hours. The OD_{600} of each culture was adjusted to 0.5 using sterile BHI. A 100μl aliquot of cell culture was added to a cuvette containing 900 μl FOX II reagent and incubated at room temperature for 30 minutes. The absorbance of each sample at 560nm was then measured, and adjusted to allow for any absorbance caused by the cells by measuring a blank of just methanol plus 100 μl cell suspension. Each culture was measured in triplicate and results

quantified using a standard curve produced using a range of hydrogen peroxide concentrations.

Over-expression and purification of *C. jejuni* His-tagged Tpx and Bcp. Primers T-*tpx*-F, T-*tpx*-R, T-*bcp*-F and T-*bcp*-R were used to amplify the full length *C. jejuni* NCTC 11168 *tpx* and *bcp* genes minus the stop codons, by PCR. These fragments were then cloned into the T7 over-expression vector pET-101/D-TOPO (Invitrogen), part of the Invitrogen Champion TOPO cloning kit, according to the manufacturer's instructions, to produce plasmids (designated pET-Tpx and pET-Bcp respectively) that express Tpx or Bcp with a C-terminal 6-his tag. *E. coli* BL21 Star™ (DE3) cells transformed with either of these plasmids were grown at 37 ºC to an OD_{600} of ~0.5 in 100ml LB medium containing 50 μg ml^{-1} carbenicillin . Expression of Tpx or Bcp was induced by addition of 1 mM final concentration IPTG into the medium followed by growth at 37 ºC for a further 3 hours, and the cells harvested by centrifugation (6,000 x g for 20 minutes at 4 ºC). Cells were resuspended in 5 ml binding buffer (20 mM sodium phosphate pH 7.4, 0.5 M NaCl, 20 mM imidazole) and cells disrupted by sonication in an MSE soniprep sonicator. Cell debris was removed by centrifugation as above and by filtration through a 0.2 μm filter. The supernatant was applied to a HisTrap™ column (GE Healthcare) and purified using the His-Tag linear gradient programme with the AKTA Prime Plus station (Amersham) according to manufacturer's instructions. Buffers used contained 20 mM imidazole (binding buffer; described above) or 500 mM imidazole (elution buffer). These were applied in a linear gradient from 0-100% elution buffer to elute His-tagged proteins from the column. Fractions containing purified Tpx or Bcp were pooled, desalted using

size exclusion chromatography into 10 mM Tris-HCl pH8 to remove NaCl and imidazole from the elution buffer, and concentrated using VIVASPIN centrifugal concentrators (Sigma). Protein concentrations were determined using the Bradford assay. N-terminal sequencing of purified proteins (Dr Arthur Moir, University of Sheffield) gave sequences of SIVNFKG and SLNIGDK, which corresponds to the first seven residues of the expected deduced Tpx and Bcp proteins respectively, minus the N-terminal methionine residues.

Nitrate reductase assays. Benzyl viologen-linked nitrate reductase assays with intact cells were carried out as described by Pittman *et al*. (30) in a 1 ml volume using a Shimadzu UV-2401 PC spectrophotometer. The final assay mixture consisted of 10 mM Tris-HCl (pH 7.5), 100 μM benzyl viologen (Sigma), and 5 mM sodium nitrate, contained in a screw-topped quartz cuvette (Hellma) fitted with a silicone seal. After addition of cells, buffer and benzyl viologen the mixture was sparged with nitrogen gas for approximately 10 min, then aliquots of a freshly-prepared sodium dithionite (Sigma) solution were injected into the cuvette until the absorbance at 578 nm was stable at approximately 1.5 units. The assay was started by the injection of anaerobic sodium nitrate into the cuvette. Rates of reductase activity were calculated using an extinction co-efficient (ε_{578}) for benzyl viologen of 8600 M^{-1} cm^{-1}. For this and the other enzyme assays below, protein concentrations were determined using the method of Markwell *et al*. (21).

Catalase assay. Catalase activity in cell-extracts was measured by directly monitoring the breakdown of hydrogen peroxide at 240 nm (42), using an extinction co-efficient

(ε_{240}) of 39.4 M^{-1} cm^{-1} in a 1ml assay mixture containing 10 mM Tris-HCl pH 7 and 50 mM hydrogen peroxide.

Dithiothreitol-linked peroxidase assays. The activity of purified Tpx or Bcp linked to dithiothreitol (DTT) oxidation was determined by monitoring the absorbance at 310 nm in a Shimadzu UV-2401PC spectrophotometer. This assay was modified from Hillas *et al*, (14). The reaction mixture contained 50mM HEPES-NaOH (pH 7), 1 mM EDTA, 1 μM pure Tpx or Bcp, 2 mM DTT and 2 mM peroxides (hydrogen peroxide, *tert*-Butyl hydroperoxide or cumene hydroperoxide) in a total volume of 1 ml at 37 ºC. The reaction was started by the addition of the substrate. All buffers and water used were chelex treated as per the manufacturer's instructions (Sigma).

NADPH-linked peroxidase activity assays. The peroxidase activity of purified Tpx and Bcp linked to NADPH oxidation via the thioredoxin reductase-thioredoxin system was determined by monitoring the decrease in absorbance at 340nm in a Shimadzu UV-2401PC spectrophotometer. Pure thioredoxin (Trx) and thioredoxin reductase (TrxR) from *E. coli* were obtained from Sigma-Aldrich (UK). The reaction mixture contained 50 mM HEPES-NaOH (pH 7.0), 20 μM NADPH, 20 μg Trx, 6.25 μg TrxR, 1 μM pure enzyme (Tpx or Bcp), and varying concentrations of peroxides (hydrogen peroxide, *tert*-butyl hydroperoxide, cumene hydroperoxide or linoleic acid hydroperoxide). Reactions were carried out in a total volume of 1 ml at 37 ºC. The reaction was started by the addition of NADPH. Linoleic acid hydroperoxide was generated by incubating 100 μM linoleic acid (Sigma) with 10 μg ml^{-1} soybean lipoxidase in 50 mM HEPES-NaOH (pH

7.0) at room temperature for 60 minutes. The concentration of linoleic acid hydroperoxide was determined spectrophotometrically using an extinction co-efficient (ε_{234}) for linoleic acid hydroperoxide of 25,000 M^{-1} cm^{-1} at 234 nm.

Cellular fractionation and detection of Tpx and Bcp by immunoblotting. For determination of the cellular location of Bcp and Tpx, *C. jejuni* periplasm and cytoplasm were isolated by polymyxin-B treatment of whole cells, as described by Sommerlad & Hendrixson (37). Cross contamination of the periplasmic and cytoplasmic fractions was assayed using markers for each fraction (cytochrome *c* content as the periplasmic marker and isocitrate dehydrogenase (ICDH) activity as the cytoplasmic marker), using the assays described by Myers and Kelly (24). For detection of Tpx and Bcp in cells, 1 ml samples were taken at defined time points throughout the growth curve, cells pelleted and then prepared for loading onto SDS gels by boiling in reduced sample buffer for 5 minutes. Loadings were normalised through OD_{600} readings carried out at the same time as the samples were pelleted. Proteins were separated by SDS-PAGE using the Mini-PROTEAN 3 apparatus (BIO-RAD, California, USA). Transfer of proteins was carried out using a Mini Trans-Blot Cell (BIO-RAD). The gel-blot sandwich was constructed according to manufacturer's instructions and the proteins transferred to a nitrocellulose membrane (Hybond-C Extra, Amersham Biosciences) at a current of 11 mA for 14 h at 4 ^{o}C. All immuno-detection steps were carried out at room temperature with constant agitation. TBS-T (25 mM Tris-HCl pH 7.4, 130 mM NaCl, 0.1% Tween 20) was used as both a base for blocking agent (5% bovine serum albumin dissolved in TBS-T) and for washing. Primary polyclonal antibodies (Anti-Tpx and Anti-Bcp, raised in rabbit from

purified *C. jejuni* Tpx and Bcp respectively, produced by Dr Simon Smith, Antibody Resource Centre, University of Sheffield) were diluted in blocking agent (1:2000) and applied to the membrane. Membranes were reacted for approximately 1 h and washed in TBS-T before the secondary antibody (monoclonal Anti-Rabbit IgG, Sigma) was diluted (1:2000) and applied to the membrane. Antibody binding was visualised by means of Enhanced Chemi-luminescence (ECL Kit, Amersham Biosciences UK), according to manufacturer's instructions. No cross reaction between anti-Tpx and Bcp or between anti-Bcp and Tpx was observed at the antibody titres used in this work. Total Lab 100 Software (Nonlinear Dynamics Ltd, UK) was used to determine the area and intensity of each band on the resulting ECL film. The product of these values was then used to calculate the band density (arbitrary units).

Real-time PCR. Wild type *C. jejuni* NCTC 11168 and 11168 *bcp* BHI-FBS cultures (25 ml) were harvested directly into an equal volume of pre-chilled 5 % (v/v) phenol made up in 100% ethanol to stabilise the RNA, then centrifuged at 6000 x g for 10 minutes at 4 ºC. Total RNA was then purified from cell pellets using the RNeasy mini kit (Qiagen, UK) according to the manufacturer's instructions. The RNA concentration and purity was determined using an Eppendorf *Bio*Photometer. cDNA synthesis was carried out using 4 µg of DNase-treated RNA primed with 0.5 µg random hexamer primers (Promega, UK). Reaction mixtures (20 µl) containing 0.5 mM dATP, dCTP, dGTP and dTTP were incubated for 2 hours at 42 ºC with 200 units BioScript reverse transcriptase (Bioline). Following synthesis, cDNA was purified using the PCR purification kit (Qiagen) to remove unincorporated dNTPs and primers. Gene specific primers were designed to

amplify internal fragments of *Cj0272* (gene downstream of *bcp*; *Cj0271*) and a control gene, *gyrA*, using PRIMER 3 software (33). A SYBR green mix was made in the ratio 13 µl Quantace sensimix (Bioline) 0.5 µl SYBR green and 4.5 µl nuclease free water (Sigma). Each reaction was carried out in a total volume of 25 µl on a 96 well optical reaction plate (Applied Biosystems). Each well contained 16 µl of the SYBR green mix (above), 12.5 pmol each primer pair and 5 µl of cDNA sample. PCR amplification was carried out in an ABI 7700 thermocycler (PE Applied Bio-systems) with the thermal cycling conditions of 50 ºC for 2 minutes followed by 95 ºC for 10 minutes followed by 40 cycles of 95 ºC for 15 seconds and 65 ºC for 1 minute. The data was analysed using the Sequence Detector System (SDS) software (PE Applied Bio-systems) and further processed in Microsoft EXCEL. A standard curve was established for each gene studied using genomic DNA to confirm that primers amplified at the same rate and to validate the experiments. Reactions minus template were carried out as negative controls.

Results

Construction and verification of mutants. To investigate the physiological role of Tpx and Bcp, we first created single *tpx* and *bcp* insertion mutants, and a *tpx/bcp* double mutant, in *C. jejuni* strain NCTC 11168, using antibiotic resistance cassettes. Directly downstream of *tpx* and transcribed in the same direction is the *napA* gene, encoding nitrate reductase (Fig. 1). Nitrate reductase activity assays using wild type and *tpx* mutant strains showed there was no loss of NapA activity in the *tpx* mutant, with values of 4.0 ± 0.2 µmol min^{-1} mg $protein^{-1}$ and 4.0 ± 0.1 µmol min^{-1} mg $protein^{-1}$ for the wild type and *tpx* mutant respectively, whereas in a *napA* mutant (30) the nitrate reductase

activity was zero. This shows there is no effect on the expression of the *napA* gene caused by insertion of the chloramphenicol resistance cassette in *tpx*. Directly downstream of the *bcp* gene is *Cj0272*, encoding a conserved hypothetical protein (Fig. 1). RT-PCR analysis of *Cj0272* expression in the *bcp* mutant showed a 2.1 ± 0.7 fold down-regulation compared to the wild-type parent, indicating a minimal downstream effect caused by insertion of the kanamycin resistance cassette into the *bcp* gene. The amplified *Cj0272* cDNA from RT-PCR reactions of both wild-type and *bcp* mutant RNA was also visible on an agarose gel. Immunoblotting of cell-free extracts of the wild-type, *tpx* and *bcp* mutant strains, using anti-TPX or anti-BCP polyclonal antibodies raised against the purified proteins, clearly showed the expected absence of Tpx and Bcp proteins in the respective mutants (Fig. 1A).

Tpx and Bcp are necessary for hyperoxic stress resistance. Growth curves of wild type *C. jejuni* 11168 and the three isogenic mutants were obtained under standard microaerobic conditions (Fig 1B, C). The *tpx* and *bcp* single mutants grew at rates comparable to wild type *C. jejuni* 11168, with a turbidity doubling time of approximately 3 hours. However, the *tpx/bcp* double mutant strain showed a significant growth defect during microaerobic growth in BHI-FBS, with a doubling time of over 7 hours, but it reached a similar cell density to the other strains after 24 hours growth. Viable counts of these growth curves showed the same overall pattern (Fig. 1C), but with the double mutant losing viability markedly in stationary phase. These strains were also grown under highly aerobic conditions to examine the effects of hyperoxic stress. As expected for the microaerophilic *C. jejuni* wild-type, this resulted in growth inhibition and a low final cell

density compared to microaerobic growth. The *tpx* and *bcp* single mutants initially showed some growth, similar to wild type cells, but their growth was retarded after just a few hours and the final cell densities reached were lower than the wild-type. No significant growth of the *tpx/bcp* double mutant occurred under these conditions (data not shown). The effect of increased oxygen on the viability of *C. jejuni* was also assessed by the transfer of early stationary phase microaerobically grown cells to highly aerobic conditions. (Fig 2A). The single *tpx* and *bcp* mutants lost viability at the same rate as wild type cells, with viability being below the level of detection after 6 hours. However, the *tpx/bcp* double mutant lost viability much more rapidly. These data highlight an important and partially redundant role for Tpx and Bcp in the protection of *C. jejuni* against oxidative stress caused by molecular oxygen.

Redundant roles of Tpx and Bcp in peroxidative and nitrosative stress resistance. Disc diffusion assays (Table 1) and cell viability assays (Fig. 2) were used to determine the roles of Tpx and Bcp in protection against peroxides, superoxide and nitrosative stress. By either method, the single *tpx* and *bcp* mutant strains appeared to be no more sensitive to hydrogen peroxide than the wild type, but the double *tpx/bcp* mutant was much more sensitive to this type of stress, showing a statistically significantly larger zone of inhibition when compared to wild type in disc diffusion assays, and with a large loss of viability in the presence of 1 mM hydrogen peroxide (Fig 2B). At 2 mM hydrogen peroxide, the double mutant showed a total loss of viability after 240 minutes incubation, although the wild-type and single mutants showed higher rates of viability loss than at 1 mM (Fig 2C). Both the *tpx* and *bcp* single mutants and the *tpx/bcp* double mutant lost

viability more rapidly than wild type cells when exposed to the organic peroxides cumene hydroperoxide (Fig 2D) and *tert*-butyl-hydroperoxide (Fig 2E). However, when these stress agents were used in disc diffusion assays, only the *tpx/bcp* double mutant showed a statistically significant increase in the size of the zone of inhibition observed (Table 1). Similarly, single *tpx* and *bcp* mutants were not more sensitive to superoxide stress mediated by methyl viologen than wild-type cells (Table 1), but the *tpx/bcp* double mutant had a significantly increased sensitivity compared to the wild type strain. An increased sensitivity to nitrosative stress with the nitrosating agent sodium nitroprusside was again also observed only in the *tpx/bcp* double mutant (Table 1).

Peroxide sensitivity assays may be affected by changes in other peroxide degrading enzyme activities, particularly catalase. In order to investigate this, we assayed catalase activity in early stationary phase cell-free extracts. The following high specific activities were found in all the strains, even when grown in the absence of hydrogen peroxide; wild-type, 15 ± 2 μmol min^{-1} mg $protein^{-1}$: *tpx*, 12 ± 2 μmol min^{-1} mg $protein^{-1}$; *bcp*, 12 ± 2 μmol min^{-1} mg protein-1; *tpx/bcp*, 14 ± 3 μmol min^{-1} mg $protein^{-1}$. Thus, differences in catalase activity cannot explain the difference in peroxide sensitivity between the single and double mutants.

Overall, as with the effect of molecular oxygen, the data in Table 1 and Fig. 2B-E suggest a high degree of redundancy in the functions of Tpx and Bcp, such that a pronounced oxidative stress phenotype that is severe enough to significantly affect growth and viability is only observed in a strain lacking both enzymes. However, more subtle effects of the absence of either enzyme may not be apparent in such experiments. The contribution of each enzyme to the protection of lipids and proteins, two key classes

of cellular macromolecules that are prone to oxidative damage, was therefore investigated.

Mutants lacking Tpx and Bcp have increased lipid peroxide levels. Reactive oxygen species can react with unsaturated membrane lipid fatty acids to form several species of lipid-peroxides, which can lead to damage to both membranes and associated proteins. Comparison of the accumulation of lipid peroxides in wild-type and mutant strains, measured using the sensitive FOX II reagent, revealed that single *tpx* or *bcp* mutants have raised lipid peroxidation levels compared to wild-type cells (Fig. 3). When grown in the presence of 1 mM hydrogen peroxide, the wild-type peroxidation level did not change significantly (P >0.05), whereas small but statistically significant increases occurred in the single *tpx* (P = 0.03) and *bcp* (P = 0.02) mutants. The overall lipid peroxide content in the *tpx/bcp* double mutant was also considerably higher than in wild type cells, although the increase in peroxide content in cells grown in the presence compared to the absence of hydrogen peroxide could not be demonstrated to be statistically significant in this case (P >0.05; Fig 3).

Evidence for increased protein carbonylation in *tpx* and *bcp* mutants. Protein carbonylation is an irreversible modification that occurs when cellular proteins are oxidised by reactive oxygen and nitrogen species. As such, the level of carbonylation of proteins can be used as a useful indicator of the degree of oxidative stress the cells have experienced (20; 38). The assay used here exploits the ability of dinitrophenylhydrazine (DNPH) to form an adduct with protein carbonyl groups (19). The DNP-carbonyl groups

can then be detected via standard immunoblotting procedures using an anti-DNP antibody, resulting in a characteristic banding pattern (Fig. 4A). That these bands represent carbonylated proteins rather than lipid or lipopolysaccharide is demonstrated by the controls in Fig. 4B. Treatment with proteinase K resulted in the disappearance or alteration of molecular weight of the majority of the bands compared to the untreated control, whereas acetone precipitation to remove lipids caused no change in the profile.

We found that when wild-type cells are grown under standard microaerobic conditions in the presence of 1 mM hydrogen peroxide, there is an increase in the carbonyl content of a number of cellular proteins compared to unstressed microaerobically grown cells, as evidenced by increased band intensities on the immunoblot (Fig 4A). Interestingly, the level of carbonylation is much higher when cells are grown aerobically versus microaerobically (Fig 4A).

There is an obvious increase in the carbonyl content of cellular proteins in the single *tpx* and *bcp* mutant strains and in the double mutant, when compared to the wild type, when cells are grown under standard microaerobic conditions (Fig 4C and 4D, (-) lanes). In the presence of 1 mM hydrogen peroxide, the level of carbonylation clearly increased in wild type cells and in both single mutants, with a further increase in the *tpx/bcp* double mutant strain (Fig 4C). The level of carbonylation also increased in the wild-type and two single mutant strains when 0.1 mM cumene hydroperoxide was used to stress the cells (Fig 4D), but again the peroxide induced level of carbonylation was clearly highest in the *tpx/bcp* double mutant (Fig 4D). Finally, under highly aerobic conditions (Fig. 4E) a more pronounced pattern is seen, with raised carbonylation levels in the single mutants compared to the wild-type, but a much more significant effect of

high oxygenation in the double mutant (Fig. 4E, last lane). Thus, aerobic conditions appear to cause significant oxidative damage to proteins in *C. jejuni* and this is particularly exacerbated in strains lacking both Tpx and Bcp.

Peroxidase activity of purified *C. jejuni* Tpx and Bcp. In order to correlate the enzymic activity of Tpx and Bcp with the phenotypes observed in the above studies with mutant strains, we determined the range of peroxide substrates that were used by the purified over-expressed enzymes. Optimal over-expression of *C. jejuni* Tpx and Bcp from pET plasmids was achieved heterologously in *E. coli* BL21* after 3 hours growth in the presence of 1 mM IPTG. The C-terminal His-tagged Tpx and Bcp proteins were purified to homogeneity using nickel-chelate affinity chromatography. Purity was confirmed by SDS-PAGE, immunoblotting and through N-terminal sequencing. The activity of the purified Tpx and Bcp were assayed in two systems, one using the non-physiological electron donor dithiothreitol (DTT), monitored directly by DTT oxidation, and the other using the proposed physiological electron donor system, thioredoxin plus thioredoxin reductase, linked to NADPH oxidation.

DTT-linked assays showed that Tpx and Bcp were both able to catalyse hydrogen peroxide dependent DTT oxidation (Fig 5, top panels), with the initial rates being approximately the same for both enzymes. Surprisingly however, only Bcp was able to oxidise DTT in the presence of the organic peroxides cumene hydroperoxide or *tert* butyl-hydroperoxide (Figs 5 middle and lower panels, right hand column). No measurable rate of cumene hydroperoxide or *tert* butyl-hydroperoxide dependent DTT oxidation was

observed when Tpx was used in the assay system (Figs 5 middle and lower panels, left hand column).

One possibility to explain the unexpected lack of Tpx activity with organic peroxides concerns the use of the artificial electron donor DTT. This assay also suffers from a variable background rate of substrate independent DTT oxidation (apparent in Fig. 5). We therefore employed the physiological electron donor thioredoxin, reduced by thioredoxin reductase and NADPH. This assay is more specific and more sensitive, but the thioredoxin system had to be comprised of commercially available enzymes derived from *E. coli*, as these enzymes are not available from *C. jejuni*. Nevertheless, both Tpx and Bcp were found to be able to reduce hydrogen peroxide linked to the oxidation of NADPH, as evidenced by a large decrease in the absorbance at 340nm (Fig 6). This activity was absolutely dependent on the presence of the substrate peroxide, thiol peroxidase, thioredoxin and thioredoxin reductase, and the rate of NADPH oxidation was higher with 100 μM H_2O_2 than with 20 μM H_2O_2 (Fig. 6). However, no NADPH oxidation occurred when purified Tpx was incubated in this assay with the organic peroxides cumene hydroperoxide or *tert* butyl-hydroperoxide (Fig 6). Only purified Bcp was able to use these organic peroxides as substrates (Fig 6), and the rate of NADPH oxidation was proportional to the peroxide concentration (Fig 6). Finally, we also synthesised linoleic acid hydroperoxide (LA-OOH, a mimic for cellular lipid peroxides) for use in this assay system, but found only a very slightly higher rate of NADPH oxidation occurred with either enzyme when 20 μM LA-OOH was provided as a substrate (Fig 6).

Aerobic growth increases the expression of Tpx and Bcp in *C. jejuni*. The purified Tpx and Bcp proteins were also used to raise polyclonal antibodies in rabbits for use in expression studies. Cultures of wild type *C. jejuni* were grown either under standard microaerobic conditions, or under highly aerated conditions (Fig. 7A). Total cellular proteins in samples of cells taken throughout the growth cycle were separated by SDS-PAGE (Fig. 7B), and immunoblotted using either anti-Tpx or anti-Bcp antibodies (Fig. 7C, D). There was virtually no Bcp produced up until 2-4 hours microaerobic growth (Fig 7D). Densitometry of the blots showed that Tpx and Bcp expression increased in both microaerobic control cultures and aerobically grown cultures as cells entered exponential phase, with maximal levels in both conditions after 2 hours growth for Tpx and 4 hours for Bcp (Fig 7C and D respectively). The amounts of the two proteins in aerobically grown cells were consistently higher than microaerobically grown cells, this being particularly evident for Bcp, which was barely detectable in early exponential phase microaerobic cultures (Fig. 7D). Levels of both Tpx and Bcp appeared to decrease somewhat after 4-6 hours microaerobic growth, but remained high in cells growing aerobically. After 24 hours growth, both Tpx and Bcp abundance decreased further still in microaerobically grown cells, but remained high in aerobically grown cells. Interestingly, there also appeared to be significant amounts of oligomerisation or aggregation of both Tpx and Bcp in aerobically grown cell samples at 24 hours, as indicated by higher molecular weight bands present on the blots at this time point (Fig 7C and D respectively). The major higher molecular weight band for each protein appeared to be equivalent to a dimer. This apparent SDS resistant aggregation did not occur at 24 hours in microaerobically grown cells. When *C. jejuni* wild-type cells were grown with 1mM

H_2O_2, no increase in expression of either Tpx or Bcp ocurred throughout the growth cycle (data not shown), unlike the results above for aerobic conditions.

Evidence that Tpx and Bcp are cytoplasmic proteins in *C. jejuni*. Tpx has been previously reported to be periplasmic in *E. coli* (4), but has been shown to use the cytoplasmic thioredoxin system as an electron donor. Moreover, neither Tpx nor Bcp from a range of bacteria, including *C. jejuni*, have obvious N-terminal signal sequences that might direct them to the periplasm. To determine the cellular location of the Tpx and Bcp proteins in *C. jejuni*, periplasmic and cytoplasmic fractions were prepared by a gentle procedure involving outer membrane disruption with the antibiotic polymyxin B (37). The fractions were then separated by SDS-PAGE (Fig. 8B), and immunoblots probed with anti-Tpx or anti-Bcp antibodies (Fig 8C and D respectively). Analysis of the cytochrome *c* content (periplasmic specific marker) and isocitrate dehydrogenase activity (ICDH; cytoplasmic specific marker) in the fractions showed that there was an inevitable but small amount of cross-contamination; the cytoplasmic fraction contained 4% of the total cytochrome *c* content, and the periplasm contained 13% of the total ICDH activity (Fig. 8A). Nevertheless, immunoblots clearly showed a much stronger signal for both Tpx and Bcp in the cytoplasmic fraction compared to the periplasmic fraction (Fig 8C and D respectively). The signal present in the periplasmic fraction can almost certainly be attributed to cytoplasmic cross-contamination.

Discussion

In this study, we have obtained evidence for a role for the closely related peroxiredoxins Tpx and Bcp in the protection of *C. jejuni* against oxidative stress, particularly that caused by excess oxygen and exogenous peroxides. Single mutations in the *tpx* or *bcp* genes resulted in even poorer growth than the wild-type under high aeration conditions. When the mutations were combined in the double mutant, growth was dramatically reduced under microaerobic conditions and was prevented almost completely under high aeration. Thus, AhpC (2) is not the only peroxiredoxin in *C. jejuni* that has a role in aerotolerance; all three such proteins are important. Our data does suggest, however, a significant degree of redundancy in the function of Tpx and Bcp, which is reinforced by the data from the disc diffusion assays using a variety of oxidative stress inducing agents. Viability assays did indicate some increased sensitivity of the single mutants to hydrogen peroxide and organic peroxides, but there are several alternative H_2O_2 defence systems in *C. jejuni*, such as catalase (13), and two cytochrome *c* peroxidases (1); our assays showed all the strains to have high catalase activities which may be able to partially compensate for the individual loss of Tpx or Bcp. However, the related peroxiredoxin AhpC is the main hydrogen peroxide scavenger in *E. coli*, with low μM K_m values (35). Although *ahpC* mutants of *C. jejuni* are apparently not hypersensitive to hydrogen peroxide (2), perhaps the function of this peroxiredoxin needs to be revisited. *tpx* and *bcp* single mutants in *H. pylori* and *E. coli* are hypersensitive to both H_2O_2 and organic peroxides (43; 17; 9; 5), and the *H. pylori* mutants were also sensitive to high oxygen conditions, but no studies have been carried out with double mutants. The superoxide stress phenotypes of the *C. jejuni* mutants also point to redundancy in the roles of Tpx and Bcp,

whereas single *tpx* and *bcp* mutants in the closely related *H. pylori* were found to be more sensitive to superoxide generators than the wild type (9), and *tpx* mutants in *E. coli* are also more sensitive to superoxide mediated killing (5). The double *tpx/bcp* mutant was also more sensitive to the nitrosating agent SNP (an NO^+ donor), most likely reflecting an indirect increase in reactive oxygen species, including peroxides, under conditions of nitrosative stress. However, some peroxiredoxins like AhpC from *H. pylori* and *M. tuberculosis* can act as peroxynitrite reductases and may therefore protect directly against nitrosative stress (3). *C. jejuni* contains at least two NO detoxifying enzymes, the haemoglobin Cgb, (11; 12) and the nitrite reductase NrfA (30).

The increase in lipid peroxidation and protein carbonylation in the *tpx* and *bcp* single mutants when compared to wild type cells grown under microaerobic conditions indicates the individual importance of both Tpx and Bcp in defending against reactive oxygen species during normal cell growth. Lipid peroxidation has not been extensively doucumented in bacteria, largely because their lipid fatty acids tend not be polyunsaturated and thus are not so susceptible to peroxidation, but an increase in lipid peroxide levels has been shown to occur in *tpx* mutants of *E. coli* (6) and in *ahpC* mutants of *Mycobacterium tuberculosis* (22). However, the polyunsaturated host cell membrane lipids will become oxidised during the inflammatory response to infection and so bacterial peroxiredoxins may also be a useful defence against host derived lipid peroxides. This may be particularly relevant in a mucosal pathogen like *C. jejuni*.

The results from the protein carbonylation experiments indicated that losing both Tpx and Bcp results in a particularly dramatic increase in the level of protein oxidation, particularly after challenge with oxygen or peroxides. Studies with single thiol peroxidase

mutants in *E. coli* (6) have shown a similar pattern of increased carbonylation. We do not know the identity of those proteins which are most heavily carbonylated, but in other bacteria like *B. subtilis* (23) the elongation factors EF-G, TufA and EF-Ts are particularly affected, which could cause global effects on protein synthesis and cell growth if their activity is compromised, thus contributing to the poor growth of the *tpx/bcp* double mutant.

The substrate specificity of Bcp appears to be similar to that found in other organisms (43; 17). In *C. jejuni*, NADPH is oxidised fastest when H_2O_2 is used as the substrate. The rates of NADPH oxidation achieved for both cumene hydroperoxide and *tert*-butyl-hydroperoxide are very similar. Thus, the *C. jejuni* Bcp acts as a general peroxidase enzyme with a broad substrate range. This is also the case for the Bcp enzymes from *H. pylori* and *E. coli* (43; 17). Rates of NADPH oxidation with linoleic acid hydroperoxide were virtually undetectable with the *C. jejuni* Bcp or Tpx enzymes although in both *H. pylori* and *E. coli*, Bcp was shown to preferentially use linoleic acid hydroperoxide as a substrate (43; 17). The fact that both single mutants exhibit an increase in lipid peroxide content suggests that Tpx and Bcp from *C. jejuni* do use lipid peroxides as substrates. Other peroxiredoxins, for example, the AhpC from *M. tuberculosis* (14), can detoxify a wide variety of lipid peroxides. However, the increase in lipid peroxides could also be indirectly due to the loss of enzymes involved in hydrogen peroxide removal, the build up of hydrogen peroxide leading to an increase in lipid peroxides.

Tpx in *C. jejuni* appears to be a dedicated hydrogen peroxide detoxifying enzyme, as no observable rates of DTT or NADPH oxidation occurred when organic peroxides

were provided as substrates. This is in contrast to Tpx enzymes from other organisms, which appear to have a wider substrate range. Tpx in *E. coli* is able to use H_2O_2, organic peroxides and lipid peroxides as substrates (4; 6), with the enzymes from *H. pylori* and *M. tuberculosis* able to use cumene hydroperoxide *in vitro* (25; 32), and the *M. tuberculosis* enzyme also being able to use H_2O_2 and *tert* Butyl-hydroperoxide (32). In these organisms, Tpx appears to act as a general peroxide detoxification enzyme, much like Bcp from *C. jejuni*. Tpx may therefore be an important scavenger of hydrogen peroxide in *C. jejuni*, as has been seen for the related peroxiredoxin AhpC in *E. coli* (35). The apparent substrate specificity for Tpx and the phenotypic results of the *tpx* mutant for sensitivity to organic peroxides is apparently in contradiction with each other, but this could be explained by the formation of some intracellular hydrogen peroxide after treatment of cells with the organic peroxides.

This study has provided evidence that the synthesis of both Tpx and Bcp is increased by atmospheric oxygen, but it is not known if this is a direct effect of molecular oxygen or an indirect effect, which could be attributed to a general increase in levels of reactive oxygen species (ROS) caused by aerobic growth. However, the fact that neither Tpx nor Bcp increased in abundance if cells were grown in the presence of external hydrogen peroxide does suggest that there is a specific oxygen effect. Interestingly, Tpx from *E. coli* is also not induced by peroxide mediated oxidative stress, but is the primary peroxiredoxin produced under anaerobic – rather than aerobic – conditions (6). After prolonged aerobic growth, aggregation of Tpx and Bcp was observed in cell samples by immunoblotting. This effect has also been reported for the peroxiredoxin AhpC of *H. pylori* and may have a protective role distinct from the antioxidant function of the protein

(8). Tpx and Bcp may be important in exponential growth, with levels decreasing as cells are entering stationary phase. The growth inhibition seen in the three mutant strains when compared to wild type cells when grown aerobically also provides further evidence for the importance of Tpx and Bcp when cells are actively dividing, particularly under non-optimal, aerobic conditions.

The localisation results strongly suggest that Tpx and Bcp are both cytoplasmic enzymes in *C. jejuni.* A cytoplasmic location is in apparent contradiction to that of Tpx in *E. coli*, which was reported to be periplasmic (4), but would be consistent with the lack of a signal sequence and use of the cytoplasmic thioredoxin system as the source of reductant for catalysis; Tpx and Bcp from *E. coli*, *M. tuberculosis*, *H. pylori*, and now *C. jejuni*, have all been reported to be thioredoxin dependent (6; 32; 25; 4; 43, 17). Perhaps the location of these enzymes in other bacteria, particularly *E. coli*, should be re-examined.

In summary, Tpx and Bcp from *C. jejuni* are enzymes with a key role in oxidative stress defence, particularly to molecular oxygen during exponential phase growth. Bcp appears to be a general peroxide reductase able to act on a wide variety of compounds, whereas Tpx is a specific H_2O_2 detoxification enzyme. Both enzymes also have a more minor indirect role in defending against superoxide and nitrosative mediated stress.

Acknowledgements

This work was supported by a UK Biotechnology and Biological Sciences Research Council studentship to JMA.

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Table 1. Effect of oxidative and nitrosative stress agents on wild-type and mutant strains measured by disc-diffusion assays

stress agent on disc	Mean diameter of inhibition zone ± SD (n = 10)			
	wild type	*tpx*	*bcp*	*tpx/bcp*
500 mM H_2O_2	26.7 ± 1.7	25.4 ± 1.2	25.6 ± 1.3	30.1 ± 1.9[a]
10% (v/v) CuOOH	34.0 ± 1.8	32.4 ± 1.1	33.8 ± 1.6	43.1 ± 3.2[a]
400 mM *tert* Butyl-OOH	36.8 ± 3.6	36.2 ± 2.1	39.0 ± 3.5	45.9 ± 3.9[a]
50 mM methyl viologen	13.7 ± 1.3	14.1 ± 1.2	14.0 ± 0.8	27.8 ± 2.9[a]
200 mM SNP	16.3 ± 1.6	16.3 ± 1.8	15.9 ± 0.7	20.9 ± 2.6[a]

[a] The difference between the *tpx/bcp* mutant and all other strains with each stress agent is significant at $P < 0.005$ using Students *t*-test. The zones of inhibition of the single *tpx* and *bcp* mutants are not significantly different to the wild-type or each other with any of the stress agents used. SNP, sodium nitroprusside.

Figure Legends

Figure 1. Growth phenotypes of *tpx* and *bcp* mutants. A. Mutation strategy for the *tpx* and *bcp* genes of *C. jejuni* NCTC 11168, and the loss of Tpx and Bcp proteins in the respective mutants, as shown by immunoblotting with anti-Tpx antibodies (upper blot) or anti-Bcp antibodies (lower blot). Lane 1 contains wild-type cell-free extract (CFE), and lane 2 contains CFE from either the *tpx* (upper blot) or *bcp* (lower blot) mutant. Lane 3 in each blot contains purified Tpx (upper blot) or Bcp (lower blot). **B,** Growth curve of the wild type (closed triangles), *tpx* (open squares), *bcp* (open diamonds) and *tpx/bcp* (open circles) strains under microaerobic conditions. **C**, corresponding viable counts of the growth curve in **B.**

Figure 2. Effect of oxidative stress on the viability of early stationary phase cells. Strains were grown overnight in BHI-FBS and viable counts were determined in triplicate on individual cell suspensions after adjusting the initial $OD_{600\ nm}$ to approx 1.0, as described in Methods. In most cases error bars are too small to be seen. **A**. aerobic conditions; **B** 1 mM hydrogen peroxide; **C**. 2 mM hydrogen peroxide; **D**. 0.1 mM cumene hydroperoxide; and **E**. 0.1 mM *tert*- butyl-hydroperoxide. Symbols are: Wild type (closed triangles), *tpx* (open squares), *bcp* (open diamonds) and *tpx/bcp* (open circles). The data shown is from one representative experiment, but was repeated twice with similar results.

Figure 3. *tpx* and *bcp* mutants have increased lipid peroxide contents.
Lipid peroxide contents of wild type *C. jejuni* NCTC 11168 and the three mutant strains were determined under unstressed (white bars) and 1 mM hydrogen peroxide-stressed

(grey bars) conditions. Cells were grown to early stationary phase and lipid-peroxide contents measured using the FOX II reagent as described in Materials and Methods.

Figure 4. **Effect of *tpx* and *bcp* mutations on protein carbonyl content**. **A**. Relative levels of protein carbonylation in wild-type cells grown microaerobically to early stationary phase with no further treatment (**m**), or exposed to 1 mM hydrogen peroxide for a further 2 hours (**H_2O_2**) or incubated with vigorous shaking at high aeration (21 % v/v oxygen) for 2 hours (**aer**). Left panel shows the Coomassie blue stained 12% (w/v) SDS-PAGE gel containing equivalent amounts of wild-type cell-free extract protein. Right panel is the corresponding immunoblot with anti-DNP antibodies. **B**, control immunoblot to confirm that the detected bands are carbonylated proteins. Aliquots of a cell-free extract from aerated cells was either left untreated, incubated with 200 μg ml^{-1} proteinase K (PK) at 37 ºC for 16 h, or treated with 2 volumes of –20 ºC acetone to precipitate protein and remove lipid. Samples were then derivatised as described in Materials and Methods, and equal loadings applied to a 12% (w/v) SDS-PAGE gel and immunoblotted with anti-DNP antibodies. **C-E**, levels of protein carbonylation detected on anti-DNP immunoblots in wild-type and mutant strains, in the presence (+) and absence (-) of (**C**), 1 mM hydrogen peroxide (**D**), 0.1 mM cumene hydroperoxide, or (**E),** after growth at high aeration under 21 % (v/v) oxygen (a), versus microaerobically (m).

Figure 5. Peroxidase activity of purified enzymes using DTT as reductant. Each panel is an absorbance time course at 310nm (due to oxidised DTT) in the absence (open symbols) or the presence (closed symbols) of the peroxide substrate shown, using

purified Tpx (left-hand column) or Bcp (right hand column). The reaction mixture contained 50 mM HEPES-NaOH (pH 7.0), 1 mM EDTA, 1 μM pure Tpx or Bcp, 2 mM DTT and 2 mM peroxides (hydrogen peroxide, cumene hydroperoxide or *tert*-Butyl hydroperoxide) in a total volume of 1 ml at 37 ºC. The reaction was started by the addition of the substrate. All buffers and water used were chelex treated as per the manufacturer's instructions (Sigma) to minimise non-specific DTT oxidation.

Figure 6. Peroxidase activity of purified enzymes using the thioredoxin system as reductant. Each panel is an absorbance time course at 340nm (due to NADPH oxidation) in the absence (open diamonds) or the presence (closed symbols) of the peroxide substrate shown, using purified Tpx (left-hand column) or Bcp (right hand column). The reaction mixture contained 50 mM HEPES-NaOH (pH 7.0), 20 μM NADPH, 20 μg *E. coli* Trx, 6.25 μg *E. coli* TrxR, 1 μM pure thiol peroxidase enzyme (Tpx or Bcp), and either 20 μM (closed triangles) or 100 μM (closed squares) peroxide substrate. Reactions were carried out in a total volume of 1 ml at 37 ºC. The reaction was started by the addition of NADPH.

Figure 7. **Effect of oxygen on the synthesis of Tpx and Bcp in wild-type cells**. **A**. Growth curves of microaerobically (m; closed triangles) and aerobically (a; open triangles) grown wild type *C. jejuni* used to measure Tpx and Bcp expression. **B**. Coomassie blue stained SDS-PAGE gel showing normalised protein loadings of cell-free extracts from each time point for microaerobic (m) and aerobic (a) cells. **C** and **D**, immunoblots (upper panels) and corresponding densitometry histograms (lower panels;

arbitrary units) of identical gels to **B,** using anti-Tpx (**C**) or anti-Bcp (**D**) as the primary antibody. Note that for the 24h samples only the density of the major Tpx and Bcp band was measured.

Figure 8. Cellular location of Tpx and Bcp proteins in *C. jejuni.* **A**. Distribution of marker proteins in cytoplasm and periplasm after fractionation of wild type *C. jejuni* cells according to the method of Sommerlad and Hendrixson (37). Open bars represents the activity of the cytoplasmic enzyme isocitrate dehydrogenase (ICDH) and grey bars represent cytochrome *c* content determined spectroscopically at 550 nm after dithionite reduction (24). **B**. Coomassie blue stained 12 % (w/v) SDS-PAGE gel showing normalised loadings of cytoplasm (C) and periplasm (P). Panel **C** is an immunoblot of an identical gel using anti-Tpx antibodies and panel **D** is an immunoblot using anti-Bcp antibodies.