Contribution of the stereospecific methionine sulfoxide reductases MsrA and MsrB to oxidative and nitrosative stress resistance in the food-borne pathogen *Campylobacter jejuni*

John M. Attack and David J. Kelly

Correspondence

David J. Kelly

d.kelly@sheffield.ac.uk

Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, UK

The microaerophilic food-borne pathogen *Campylobacter jejuni* is exposed to highly variable oxygen concentrations during its life cycle and employs a variety of protection mechanisms to resist oxidative stress. However, not all of the enzymes that mediate such protection have yet been identified. Two genes in strain NCTC 11168, *Cj0637c* and *Cj1112c*, are predicted to encode unrelated methionine sulfoxide reductases, which may repair oxidized methionine residues in proteins and thus contribute to oxidative stress defence. *Cj0637* and *Cj1112* were overexpressed, purified and shown by a coupled thioredoxin–thioredoxin reductase–NADPH assay to catalyse the stereospecific reduction of the *S* and *R* diastereoisomers, respectively, of the model compound methyl *p*-tolyl sulfoxide. *Cj0637* is thus identified as MsrA and *Cj1112* as MsrB. The contribution of these enzymes to oxidative and nitrosative stress resistance in *C. jejuni* was assessed by phenotypic analysis of a set of isogenic *msrA*, *msrB* and *msrA/B* insertion mutants. As RT-PCR data suggested a polar effect on *Cj1112c* in the *msrB* mutant, an *msrB/msrB*⁺ merodiploid complementation strain was also constructed. The *msrA/B* strain was severely growth inhibited under standard microaerobic conditions, whereas the *msrA* and *msrB* strains grew normally. Agar plate disc diffusion assays showed that all mutants displayed increased sensitivity to hydrogen peroxide, organic peroxide, superoxide, and nitrosative and disulphide stress, but quantitative cell viability assays showed that the *msrA/B* double mutant was markedly more sensitive to both oxidative and nitrosative stress. All of the stress-sensitivity phenotypes observed for the *msrB* mutant were restored to wild-type in the *msrB/msrB*⁺ merodiploid. It is concluded that MsrA and MsrB make a significant contribution to the protection of *C. jejuni* against oxidative and nitrosative stress.

Received 16 April 2008

Revised 15 May 2008

Accepted 16 May 2008

INTRODUCTION

Campylobacter jejuni is a microaerophilic human pathogen that is a significant cause of serious food-borne gastrointestinal disease worldwide (Tauxe, 1992; Park, 2002). *C. jejuni* is a commensal in many bird species, including poultry, and contaminated chicken meat is a major source of infection (Ketley, 1997). The genome sequence of *C. jejuni* strain NCTC 11168 was published in 2000 (Parkhill *et al.*, 2000), and several other strains have been recently sequenced including strains 81116 (Pearson *et al.*, 2007) and 81-176 (Hofreuter *et al.*, 2006). Oxidative and nitrosative stresses are major factors that pathogens must combat in order to survive both outside and inside the host. Most strains of *C. jejuni* are routinely cultured in gas

atmospheres containing 3–10 % (v/v) oxygen and 5–10 % (v/v) carbon dioxide. However, during its life cycle *C. jejuni* must be exposed to highly variable oxygen concentrations, and it thus presents an interesting paradox: although oxygen sensitive, it nevertheless must be able to survive high environmental oxygen tensions, resist the oxidative stresses encountered *in vivo*, and adapt to the severe oxygen limitation of the gut. The relationship between *C. jejuni* and oxygen is therefore one of the major defining features of the biology of this food-borne pathogen, yet it is also one of the least studied and understood.

Incomplete reduction of oxygen to water in oxidative phosphorylation results in the generation of several reactive oxygen species (ROS), including the superoxide anion (O₂^{•−}), hydrogen peroxide (H₂O₂) and the hydroxyl radical (HO[•]) (Storz & Imlay, 1999). ROS and reactive nitrogen intermediates (RNI) are released by the immune system to

Abbreviations: Met-SO, methionine sulfoxide; RNI, reactive nitrogen intermediates; ROS, reactive oxygen species; SNP, sodium nitroprusside; Trx, thioredoxin; TrxR, thioredoxin reductase.

combat invading micro-organisms (Burney *et al.*, 1999; Poole & Hughes, 2000). RNI include nitric oxide (NO) (Hughes, 1999), and this can react with superoxide to yield the highly toxic peroxynitrite ion (ONOO⁻) (Burney *et al.*, 1999). Bacteria have evolved a multi-layered defence against these stresses, including superoxide dismutase (Pesci *et al.*, 1994), catalase (Storz & Imlay, 1999; Chelikani *et al.*, 2004), cytochrome *c* peroxidases (Attack & Kelly, 2007), peroxiredoxins such as alkyl-hydroperoxide reductase and thiol peroxidase (Poole *et al.*, 2000; Bryk *et al.*, 2000; Wood *et al.*, 2003; Poole, 2005), and bacterial globins (Poole & Hughes, 2000). Inactivation of genes encoding catalase, superoxide dismutase or the peroxiredoxin AhpC in *C. jejuni* results in an increase in sensitivity to various oxidative stresses (Grant & Park, 1995; Purdy *et al.*, 1999; Baillon *et al.*, 1999), and loss of the globin Cgb or the periplasmic nitrite reductase Nrfa results in an increased sensitivity to RNI (Elvers *et al.*, 2004; Pittman *et al.*, 2007). These studies underline the importance to the fitness of *C. jejuni* of combating both ROS and RNI.

As well as combating the causes of oxidative stress, cells must also repair the damage caused by ROS and RNI. The oxidation of methionine residues by toxic oxygen intermediates is a major source of oxidative damage in cells (Weissbach *et al.*, 2002). Formation of methionine sulfoxide (Met-SO) can lead to protein conformational changes, causing inactivation (Skaar *et al.*, 2002). Removal of methionine from the N terminus of many newly synthesized proteins is essential for folding and activity. Oxidized methionine is a much poorer substrate for methionine aminopeptidase than reduced methionine, and thus many newly synthesized proteins would be unable to fold if cells did not have effective mechanisms to convert Met-SO residues back to methionine (Chang *et al.*, 1989; Solbiati *et al.*, 1999). Methionine is oxidized to two diastereoisomers of Met-SO, Met-(S)-SO and Met-(R)-SO, which differ due to the asymmetry of the sulphur atom in the lateral chain (Ezraty *et al.*, 2005). Oxidized methionine residues are repaired by methionine sulfoxide reductases (Msr), of which there are two major types: MsrA is specific for the S isomer and MsrB for the R isomer (Brot *et al.*, 1981, 1984; Sharov *et al.*, 1999; Brot & Weissbach, 2000; Grimaud *et al.*, 2001; Weissbach *et al.*, 2002). MsrA and MsrB are very highly conserved enzymes in both eukaryotes and prokaryotes, but remarkably, even though their substrates are almost identical, these proteins share no similarity at either the sequence or structural levels (Kauffmann *et al.*, 2002; Lowther *et al.*, 2002).

The importance of Msr activity has been demonstrated experimentally in several eukaryotes and a few bacteria, including *Escherichia coli* (St John *et al.*, 2001; Grimaud *et al.*, 2001), *Staphylococcus aureus* (Moskovitz *et al.*, 2002), *Xanthomonas campestris* (Vattanaviboon *et al.*, 2005), *Mycobacterium smegmatis* (Douglas *et al.*, 2004), *Lactobacillus reuteri* (Walter *et al.*, 2005), *Helicobacter pylori* (Alamuri & Maier, 2004) and *Neisseria gonorrhoeae* (Skaar *et al.*, 2002). Where studied, Msr activity seems to

be required for full resistance to oxidative and nitrosative stress agents and, where investigated in the case of pathogens, for colonization and virulence.

C. jejuni NCTC 11168 contains putative *msrA* and *msrB* genes with unknown roles. The deduced MsrA is encoded by *Cj0637c*, and MsrB by *Cj1112c* (Parkhill *et al.*, 2000). In this study we have overexpressed and purified both Cj0637 and Cj1112 from *C. jejuni* NCTC 11168 and have shown that they catalyse the stereospecific reduction of the S and R diastereoisomers, respectively, of methyl *p*-tolyl sulphoxide. We have investigated the *in vivo* roles of both MsrA and MsrB through generation of single and double knockout mutants and phenotypic and complementation analysis of their responses to oxidative and nitrosative stress. The data indicate that these enzymes play an important role in addition to other known components of the oxidative and nitrosative stress response in *C. jejuni*.

METHODS

Bacterial strains, media, and culture conditions. *C. jejuni* strain NCTC 11168 was grown at 37 °C in a microaerobic atmosphere [10 % (v/v) O₂, 5 % (v/v) CO₂, 85 % (v/v) N₂] in a MACS-VA500 growth cabinet (Don Whitley Scientific) on Columbia agar (Oxoid) containing 5 % (v/v) lysed horse blood, and amphotericin B and vancomycin (10 µg ml⁻¹ of each). Chloramphenicol and kanamycin were added to plates at final concentrations of 30 and 50 µg ml⁻¹, respectively, to select for *C. jejuni* *msrA* and *msrB* 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) fetal calf serum (BHI-FCS) contained in 250 ml shake flasks in the microaerobic atmosphere described above and shaken orbitally at 200 r.p.m. *E. coli* was grown routinely at 37 °C with shaking at 200 r.p.m. in Luria-Bertani (LB) medium supplemented with appropriate antibiotics. Growth of *C. jejuni* and *E. coli* was monitored by measuring the OD₆₀₀ 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* (Sambrook *et al.*, 1989).

Transformation of *C. jejuni*. Cells were harvested from 1-day-old Columbia blood agar plate cultures into 1 ml BHI broth and centrifuged at 3500 g for 20 min at room temperature. The cell pellet was resuspended in 1 ml ice-cold WASH buffer [272 mM sucrose, 15 % (w/v) glycerol in water, filter-sterilized] and centrifuged at 3500 g for 10 min at 4 °C. This step was repeated three times and the pellet was resuspended in a final volume of 200 µl WASH buffer. Plasmid DNA (5–15 ng) was added to 100 µl cells, mixed and transferred to a pre-chilled electroporation cuvette (Bio-Rad), and placed immediately in a Gene Pulser chamber (Bio-Rad) followed by electroporation with a pulse of 25 F, 2.5 kV and 200 Ω, giving time constants of 4–5 ms. The transformation mixture was supplemented with 100 µl BHI broth and plated out onto non-selective Columbia blood agar plates. The plates were incubated for 24 h at 37 °C, then the growth was harvested into 200 µl BHI. Aliquots of 100 µl were then plated out onto selective Columbia blood agar plates containing appropriate antibiotics (see below) and incubated for 4–5 days at 37 °C.

Construction of *C. jejuni* *msrA* and *msrB* mutants. For construction of an *msrA* mutant strain, primers *msrA*-F (5'-GAT GCC AAA GAT AAG GCT-3') and *msrA*-R (5'-ATC CAA GAA GCG TAG AAC-3') were used to amplify by PCR a 2248 bp fragment of the *C. jejuni* NCTC 11168 genome containing the full-length *msrA* gene plus 5' and 3' flanking regions, and the gene was cloned into cloning vector pGEM-T easy to produce plasmid pGEM-*msrA*. The *msrA* gene was insertionally inactivated at a unique *Bst*Z171 site in the centre of the *msrA* gene using a chloramphenicol-resistance cassette derived from pAV35 (a gift from Professor Julian Ketley, University of Leicester), to produce plasmid pGEM-*msrA*-CAT. For construction of an *msrB* mutant strain, primers *msrB*-F (5'-GGT AGA TTT TTA AAG CGA TAA CT-3') and *msrB*-R (5'-ACA AGC ATA GGA AAT GCC AT-3') were used to amplify by PCR a 1400 bp fragment of the *C. jejuni* NCTC 11168 genome containing the full-length *msrB* gene plus 5' and 3' flanking regions, and the gene was cloned into cloning vector pGEM-T easy to produce plasmid pGEM-*msrB*. The *msrB* gene was insertionally inactivated at a unique *Swa*I site using the *aphAIII* gene (Kan^R cassette), producing plasmid pGEM-*msrB*-KAN. The Kan^R cassette is derived from plasmid pJMK30, and contains its own promoter so as to minimize any polar effects on downstream genes (van Vliet *et al.*, 1998). Transformation of *C. jejuni* NCTC 11168 with plasmids pGEM-*msrA*-CAT and pGEM-*msrB*-KAN was carried out by electroporation as described above, and transformants were selected on Columbia 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 restreaked onto Columbia blood agar plates containing the appropriate antibiotics, and correct insertion of the antibiotic-resistance cassettes into the target genes was verified by extraction of chromosomal DNA by MicroLYSIS (Web Scientific) according to the manufacturer's instructions. PCR with gene-specific primers for *msrA* (forward primer T-*msrA*-F, 5'-CAC CAT GAA AAA TAT CGT TTT AGG TGG T-3'; reverse primer T-*msrA*-R, 5'-GTC TGA TTG TAT TTT TTG CAG TTT-3') and *msrB* (forward primer T-*msrB*-F, 5'-CAC CAT GAA AGA ATT AAA TGA AGA AGA AAA-3'; reverse primer T-*msrB*-R, 5'-ATC CTT AGT TTT TAC AAA TTC CAA A-3') was used to confirm allelic exchange by double crossover, by an increase in the size of the PCR product of ~0.8 or ~1.4 kb for the chloramphenicol or kanamycin cassette insertions, respectively. Strains were designated 11168 *msrA* and 11168 *msrB*, respectively. A *C. jejuni* 11168 *msrA/msrB* double mutant was constructed by electroporation of *C. jejuni* 11168 *msrA* with pGEM-*msrB*-KAN and selection on Columbia blood agar plates containing both chloramphenicol and kanamycin.

Complementation of *msrB*. For construction of a complemented *msrB* strain, a complementation construct was produced by cloning the *msrB* gene plus the upstream ribosome-binding site into the *Campylobacter* complementation vector pRRC (Karlyshev & Wren, 2005). The *msrB* gene product was produced by PCR using primers C-*msrB*-F (5'-GCT CTA GAT ACA AGG GCA GAT CAT GAA AGA-3') and C-*msrB*-R (5'-GCT CTA GAC TAT CAA TCC TTA GTT TTT ACA-3'), and cloned into the *Xba*I site of pRRC to produce plasmid p-*msrB*-COMP. This was then transformed into *C. jejuni* strain 11168 *msrB* by electroporation and selection on Columbia blood agar plates containing both chloramphenicol and kanamycin. Confirmation of the insertion of the complementation cassette at one of the rRNA loci was carried out by PCR using primers C-*msrB*-F and C-seq-R (5'-CTC TTG CAC ATT GCA GTC CTA C-3').

Disc diffusion assays. Cultures (50 ml) of *C. jejuni* NCTC 11168 and isogenic mutant strains were grown microaerobically at 37 °C to early stationary phase, and the OD₆₀₀ values were adjusted to 1.0 with BHI. A 20 ml volume of each culture was added separately to 400 ml cooled Mueller-Hinton agar (Oxoid) containing amphotericin and vancomycin at final concentrations of 10 µg ml⁻¹. Following pouring of plates, a sterile 8 mm paper disc made from Whatman No. 1 paper

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 were as follows: 500 mM H₂O₂, 10% (w/v) cumene hydroperoxide, 50 mM methyl viologen, 200 mM sodium nitroprusside (SNP), 400 mM diamide. Plates were incubated microaerobically at 37 °C for 3 days and the diameters of the zones of inhibition created around the discs were measured. At least 10 replicate plates were set up for each agent.

Viability assays. Cultures (50 ml) of *C. jejuni* NCTC 11168 and isogenic mutant strains were grown microaerobically at 37 °C to early stationary phase and the OD₆₀₀ values adjusted to 1.0 with BHI. The agents being tested were added to the following final concentrations: 1 mM H₂O₂, 2 mM diamide, 2 mM SNP and 0.5 mM spermine NONOate (Sigma-Aldrich). 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. Aliquots (5 µl) of each dilution were plated in triplicate onto blood agar plates and incubated at 37 °C for 3 days, after which time the colonies were counted.

Overexpression and purification of *C. jejuni* His-tagged MsrA and MsrB.

Primers T-*msrA*-F and T-*msrA*-R were used to amplify the full-length *C. jejuni* NCTC 11168 *msrA* gene minus the stop codon, and primers T-*msrB*-F and T-*msrB*-R were used to amplify the full-length *msrB* gene minus the stop codon by PCR. These amplicons were then cloned into the T7 overexpression vector pET-101/D-TOPO (Invitrogen), part of the Invitrogen Champion TOPO cloning kit, according to the manufacturer's instructions. Plasmids were designated pET-MsrA and pET-MsrB, respectively, and produce recombinant proteins with C-terminal 6-His tags. *E. coli* BL21 Star (DE3) cells containing plasmid pET-MsrA or plasmid pET-MsrB were grown at 37 °C to OD₆₀₀ ~0.5 in 100 ml LB medium containing 50 µg carbenicillin ml⁻¹. Expression of MsrA and MsrB was induced by addition of 1 mM IPTG (final concentration) into the medium followed by growth at 37 °C for a further 3 h, and the cells were harvested by centrifugation (6000 g for 20 min at 4 °C). Cells were resuspended in 5 ml binding buffer (20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 20 mM imidazole) and disrupted by sonication in an MSE Soniprep sonicator. Cell debris was removed by centrifugation as described 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 program with the AKTA Prime Plus station (Amersham) according to the 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 to 100% elution buffer to elute His-tagged proteins from the column. Fractions containing purified MsrA or MsrB were pooled, desalted using size-exclusion chromatography into 10 mM Tris/HCl, pH 8, 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 protein was carried out by Dr Arthur Moir, University of Sheffield, using the automated Edman degradation method.

NADPH-linked enzyme activity assays. The reductase activity of purified MsrA and MsrB linked to NADPH oxidation via the thioredoxin reductase-thioredoxin system was determined by monitoring the decrease in A₃₄₀ in a Shimadzu UV-2401PC spectrophotometer. Pure thioredoxin (Trx) and thioredoxin reductase (TrxR) from *E. coli* were obtained from Sigma-Aldrich. The reaction mixture contained 50 mM HEPES-NaOH (pH 7.0), 20 mM NADPH, 20 µg Trx, 6.25 µg TrxR, 1 µM pure enzyme (MsrA or MsrB), and varying concentrations of (S) or (R) methyl *p*-tolyl sulphoxide (Sigma). Reactions were carried out in a total volume of 1 ml at 37 °C. The reaction was started by the addition of NADPH.

RT-PCR. Wild-type *C. jejuni* NCTC 11168 and 11168 *msrB* BHI-FCS cultures (25 ml) were harvested directly into an equal volume of pre-chilled ethanol containing 5% (v/v) phenol to stabilize the RNA. Samples were centrifuged at 6000 g for 10 min at 4 °C. Total RNA was then purified from cell pellets using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The RNA concentration and purity were determined using an Eppendorf BioPhotometer. cDNA synthesis was carried out using 4 µg starting material primed with 0.5 µg random hexamers (Promega). Reaction mixtures (20 µl) containing 0.5 mM dATP, dCTP, dGTP and dTTP were incubated for 2 h at 42 °C with 200 U BioScript reverse transcriptase (Bioline). Following synthesis, cDNA was purified using the PCR purification kit (Qiagen) to remove unincorporated dNTPs and primers. Gene-specific primers (forward primer RT-marC-F, 5'-GGG TTC TGA GCT TTA TTT GAT GTT-3'; reverse primer RT-marC-R, 5'-CAT CAC TCA TAG CCA AAA ATT GAG-3') were designed to amplify internal fragments of *marC* (the gene downstream of *msrB*; *Cj1112c*) and a control gene, *gyrA* (forward primer RT-gyrA-F, 5'-TAT AGG TCG TGC TTT GCC TGA-3'; reverse primer RT-gyrA-R, 5'-TAT CTC CAT GTG GGT GAT AAC G-3'), using PRIMER 3 software (Rozen & Skaletsky, 2000). A SYBR green mix was made with 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 SYBR green mix, 12.5 pmol of each primer pair and 5 µl cDNA sample. PCR amplification was carried out in an ABI 7700 thermocycler (Perkin-Elmer Applied Biosystems) with the thermal cycling conditions of 50 °C for 2 min, followed by 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 65 °C for 1 min. The data were analysed using the Sequence Detector System (SDS) software (Perkin-Elmer Applied Biosystems) and further processed using 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. No-template reactions were carried out as negative controls.

RESULTS

Cj0637c and *Cj1112c* encode methionine sulfoxide reductases specific for the (S) and (R) isomers, respectively, of the model compound methyl *p*-tolyl sulfoxide

Optimal overexpression of soluble *C. jejuni* Cj0637 and Cj1112 from the pET101 vector was achieved heterologously in *E. coli* BL21 Star (DE3) cells after 3 h growth at 37 °C in the presence of 1 mM IPTG. The C-terminal His-tagged proteins were purified using nickel-chelate affinity chromatography of crude cell-free extracts, and concentrated to homogeneity (MsrB) or near homogeneity (MsrA), as judged by SDS-PAGE (Fig. 1). N-terminal sequencing of the purified proteins gave sequences of M K N I V L G and M K E L N E E, which correspond to the first seven residues of *C. jejuni* NCTC 11168 MsrA and MsrB proteins, respectively. To assay the enzymic activity of these proteins, we employed the model substrate methyl *p*-tolyl sulfoxide, which is commercially available in the pure form as both the S and the R diastereoisomer. We also used the likely physiological electron donor Trx, reduced by TrxR and NADPH, so that NADPH oxidation could be observed directly at 340 nm. This assay is specific and sensitive, but the Trx system had to be formed of

commercially available enzymes derived from *E. coli*, as these enzymes are not available from *C. jejuni*. Nevertheless, when purified Cj0637 was used in the reaction, NADPH oxidation only occurred when the (S) isomer of methyl *p*-tolyl sulfoxide was provided (Fig. 1a). The rate of oxidation of NADPH was similar if the substrate concentration was varied 10-fold between 0.3 µM and 3 µM (Fig. 1a), suggesting a K_m well below 0.3 µM. Component assays showed that the activity observed with the (S) isomer was fully dependent on the presence of Trx and TrxR in the assay (data not shown). No observable rate of NADPH oxidation occurred when a large excess (30 µM) of the (R) isomer of methyl *p*-tolyl sulfoxide was provided (Fig. 1a).

When purified Cj1112 was used in this assay system, methyl *p*-tolyl sulfoxide-dependent NADPH oxidation was also detected, but with the opposite substrate stereospecificity; measurable rates of NADPH oxidation only occurred when the (R) isomer of methyl *p*-tolyl sulfoxide was provided, with no oxidation occurring with an excess (30 µM) of the (S) isomer (Fig. 1b). The rate of NADPH oxidation by Cj1112 was lower than that of Cj0637 and appeared to be substrate-dependent between 0.3 and 3 µM, suggesting a substrate affinity lower than that of Cj0637, but this was not investigated further. These data identify Cj0637 as MsrA (with specificity for the S diastereoisomer) and Cj1112 as MsrB (specific for the R diastereoisomer). The Trx system-based assay was also used to determine whether Met-SO reductase activity could be detected in cell-free extracts of *C. jejuni*, but this was unsuccessful.

Construction, verification and RT-PCR analysis of *msrA* and *msrB* mutants

To investigate the physiological role of MsrA and MsrB, we first created *msrA* and *msrB* single-null mutants, and an *msrA/B* double mutant in the *C. jejuni* strain NCTC 11168. These mutants were all easily obtainable by antibiotic-resistance marker selection on blood agar plates, indicating that the *msr* genes are not essential for the viability of *C. jejuni*, although colonies of the double mutant grew noticeably more slowly. The mutants were verified by PCR of genomic DNA using gene-specific primers, which showed the expected presence of the appropriate antibiotic resistance cassette in each gene (Fig. 2a).

The gene directly downstream of *msrA* is transcribed from the opposite DNA strand and so the creation of an *msrA* mutant by insertional inactivation would have no polar effect (Fig. 2a). Directly downstream of the *msrB* gene, however, is *Cj1111c* (encoding a putative membrane protein, MarC) in the same transcriptional orientation (Fig. 2a). RT-PCR analysis of *Cj1111c* expression in the *msrB* mutant showed a 16.5 ± 0.7 -fold downregulation compared with the wild-type parent, indicating a significant polar effect caused by insertion of the kanamycin-resistance cassette into the *msrB* gene, despite the cassette

Fig. 1. Purification and Trx-linked reductase activity of (a) MsrA and (b) MsrB enzymes. The upper figures in each panel show SDS-PAGE gels of the purified and concentrated enzymes. Lanes: M, protein standards; 1, purified protein after passage through Hi-TRAP column and desalting but before concentration; 2, flow through after Vivaspin concentration; 3, concentrated proteins used for assays. Middle and lower figures in each panel are absorbance time-courses at 340 nm (due to NADPH oxidation) in the absence (◆) or presence (open symbols) of the isomer of methyl *p*-tolyl sulphoxide shown, using purified MsrA (left panels, a) or MsrB (right panels, b). The reaction mixture contained 50 mM HEPES-NaOH (pH 7.0), 20 μ M NADPH, 20 μ g *E. coli* Trx, 6.25 μ g *E. coli* TrxR, 1 μ M pure enzyme (MsrA or MsrB), and 0.3 μ M (Δ), 3 μ M ($\square$) or 30 μ M ($\circ$) of the (*R*) or (*S*) isomer of methyl *p*-tolyl sulphoxide. Reactions were carried out in a total volume of 1 ml at 37 °C. The reaction was started by the addition of NADPH.

being in the same transcriptional orientation as *Cj1111c*. This meant that construction of a complemented *msrB* strain was essential to correctly attribute any phenotypes seen in the *msrB* mutant to the loss of this protein alone and not to any polar effects caused by loss of the *Cj1111c* protein. Complementation was achieved using the integrative vector pRRC (Karlyshev & Wren, 2005), with expression of the wild-type *msrB* allele under the control of the chloramphenicol-resistance gene on this plasmid.

Both MsrA and MsrB are required for full resistance to oxidative and nitrosative stress in *C. jejuni*

Microaerobic growth curves of wild-type *C. jejuni* 11168 and the three isogenic mutant strains in BHI-FCS media are shown in Fig. 2(b). Both the *msrA* and the *msrB* single mutant grew at an identical rate to wild-type cells (turbidity doubling time ~ 3 h). In contrast, the *msrA/B* double mutant showed a significant retardation of growth under these conditions, with the doubling time increased to ~ 15 h.

In order to determine the phenotypic effects of the removal of methionine sulphoxide reductase activity from the cells, we assessed the resistance of the wild-type and mutant strains to oxidative and nitrosative stress. A variety of agents that induce different types of stress were used in both disc diffusion assays and quantitative viability assays (kill curves). Disc diffusion assays were carried out with hydrogen peroxide and cumene hydroperoxide, to determine the effect of peroxide-mediated stress (Fig. 3). The zones of inhibition for both of the single mutants and for the double mutant were significantly larger than the zones for wild-type cells with both H_2O_2 and cumene hydroperoxide ($P < 0.0001$; Fig. 3a, b). However, there was no significant difference among any of the three mutants for either agent. When H_2O_2 was used in viability assays, only the *msrA/B* double mutant showed an increased loss in viability when compared with wild-type or single mutant strains (Fig. 4a).

Methyl viologen (paraquat) is a redox cycling agent that is known to generate intracellular superoxide, and was used

Fig. 2. (a) Mutation strategy and PCR confirmation of cassette insertions in the *msrA* and *msrB* genes of *C. jejuni* NCTC 11168. On the agarose gel images, lane 1 contains amplicons of the wild-type *msrA* (498 bp; upper gel image) or *msrB* (360 bp; lower gel image) genes, resulting from PCR of wild-type genomic DNA with the primer pairs T-*msrA*-F and T-*msrA*-R or T-*msrB*-F and T-*msrB*-R. Lane 2 contains amplicons of the mutated *msrA::cat* (upper gel image) or *msrB::kan* (lower gel image) genes resulting from PCR of the respective mutant genomic DNA with the same primer pairs. An increase in size in lane 2 of ~900 bp in the top panel (*msrA*) and ~1400 bp in the bottom panel (*msrB*) indicates the correct insertion of the *Cm^R* and *Kan^R* cassettes, respectively. (b) Growth curves of the wild-type (▲), *msrA* (○), *msrB* (□) and *msrA/B* (◇) strains under microaerobic conditions in BHI-FCS media, as described in Methods.

in disc diffusion assays to assess the effect of superoxide-mediated stress (Fig. 3c). Both of the single mutants showed a significant increase in the size of the zones of inhibition when compared with the wild-type ($P<0.0001$). However, the double mutant showed a large increase in the zone of inhibition when compared with the two single mutants: the average size of the double mutant growth inhibition zone was almost double that of the two single mutants (Fig. 3c).

SNP is a nitrosating agent and was used in both disc diffusion assays and in kill curves. The disc diffusion assays showed growth of both of the single mutants to be significantly inhibited when compared with the wild-type ($P=0.012$ for *msrA* and $P<0.0001$ for *msrB*), with the *msrA/B* double mutant zone of inhibition being larger still ($P<0.0001$; Fig. 3d). The same phenotypes were seen when SNP was used in viability assays (Fig. 4b). Both single mutants lost viability more rapidly than wild-type cells over the 240 min time-course. The *msrA::cat* and *msrB::kan* mutants both exhibited approximately a 5-log reduction in viability over the time-course. The *msrA/B*

double mutant was killed much faster, with a complete loss of viability after 180 min (Fig. 4b). The degradation of SNP can produce sodium cyanide, so in order to ensure that this was not responsible for the effects observed, control kill curve experiments were performed with the same concentrations of sodium cyanide. No significant loss of viability was observed for either wild-type or mutant strains (data not shown).

Spermine NONOate is a direct NO releaser and was used as an additional reagent to SNP to expose the cells to nitrosative stress. Neither of the single mutants showed increased loss of viability compared with the wild-type (Fig. 4c), but the *msrA/B* double mutant showed a significantly increased loss in viability of over 3 logs compared with wild-type cells (Fig. 4c).

Diamide is a reagent that oxidizes thiols and causes a general increase in disulphide bond formation in proteins. Diamide was used to study the comparative effects of disulphide stress in the *msr* mutants. The zones of diamide-induced growth inhibition of the two single mutants and

Fig. 3. Disc diffusion assays using (a) 500 mM hydrogen peroxide, (b) 10 % (v/v) cumene hydroperoxide (CuOOH), (c) 50 mM methyl viologen (MV), (d) 200 mM SNP, and (e) 400 mM diamide. The differences in the sizes of the zones of inhibition between the wild-type and the three mutant strains are all statistically significant for all agents tested at $P < 0.05$ (see key). When the wild-type and the complemented *msrB/msrB*⁺ strain are compared using Student's *t* test, the difference is not significant for all agents tested. Error bars, SD based on 10 replicate disc measurements.

the double mutant increased significantly when compared with wild-type cells ($P < 0.0001$ for all three strains compared with wild-type) and to approximately the same degree. There was no significant difference in the sizes of the zones of inhibition among any of the three mutant strains (Fig. 3e). When diamide was used in viability assays,

only the *msrA/B* double mutant showed an increased loss of viability when compared with the wild-type. Wild-type cells and the *msrA::cat* and *msrB::kan* single mutants were all totally killed after 240 min growth, whereas the double mutant was totally killed after just 180 min (Fig. 4d).

Fig. 4. Effect of oxidative or nitrosative stress on the viability of stationary phase cells. Viable counts were carried out in triplicate on cell suspensions after addition of the agents shown (error bars are plotted but in most cases are too small to be visible). (a) Hydrogen peroxide (1 mM), (b) 2 mM diamide, (c) 0.5 mM SNP, (d) 2 mM Spermine-NONOate. ▲, Wild-type *C. jejuni* NCTC 11168; ○, *msrA*; □, *msrB*; ◇, *msrA/B* double mutant.

Complementation of the *msrB* mutant restores a wild-type phenotype

As described above, RT-PCR analysis of the *msrB::kan* mutant showed that the gene downstream of *msrB*, *Cj1111c*, was downregulated over 16-fold in the mutant relative to the wild-type. This is a significant polar effect caused by the insertion of the Kan^R cassette into the *msrB* locus. *Cj1111c* encodes a MarC homologue, and has sequence similarity with multiple antibiotic exporters from a number of bacteria (determined by analysis using the NCBI BLAST program). Given this predicted function, it is unlikely that loss of the Cj1111 protein would result in the phenotypes seen in the *msrB::kan* mutant. This is reinforced by the *msrB::kan* phenotype being identical to the *msrA::cat* mutant phenotype, in which polar effects are not an issue.

Transformation of the *msrB::kan* mutant with the vector *p-msrB-COMP* resulted in the generation of a complemented strain designated *msrB/msrB⁺*. Phenotypic characterization of this complemented strain using various oxidative and nitrosative stress agents showed that the provision of a functional copy of the *msrB* gene in the mutant strain restored its phenotype to one closely resembling that of the wild-type strain. Disc assays with hydrogen peroxide, cumene hydroperoxide, methyl viologen, SNP and diamide showed that in each case the zones of inhibition of the *msrB/msrB⁺* strain were not significantly different from those of the wild-type (Fig. 3). We selected SNP for use in viability assays with the wild-type, *msrB* and *msrB/msrB⁺* strains (Fig. 5), and this showed that the viability of the *msrB/msrB⁺* strain was similar to that of the wild-type, whereas the viability of the *msrB* mutant strain decreased 10⁵-fold, as seen above (compare Figs 4b and 5).

Fig. 5. Kill curve using SNP to demonstrate complementation of *msrB*. Viable counts were carried out in triplicate on cell suspensions after addition of 2 mM SNP (error bars are plotted but in most cases are too small to be visible). ▲, Wild-type *C. jejuni* NCTC 11168; □, *msrB*; △, *msrB/msrB⁺* complementation strain.

DISCUSSION

The importance of methionine sulfoxide reductases as components of the response to oxidative stress in prokaryotes has been recognized in recent years, but their significance in relation to other enzymic forms of defence has been evaluated in relatively few species. In this work we have shown that Cj0637 in *C. jejuni* encodes an MsrA protein, which is highly specific for the (S) isomer of Met-SO (more specifically the model compound methyl *p*-tolyl sulfoxide). No activity is seen when excess (R) isomer is provided as the substrate. This specificity has been seen with all other MsrA enzymes studied, such as those from *E. coli* (Moskovitz *et al.*, 2000), *H. pylori* (Alamuri & Maier, 2006) and *Neisseria meningitidis* (Olry *et al.*, 2002), as well as in the eukaryotic proteins, such as that from *Caenorhabditis elegans* (Lee *et al.*, 2005). However, far fewer bacterial MsrB proteins have been studied biochemically, a notable exception being the enzyme from *Staphylococcus aureus* (Moskovitz *et al.*, 2002). Our results show that in *C. jejuni*, Cj1112 is a reductase specific for the (R) isomer of Met-SO (or methyl *p*-tolyl sulfoxide), and can therefore be classed as an MsrB enzyme. While both MsrA and MsrB have been found to be able to reduce both free and peptide-bound Met-SO, the activity of MsrB against free Met-(R)-SO is usually lower (Lowther *et al.*, 2002; Lin *et al.*, 2007). This was also found to be the case with the *C. jejuni* MsrB enzyme in this study. *C. jejuni* MsrA also appeared to have a higher substrate affinity than MsrB. The activity of both MsrA and MsrB could be assayed with the Trx system and this would be consistent with a cytoplasmic location for both enzymes. Neither protein appears to have an obvious signal sequence, which is also consistent with a function in the cytoplasm, although this needs to be confirmed experimentally. There is evidence that some Msr enzymes may be extracytoplasmic, such as the secreted form of PilB in *N. gonorrhoeae* (Skaar *et al.*, 2002). In addition to MsrA and MsrB, there is emerging evidence that many bacteria may also contain additional, unrelated enzymes with Met-SO reductase activity, exemplified by the YebR protein of *E. coli* (Lin *et al.*, 2007). This is a GAF domain protein which can reduce, and is specific for, free Met-(R)-SO but does not interact with peptides containing Met-SO. It may have an important signalling role during oxidative stress, or to ensure adequate methionine pools under such conditions (Lin *et al.*, 2007). However, BLAST searches indicate that *C. jejuni* does not contain a homologue of YebR.

From mutant studies, we have demonstrated that in *C. jejuni* NCTC 11168 both MsrA and MsrB participate in protection against oxidative and nitrosative stress. The successful complementation of the *msrB::kan* mutant showed that a wild-type sensitivity phenotype was restored in the complemented strain when studied using disc assays with all agents, and with kill curves using SNP. Thus, despite evidence of a polar effect on the downstream *Cj1111c* gene, we can conclude that the phenotypes of the *msrB* mutant reflect the consequences of the inactivation of

this gene. Successful complementation of *msr* mutants has also been demonstrated in *H. pylori* (Alamuri & Maier, 2004) and in *X. campestris* (Vattanaviboon *et al.*, 2005).

The observation that the *msrA/B* double mutant shows a severe decrease in growth rate, whereas the single mutants grow similarly to the wild-type, indicates that loss of one methionine sulfoxide reductase activity in the cell can be tolerated under microaerobic conditions. Loss of both MsrA and MsrB presumably leads to such a serious accumulation of Met-SO in proteins that growth is affected significantly. However, both hydrogen peroxide and cumene hydroperoxide lead to an increased killing of both of the single mutants as well as the double mutant when used in disc assay studies, implying that a significant amount of damage to methionine residues is caused by these agents, leading to an increase in killing when one or both isomers of Met-SO are unable to be reduced. The effects do not appear to be cumulative, with the amount of killing of the double mutant being the same as for the two single mutants, implying that the loss of either Msr activity is a significant detriment to the cell. Sensitivity to peroxide is seen in *msrA* and *msrB* mutants in *N. gonorrhoeae* (Skaar *et al.*, 2002), *H. pylori* (Alamuri & Maier, 2004) and *X. campestris* (Vattanaviboon *et al.*, 2005). In *C. jejuni*, however, when hydrogen peroxide was used in cell viability (kill curve) assays only the *msrA/B* double mutant was significantly affected. As kill curves were carried out using stationary-phase cells, this could imply that both MsrA and MsrB are preferentially produced during stationary phase, thus leading to a less obvious phenotype in either of the single mutants. This seems to be the case in *X. campestris* (Vattanaviboon *et al.*, 2005) and *H. pylori* (Alamuri & Maier, 2006), but will require validation by expression analysis of the *msr* genes throughout the growth curve. Known regulators of the oxidative stress response in *C. jejuni* include PerR and Fur (van Vliet *et al.*, 1998; Holmes *et al.*, 2005), but there is as yet no evidence of a role for these regulators in controlling expression of *msrA* or *msrB*. NssR is a DNA-binding protein that controls expression of a small regulon of genes in response to nitrosative stress (Elvers *et al.*, 2005), but *msrA* and *msrB* are not part of this regulon.

The *C. jejuni* *msrA/B* double mutant is peroxide stress-hypersensitive, as no repair of oxidized methionine residues can take place. It should be noted, of course, that like many bacteria, *C. jejuni* contains a plethora of enzymes able to detoxify peroxides, such as catalase (Grant & Park, 1995), cytochrome *c* peroxidases (Atack & Kelly, 2007), AhpC (Baillon *et al.*, 1999), and Tpx and Bcp (Atack *et al.*, 2008). Although these appear to be able to detoxify hydrogen peroxide, peroxide exposure must cause some oxidation of methionine residues to account for the effects seen. In the *msrA/B* double mutant, an accumulation of oxidized methionines is clearly associated with loss of viability. These experiments perhaps show the limitations of both disc assays and kill curves; while disc assays are crude and use vast excesses of stress agent, they do indeed

show phenotypes that would not be seen using viable counts with much lower concentrations of stress agents. Conversely, kill curves provide a much more sensitive level of phenotype detection.

Superoxide stress mediated by methyl viologen causes an increase in killing of both single mutants when compared with wild-type cells, but this is increased markedly in the *msrA/B* double mutant. Superoxide dismutase of *C. jejuni* has been studied (Purdy & Park, 1994; Purdy *et al.*, 1999), but presumably this activity is not sufficient to prevent superoxide-mediated oxidation of methionine residues to such a degree as to cause the mutant phenotypes seen here. Mutants in a range of other bacteria lacking Msr activity, such as *X. campestris* (Vattanaviboon *et al.*, 2005), *N. gonorrhoeae* (Skaar *et al.*, 2002) and *H. pylori* (Alamuri & Maier, 2004), have been shown to be more sensitive to superoxide stress. However, it is thought that superoxide does not oxidize methionine residues directly, but rather does so through dismutation to hydrogen peroxide (carried out by metal ions) and through formation of the hydroxyl radical HO[•] (Vogt, 1995). Therefore it is likely that the increased killing of both single mutants and the double *msrA/B* mutant in *C. jejuni* is through formation of these species, possibly mostly by the hydroxyl radical, since if the damage to methionine residues was mainly by hydrogen peroxide, one would expect the killing phenotypes seen with hydrogen peroxide and superoxide to be very similar. It is also thought that superoxide causes damage to methionine residues through formation of the highly reactive peroxynitrite radical after interacting with nitric oxide (Vogt, 1995).

SNP (a nitrosating agent, which is primarily an NO⁺ donor) caused a significant loss of viability and increased zones of killing in both single mutants, and had a much magnified effect in the double *msrA/B* mutant. SNP also shows this effect in kill curve assays. However, only the double mutant is affected if the NO releaser spermine NONOate is used in kill curve experiments. Presumably, cells have sufficient mechanisms present to remove free nitric oxide before it is able to oxidize methionine residues, but if NO groups are donated directly to proteins and allowed to damage residues in this way, it is more difficult for the cells to repair them before this leads to a loss in viability. In *E. coli*, *msrA* mutants have been shown to be hypersensitive to nitrosative stress agents (St. John *et al.*, 2001), most likely through their role in the formation of peroxynitrite. Indeed, *H. pylori* Msr is upregulated upon addition of peroxynitrite (Alamuri & Maier, 2006), indicating an important role of Met-SO repair when cells experience nitrosative stress. Our results indicate a significant contribution of MsrA and MsrB to nitrosative stress resistance in *C. jejuni*, in addition to the previously established roles for the globin Cgb (Elvers *et al.*, 2004) and the nitrite reductase NrfA (Pittman *et al.*, 2007).

Diamide, a thiol-specific oxidizing agent that increases disulphide bond formation in proteins, causes a significant increase in the size of the zone of inhibition in both *C.*

jejuni single mutants and the double *msrA/B* mutant, to approximately the same degree. There is no cumulative effect in the double mutant. However, as with hydrogen peroxide, when diamide is used in kill curve assays, only the *msrA/B* double mutant shows an increase in killing compared with wild-type cells. This again is likely to be due to the growth phase-dependent expression of the Msr enzymes, as seen in other bacteria (Alamuri & Maier, 2004; Vattanaviboon *et al.*, 2005), and again could highlight the limitations of both methods as mentioned above. The systems present to remove peroxides and deal with stress agents such as diamide appear to be more robust than those that deal with superoxide and nitrosative stress agents. This conclusion is drawn from the phenotypes of the single and double mutants when using both disc assays and kill curves. When peroxide stress agents and diamide are used, both the single mutants and the double mutant show a similar increase in the zone of inhibition, with no cumulative effect seen in the double mutant. When used in kill curves, only the double mutant exhibits an increased loss in viability. However, with methyl viologen and diamide, a much more sensitive phenotype is seen in the double mutant in both disc assays and kill curves.

Msr enzymes have also been shown to be necessary for host colonization and persistence in *Lactobacillus reuteri* and *H. pylori* (Alamuri & Maier, 2004; Walter *et al.*, 2005), which is not only due to protection against oxidative stress but also through a possible role in the maintenance and expression of adhesins, allowing persistence and biofilm formation (Wizemann *et al.*, 1996). Host colonization and persistence studies of the three mutant strains studied here would also shed further light on the *in vivo* role of MsrA and MsrB from *C. jejuni*. Growth phase-dependent and stress-induced expression of the cognate genes should also be addressed in future studies.

ACKNOWLEDGEMENTS

J. M. A. was supported by a DTA studentship from the Biotechnology and Biological Sciences Research Council. We thank Ben Harding, Emma Alty and Laura Smith for technical help.

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Edited by: T. Abee