A sulphite respiration system in the chemoheterotrophic human pathogen 
Campylobacter jejuni

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The ability to use sulphite as a respiratory electron donor is usually associated with free-living chemolithotrophic sulphur-oxidizing bacteria. However, this paper shows that the chemoheterotrophic human pathogen Campylobacter jejuni has the ability to respire sulphite, with oxygen uptake rates of $23 \pm 8$ and $28 \pm 15$ nmol O$_2$ min$^{-1}$ (mg cell protein)$^{-1}$ after the addition of 0.5 mM sodium sulphite or metabisulphite, respectively, to intact cells. The C. jejuni NCTC 11168 Cj0004c and Cj0005c genes encode a monohaem cytochrome c and molybdopterin oxidoreductase, respectively, homologous to the sulphite : cytochrome c oxidoreductase (SOR) of Starkeya novella. Western blots of C. jejuni periplasm probed with a SorA antibody demonstrated cross-reaction of a 45 kDa band, consistent with the size of Cj0005. The Cj0004c gene was inactivated by insertion of a kanamycin-resistance cassette. The resulting mutant showed wild-type rates of formate-dependent respiration but was unable to respire with sulphite or metabisulphite as electron donors. 2-Heptyl-4-hydroxyquinoline-N-oxide (HQNO), a cytochrome bc$_1$ complex inhibitor, did not affect sulphite respiration at concentrations up to 25 mM, whereas formate respiration (which occurs partly via a bc$_1$ dependent route) was inhibited 50%, thus suggesting that electrons from sulphite enter the respiratory chain after the bc$_1$ complex at the level of cytochrome c. Periplasmic extracts of wild-type C. jejuni 11168 showed a symmetrical absorption peak at 552 nm after the addition of sulphite, demonstrating the reduction of cytochrome c. No cytochrome c reduction was observed after addition of sulphite to periplasmic extracts of the Cj0004c mutant. A fractionation study confirmed that the majority of the SOR activity is located in the periplasm in C. jejuni, and this activity was partially purified by ion-exchange chromatography. The presence of a sulphite respiration system in C. jejuni is another example of the surprising diversity of the electron-transport chain in this small-genome pathogen. Sulphite respiration may be of importance for survival in environmental microaerobic niches and some foods, and may also provide a detoxification mechanism for this normally growth-inhibitory compound.

INTRODUCTION

Campylobacter jejuni is a Gram-negative, spiral-shaped bacterium that causes acute diarrhoeal illness, and is now established as the leading cause of acute bacterial gastroenteritis in both industrialised and developing countries (Friedman et al., 2000). The most common route of infection is by ingestion of contaminated food, milk or water, with poultry being a particularly common source of contamination (Friedman et al., 2000), as C. jejuni is a commensal of the gastrointestinal tract of many species of birds. Acute symptoms of C. jejuni infection in humans include diarrhoea, fever and abdominal pain. Complications arising after infection are rare although there is association with reactive arthritis and certain neurological disorders such as Miller–Fisher and Guillain–Barre syndromes (Nachamkin et al., 2000; Skirrow & Blaser, 2000). Several factors contribute to the pathogenicity of C. jejuni, including environmental survival, adhesion to host mucosa, the ability to invade host cells and the production of several toxins (Pickett, 2000). Until recently, many aspects of the physiology of this food-borne pathogen were poorly defined. The publication of the genome sequence (Parkhill et al., 2000) has stimulated a renewed interest in studying the physiological characteristics of C. jejuni, which are crucial to understanding its mechanisms of pathogenicity and ability to survive in the environment.

C. jejuni is an obligate microaerophile which appears to have a surprisingly complex and highly branched respiratory

Abbreviation: SOR, sulphite : cytochrome c oxidoreductase.
chain for a relatively small-genome pathogen, allowing the use of a wide variety of electron donors such as formate, hydrogen, D-lactate, succinate, malate and NAD(P)H, and alternative electron acceptors to oxygen, including fumarate, nitrate, nitrite, N- or S-oxides and hydrogen peroxide (Kelly, 2001; Sellars et al., 2002; Myers & Kelly, 2005). The addition of sulphite to intact cells of C. jejuni was found by Hoffman & Goodman (1982) to produce a proton pulse (H+/O ratio = 1.58), and sulphite was thus proposed to serve as a potential electron donor. However, the nature of the sulphite oxidation system was not elucidated and no further studies have been carried out. The potential presence of a sulphite respiration system in C. jejuni may be of importance in survival of this pathogenic organism in microaerobic aquatic niches, rich in sulphite, and in some foods, as sulphite is commonly added to processed foods as a preservative. Interestingly, sulphite is also released in the human body by neutrophils in response to stimulation with lipopolysaccharide as part of the host defence, as sulphite is a well-known antimicrobial (Mitsuhashi et al., 1998).

Sulphite oxidation can support chemolithotrophic and phototrophic growth in a diverse range of bacteria and archaea (Wood, 1988; Sorokin, 1995; Friedrich, 1998) and is known to occur either by direct oxidation, usually utilizing a molybdenum-containing sulphite:cytochrome c oxidoreductase (SOR) or by indirect, AMP-dependent oxidation via the intermediate adenylsulphate (Wood, 1988; Kappler & Dahl, 2001). In this study, we demonstrate the ability of the chemoheterotrophic pathogen C. jejuni to utilize sulphite and metabolisulphite as respiratory electron donors, identify the proteins constituting the sulphite oxidase and elucidate the pathway for electron transport from sulphite to oxygen.

**METHODS**

**Bacterial strains, media and culture conditions.** Cultures of C. jejuni strain NCTC 11168 were routinely grown at 37 °C in microaerobic conditions [5% (v/v) O2, 10% (v/v) CO2 and 85% (v/v) N2] in a MABS-VA500 Incubator (Don Whitley Scientific) on Columbia agar containing 5% (v/v) lysed horse blood and 10 μg ml⁻¹ each of amphotericin B and vancomycin. Cells were subcultured onto fresh medium every 2–3 days to maintain actively dividing cells. Liquid cultures were grown in brain heart infusion (BHI) broth supplemented with 5% (v/v) fetal calf serum (BHI-FCS) and the above antibiotics at 37 °C. Cultures were maintained in the MABS-VA500 Incubator with continuous orbital shaking at 200 r.p.m.

**Insertional inactivation of Cj0004c.** PCR primers (forward primer 5'-TGAAGGTATAGGAATGATG-3', reverse primer 5'-GGAGTTCTTATTTCTAAAGC-3') were designed to amplify a 1.6 kb product containing the Cj0004c gene, which was cloned into pGEM T-easy (Promega) and insertionally inactivated at a unique EcoNI site using the aphAIII (kanamycin resistance) gene containing its own promoter, derived from pJKM30 (van Vliet et al., 1998), producing plasmid pDM4. As the downstream gene (gyrB) is transcribed in the opposite orientation to Cj0004c, there is no polarity effect of this insertion. Plasmid pDM4 was then electroporated into C. jejuni 11168, with selection on blood agar plates containing 30 μg kanamycin ml⁻¹. The above primers were used in a PCR using genomic DNA from the wild-type 11168 strain or from a kanamycin-resistant transformant, in order to verify the correct mutant construction.

**Measurement of respiration rates by oxygen uptake.** Substrate oxidation was determined as described previously (Hughes et al., 1998), by measuring the change in dissolved oxygen concentration of cell suspensions in a Clark-type oxygen electrode linked to a chart recorder and calibrated using air-saturated 25 mM phosphate buffer (pH 7.5) (220 nmol dissolved O2 ml⁻¹ at 37 °C). A zero-oxygen baseline was determined by the addition of sodium dithionite. The cell suspension was maintained at 37 °C and stirred at a constant rate. Substrates and inhibitors were added by injection through a fine central pore in the airtight plug. Rates were expressed in nmol O2 utilized min⁻¹ (mg cell protein)⁻¹.

**Analysis of cytochrome spectra, and cytochrome c quantification.** Cytochrome spectra were obtained at room temperature using a Shimadzu UV-2101PC double-beam scanning spectrophotometer. Spectra were scanned from 400 nm to 700 nm. Reduced minus oxidized spectra were obtained by adding a few grains of sodium dithionite or ammonium persulphate as reductant or oxidant, respectively (Jones & Poole, 1985). Reduction of cytochromes by physiological substrates was performed in a sealed cuvette with addition of substrate by injection through a seal in the lid. These spectra were scanned against an ammonium-persulphate-oxidized baseline. The amount of cytochrome c in cell fractions was quantified by the increase in absorbance at 550 nm after reduction of the sample by excess sodium dithionite, using an absorption coefficient of 20 mM⁻¹ cm⁻¹.

**Preparation of periplasmic proteins from C. jejuni.** Cells were grown in the microaerobic cabinet at 37 °C in 200 ml BHI-FCS broth overnight. The cell suspension was centrifuged (15 000 g, 20 min at 4 °C) and the resulting pellet was resuspended in 10 ml 20% (w/v) sucrose, 30 mM Tris/HCl pH 8 at room temperature. EDTA was added to a final concentration of 1 mM and the suspension was poured into a 100 ml conical flask and stirred at 180 r.p.m. in a 25 °C constant-temperature room for 10 min. The suspension was then centrifuged (10 000 g, 10 min at 4 °C) and the pellet was resuspended in ice-cold 10 mM Tris/HCl pH 8 to a volume of 10 ml and stirred at 180 r.p.m. in a 4 °C constant-temperature room for 10 min. The suspension was then centrifuged again (18 000 g, 15 min at 4 °C) and the supernatant collected as the periplasmic fraction. The pellet was also used for further fractionation of cytoplasm and membrane as described below.

**Preparation of C. jejuni cell-free extract.** A 200 ml C. jejuni culture in BHI-FCS was grown for 16 h at 37 °C with shaking in a microaerobic atmosphere. The cells were harvested by centrifugation (10 000 g, 15 min, 4 °C) and resuspended in 5 ml 10 mM Tris/HCl pH 8. The cell suspension was sonicated (20 kHz, 6 μm amplitude) and centrifuged (20 000 g, 20 min, 4 °C) to remove cell debris.

**Localization of SOR activity.** A 1-litre culture of C. jejuni was grown overnight in BHI-FCS and harvested by centrifugation (10 000 g, 15 min, 4 °C). The whole cell pellet was resuspended in 60 ml 20% (w/v) sucrose, 30 mM Tris/HCl pH 8 and subjected to the osmotic-shock procedure described above, but with a resuspension volume of 60 ml. After decanting the periplasmic fraction, the pellet was resuspended in 5 ml 10 mM Tris/HCl pH 8 and sonicated (20 kHz, 6 μm amplitude) to release the cell contents. This suspension was subjected to low-speed centrifugation (20 000 g, 20 min, 4 °C) to remove cell wall debris and then the supernatant was ultracentrifuged (100 000 g, 80 min, 4 °C) to separate membrane and cytoplasmic fractions. The membrane pellet was resuspended in 3 ml 10 mM Tris/HCl pH 8.
Enzyme assays. SOR activity was assayed by a method based on that described by Kappler et al. (2000), using a Shimadzu UV-2101PC double-beam scanning spectrophotometer at a wavelength of 550 nm. Rates were obtained by measuring the increase in absorbance of cytochrome c after adding 0.5 mM sodium sulphite to a cuvette containing the following: 900 µl 10 mM Tris/HCl pH 8, 100 µl 10 mg horse heart cytochrome c ml⁻¹, 10–100 µl cell fraction. An absorption coefficient of 20 mM⁻¹ cm⁻¹ at 550 nm was used.

Isocitrate dehydrogenase activity was assayed according to Leyland & Kelly (1991) in a Shimadzu UV-2101PC spectrophotometer at a wavelength of 340 nm. Rates were obtained by adding 100 µl 50 mM sodium isocitrate to a cuvette containing the following: 100 µl 50 mM Tris/HCl pH 8; 100 µl 10 mM NADP; 100 µl 10 mM MgCl₂; 500–590 µl distilled H₂O; 10–100 µl cell fraction. The specific activity of isocitrate dehydrogenase was calculated using an absorption coefficient of NADPH of 6.22 mM⁻¹ cm⁻¹ at 340 nm.

Partial purification of SOR activity. Ion-exchange chromatography was utilized for partial purification of the periplasm. A column packed with 30 ml DEAE-Sepharose (Pharmacia) was connected to a Bio-Rad Biologic HP system. A 50 ml sample of periplasm was loaded onto the anion-exchange column and equilibrated with 50 mM Tris/HCl pH 8, 100 µM NADP, 100 µM MgCl₂, 500–590 µl distilled H₂O, 10–100 µl cell fraction. The specific activity of isocitrate dehydrogenase was calculated using an absorption coefficient of NADPH of 6.22 mM⁻¹ cm⁻¹ at 340 nm.

SDS-PAGE and Western blotting. SDS-PAGE was carried out according to the method of Laemmli (1970) and gels were stained for protein using Coomassie brilliant blue R. For Western blots, periplasmic extracts were prepared as described above and denatured by heating (100°C, 5 min) in the presence of SDS sample buffer. The protein was loaded and separated by SDS-PAGE (12%, w/v, acrylamide), then electroblotted onto a PVDF membrane (Bio-Rad) in a Bio-Rad mini PROTEAN II cell at 11 mA for 16 h. Immuno-detection was performed using an ECL detection kit (Amersham Biosciences) according to the instructions of the manufacturer. The primary antiserum for detection of Cj0005 was kindly donated by Christiane Dahl (Institut fur Mikrobiologie und Biotechnologie, Bonn, Germany). The antiserum was prepared in rabbit and was raised against SorA of Starkeya novella, as described by Kappler et al. (2000). The working concentration for this serum was a 1 : 10 000 dilution. An anti-rabbit-horseradish peroxidase conjugate (Amersham Biosciences) was used as a secondary antibody (1 : 2000 dilution).

Sequence alignment and phylogenetic analysis. Multiple protein sequence alignments and phylogenetic analyses were performed using CLUSTAL X (Thompson et al., 1997) with output generated by ESPript (Gouet et al., 1999). Phylogenetic trees were viewed in TreeView (Page, 1996).

Determination of protein concentration. This was done by the Lowry method.

RESULTS

Oxygen-linked respiration of formate, sulphite and metabisulphite by C. jejuni

C. jejuni has been predicted to utilize several types of electron donor to support respiration (Myers & Kelly, 2005). Fig. 1(a) shows the ability of C. jejuni to use formate as a respiratory electron donor. Addition of 5 mM sodium formate to late-exponential-phase wild-type 11168 cells produced a mean oxygen respiration rate of 478 ± 118 nmol O₂ min⁻¹ mg⁻¹ (mean ± SD, n = 3 biological replicates). Fig. 1(b) shows the ability of C. jejuni to respire using sulphite as an electron donor. Addition of 0.5 mM sodium sulphite to C. jejuni cells produced a mean oxygen respiration rate of 23 ± 8 nmol O₂ min⁻¹ mg⁻¹ (n = 3). Concentrations above 0.5 mM sulphite were found to produce a background rate of oxygen consumption in the absence of cells due to chemical oxidation of sulphite; 0.5 mM sulphite was tested without cells and shown to produce no residual oxygen consumption. Fig. 1(c) shows the ability of C. jejuni to respire using 0.5 mM sodium metabisulphite (Na₂S₂O₅), which produced an oxygen respiration rate of 28 ± 15 nmol O₂ min⁻¹ mg⁻¹ (n = 3). The actual electron donor here is likely to be the bisulphite anion HSO₃⁻, which is produced by reaction of metabisulphite with water.

Identification of a putative SOR in C. jejuni

Examination of the C. jejuni NCTC 11168 genome sequence (Parkhill et al., 2000) for genes encoding potential components of a sulphite respiration system revealed a putative periplasmic molybdoenzyme encoded by Cj0005c with a molecular mass of 46 kDa and an associated 13 kDa periplasmic molybdoenzyme containing a c-type haem-binding motif (CXXCH) encoded by Cj0004c, as the most likely candidates. Cj0005 shows 32% sequence identity to the well-characterized SorA subunit found in the chemolithothroph Starkeya novella (formerly Thiobacillus novellus). The sorA genes of St. novella encode a periplasmic αβ heterodimeric SOR, consisting of a 40.6 kDa subunit containing a molybdopterin cofactor (SorA), where sulphite oxidation takes place, and an 8.8 kDa mono-haem cytochrome c₅₅₂ (SorB) subunit which acts as a specific electron acceptor (Kappler et al., 2000, 2001). A relationship between Cj0005 and SorA was previously noted by Kappler & Dahl (2001). However, Cj0005 also shows significant amino-acid identity to several uncharacterized molybdoproteins in chemoheterotrophs, including 46% amino-acid identity to SO0715, a putative 45 kDa molybdoprotein containing a Mo-Co dimerization domain encoded in Shewanella oneidensis, and 45% amino-acid identity to VPA1143, a putative 48 kDa molybdoprotein encoded in the pathogen Vibrio parahaemolyticus. The close phylogenetic relationship of C. jejuni Cj0005 to SorA of St. novella and other homologues in Sh. oneidensis and V. parahaemolyticus is shown in the phylogenetic tree in Fig. 2(a), which demonstrates the relatedness between a broad range of known molybdoenzymes including examples from the nitrate and DMSO reductase families from a number of different prokaryotes. The putative SOR proteins, including Cj0005, form a well-defined group, which clusters with St. novella SorA. Interestingly, C. jejuni also encodes an additional molybdoenzyme (Cj0379) which is distantly related to this group. Fig. 2(b) shows the gene arrangement around the putative sorA genes. Each of the genes encoding the
homologues appears to be part of an operon. A single 11.5 kDa (pre Sec-cleavage) cytochrome c (SorB) or putative 13 kDa cytochrome c (Cj0004) subunit is encoded downstream of the SorA/Cj0005-molybdoproteins of St. novella and C. jejuni respectively, while a putative 24 kDa dihaem cytochrome c (VPA1144) is encoded downstream of the V. parahaemolyticus SOR-molybdoprotein, and two separate 14 and 11 kDa cytochrome c subunits (SO0714 and S00716) are encoded upstream and downstream of the SOR-molybdoprotein of Sh. oneidensis. Fig. 2(c) shows a multiple sequence alignment of the SorA-downstream associated cytochrome subunits in these bacteria. As expected there is a high degree of amino acid conservation in the CXXCH motif region of these cytochromes, but while SO0716 and the C-terminal half of VPA1144 show 34% amino-acid sequence identity to Cj0004 over 92 and 79 residues, respectively, Cj0004 is only 23% identical to SorB of St. novella. These comparisons indicate that the c-type cytochromes that act as the specific electron acceptor for SorA are much more conserved than is SorA itself.

**A Cj0004c mutant is specifically deficient in sulphite and metabisulphite respiration**

In order to determine if the molybdoprotein encoded by Cj0005c was responsible for the SOR activity in C. jejuni, an attempt was made to construct a mutant strain. A plasmid vector containing a kanamycin-resistance cassette flanked by DNA from the Cj0005c ORF was constructed, transformed by electroporation into C. jejuni, and kanamycin-resistant transformants were isolated. However, PCR analysis showed that, for unknown reasons, the transformants were wild-type for the Cj0005c gene, showing the mutagenesis to be unsuccessful. The Cj0004c gene was therefore cloned and inactivated by the insertion of the kanamycin-resistance cassette at a unique EcoNI site (Fig. 3a). Electroporation of this plasmid into C. jejuni resulted in kanamycin-resistant colonies, which were confirmed by PCR to be the result of a successful allelic exchange, verified by the 1.5 kb increase in band size of the PCR product when using the same primers as for the cloning of the Cj0004c gene (Fig. 3b). One colony was picked and the strain designated JDM4. SDS-PAGE and Western blotting of periplasmic fractions using antibody raised against the SorA subunit of St. novella demonstrated the presence of a cross-reacting protein in the region of the 46 kDa molecular mass of the putative oxidoreductase, Cj0005 (Fig. 3c). The Cj0004c mutant was shown to be still expressing this protein, as would be expected (Fig. 3c). However, SDS-PAGE followed by a haem stain to detect c-type cytochromes showed the absence of a low-abundance 13 kDa haem-staining band in the periplasm of the mutant strain that was present in the wild-type parent (Fig. 3d), consistent with the expected loss of the Cj0004 protein.

**Fig. 1.** (a–c) Oxygen consumption catalysed by wild-type 11168 and Cj0004c mutant cells. Typical oxygen consumption rates at 37 °C of late-exponential-phase BHI-broth-grown wild-type 11168 (WT) and Cj0004c mutant (JDM4) (each 1 mg cell protein), after the addition of the following electron donors: (a) 5 mM sodium formate; (b) 0.5 mM sodium sulphite, (c) 0.5 mM sodium metabisulphite. (d) Inhibition of wild-type 11168 sulphite- and formate-stimulated oxygen respiration rates at 37 °C (2 mg cell protein), after the addition of 2-heptyl-4-hydroxyquinoline N-oxide (HQNO), a bc₁ complex inhibitor. △, Percentage of oxygen respiration when stimulated by 5 mM formate; ■, percentage of oxygen respiration when stimulated by 0.5 mM sodium sulphite. For this experiment, cells were obtained from blood agar plates and resuspended in 25 mM phosphate buffer pH 8. Respiration rates with formate and sulphite were lower than with broth-grown cells; 100% values were 13.4 and 5 nmol O₂ min⁻¹ mg⁻¹ respectively.
Fig. 1(a) shows that the Cj0004c mutant (JDM4) was unaffected in its ability to respire formate, with a similar rate to wild-type of $498 \pm 70$ nmol O$_2$ min$^{-1}$ mg$^{-1}$ ($n=3$). However, the addition of sulphite (Fig. 1b) or metabisulphite (Fig. 1c) to Cj0004c mutant cells resulted in no oxygen uptake, showing that Cj0004 is essential for sulphite- and metabisulphite-dependent respiration.

**The electron transport pathway from sulphite to oxygen**

Fig. 1(d) shows the rate of oxygen respiration in intact cells of 11168 with either 5 mM formate or 0-5 mM sodium sulphite as the electron donor, after the addition of increasing concentrations of the cytochrome $bc_1$ complex inhibitor 2-heptyl-4-hydroxyquinoline-N-oxide (HQNO).
Concentrations of HQNO of 20–25 μM resulted in ≥50% inhibition of oxygen respiration with formate as the electron donor. At the same concentrations of HQNO, the oxygen respiration rate was not inhibited with sulphite as the electron donor. The more specific cytochrome bc$_1$ complex inhibitors antimycin A and myxothiazol could not be used because even at high concentration they did not inhibit the formate respiration of $C. jejuni$. This is probably due to a problem with membrane permeability.

Fig. 4(a) shows the change in absorbance in the 550 nm region of the visible spectrum after the addition of 0?5 mM sulphite to a periplasmic extract of wild-type cells. A symmetrical peak at 552 nm was observed, indicating the reduction of cytochrome c in the extract. Full reduction by dithionite resulted in a larger symmetrical peak. Fig. 4(b) shows the same experiment with the periplasm of the $Cj0004c$ mutant. No change in absorbance was found in the 550 nm region after the addition of 0·5 mM sulphite, indicating that cytochrome c was not reduced by sulphite in this strain. The data in Figs 1 and 4 suggest that electrons from sulphite feed directly into the respiratory chain from $Cj0004c$ at the level of cytochrome c, bypassing the bc$_1$ complex.

**Induction, localization and partial purification of SOR activity**

Growth of $C. jejuni$ 11168 in BHI broth containing 5 mM sodium sulphite increased the rate of SOR activity (measured as sulphite : horse heart cytochrome c reductase activity) in late-exponential-phase cell-free extracts approximately twofold, from 142±5 nmol cyt c min$^{-1}$ mg$^{-1}$ (mean ± SD, $n=3$) to 290±31 nmol cyt c min$^{-1}$ mg$^{-1}$ ($n=3$), indicating partial inducibility of the enzyme. The
The deduced sequence of the Cj0005 protein is predicted to contain a twin-arginine motif at the N-terminus, suggesting export to the periplasm via the TAT system. The smaller Cj0004 protein is predicted to contain a typical Sec signal peptide, and this is also expected to be periplasmic. A cell fractionation was carried out to determine the cellular location of the SOR activity (Fig. 5). The distribution of activity was compared to that of markers for the periplasm (dithionite-reducible total cytochrome c) and cytoplasm (isocitrate dehydrogenase activity). The results of a typical fractionation showed 51% activity of SOR in the periplasm, 22% in the cytoplasm and 28% in the membrane fractions. The periplasm was contaminated with 23% of the cytoplasmic marker, and the cytoplasmic fraction was contaminated with 7% of the periplasmic marker in this experiment. The fractionation was repeated three times on separate cultures with similar results. Partial purification of SOR activity from the periplasm by anion-exchange chromatography is shown in Fig. 6(a). Fractions 13–18 were found to contain the highest level of activity, with fraction 16 (95 mM NaCl) being the peak fraction. These fractions were subjected to SDS-PAGE and then stained with Coomassie blue, which revealed the 46 kDa Cj0005 protein (Fig. 6b). Fractions 13–18 were also run on SDS-PAGE, blotted onto PVDF membrane and the membrane reacted with an antibody raised against SorA of St. novella. The antibody cross-reacted with a 46 kDa band in fractions 16, 17 and 18 (Fig. 6b), the same size as Cj0005 observed in blots of whole periplasm (Fig. 3). Fraction 17 produced the strongest cross-reaction on the immunoblot.

**DISCUSSION**

This study is believed to be the first characterization of a sulphite respiration system from a chemoheterotrophic pathogenic bacterium, rather than the typical chemolithotrophic species with which this ability is more commonly associated (Wood, 1988). The results obtained show unequivocally that *C. jejuni* has the ability to respire sulphite and imply that the proton pulse detected by Hoffman & Goodman (1982) after the addition of sulphite to intact cells was the result of proton translocation accompanying the transfer of electrons from sulphite to oxygen catalysed by a specific sulphite oxidase.

Sulphite is an extremely widespread inorganic anion in many environments, particularly low-oxygen niches in soil or water, where it is more stable than in aerobic conditions, and is thus available for microbial oxidation or reduction by a variety of pathways (Wood, 1988). The possession of a sulphite respiration system may thus contribute to the survival of *C. jejuni* in such environments, particularly as this bacterium has a very high-affinity cb-type cytochrome c oxidase which could allow sulphite respiration at extremely low oxygen concentrations. However, for many chemoheterotrophic bacteria sulphite, and particularly metabisulphite (which in aqueous solutions is rapidly converted to the bisulphite anion, HSO₃⁻), are well-known growth-inhibitory compounds. Nevertheless, Bolton *et al.* (1984) showed enhanced growth and aero-tolerance of *C. jejuni* by addition of a mixture of ferrous sulphate, sodium metabisulphite and sodium pyruvate (FBP) as a growth supplement in laboratory media. FBP is thought to function as a system for reducing oxidative stress by removing free radicals from the medium. Our demonstration that *C. jejuni* can respire sulphite, and metabisulphite provides an explanation for their relative lack of toxicity for this bacterium and suggests a
growth-enhancing role for sulphite/bisulphite as an electron donor. Interestingly, growth of the closely related gastric pathogen Helicobacter pylori was found to be inhibited by the addition of sulphite or metabisulphite to the media (Jiang & Doyle, 2000) but FP growth supplement, which did not contain metabisulphite, did enhance the growth of H. pylori. Unlike C. jejuni, the genome of H. pylori does not encode a homologue of the SOR system. Thus, sulphite detoxification could be an additional function of this type of sulphite respiration system. This might be important in vivo, where, for example, there is evidence that sulphite can be produced by neutrophils in response to stimulation by lipopolysaccharide (Mitsuhashi et al., 1998).

Analysis of the Cj0004c/Cj0005c operon has demonstrated that C. jejuni encodes a SOR enzyme for sulphite oxidation, which is very similar to that of St. novella. The latter has been studied in detail and shown to be heterodimeric, and clearly distinct from the eukaryotic SOR enzymes, which are homodimeric proteins containing haem b and with a molybdopterin cofactor residing in each subunit (Kappler et al., 2000). The MPT cofactor is unusual for a prokaryote, with the majority of known bacterial molybdooenzymes containing the dinucleotide form of the pterin cofactor (Hille, 1996; Kisker et al., 1997, 1999). The St. novella SOR $K_m$ value for sulphite was 27 μM and a periplasmically located cytochrome $c_{550}$ has been identified as the natural electron acceptor, with a low $K_m$ value of 2.5 μM (Kappler et al., 2000). While the corresponding SorA molybdoenzymes of C. jejuni and St. novella are clearly homologous, the SorB monohaem cytochrome subunits of the two bacteria are not closely related, and it seems that SorA homologues in other bacteria may also be associated with distinct cytochrome subunits serving the same function. Although we were unable to mutate Cj0005c, several lines of evidence showed that the cytochrome encoded by Cj0004c was essential for sulphite respiration. The Cj0004c mutant possessed no detectable rate of oxygen respiration after the addition of sulphite or metabisulphite as an electron donor. Additionally, no cytochrome c reduction was detected upon addition of sulphite to periplasmic extracts. The mutation did not affect the expression of the large molybdopterin-containing subunit, Cj0005, which is thought to contain the active site responsible for the oxidation of sulphite. A more complete analysis involving attempts to remutagenize Cj0005c and complement both genes should be carried out, although genetic tools for C. jejuni are still somewhat limited.

SOR activity is located mainly in the periplasm of C. jejuni. Cell fractionation of C. jejuni is not as reliable as with ‘model’ bacteria like Escherichia coli, and the smaller percentages of activity detected in other cell compartments are probably due to an unavoidable lysis of some cells during the osmotic shock procedure and the enzyme non-specifically binding to the membrane fraction. A periplasmic
location would be in agreement with the predicted topology of the subunits and the signal sequences contained on the N-terminus of each subunit. The TAT system has been shown to export folded or partially folded proteins with the cofactors previously inserted in the cytoplasm (Sargent et al., 1998; Berks et al., 2003). The smaller cytochrome c subunit would be exported via the Sec pathway as an unfolded polypeptide with the haem group inserted in the periplasm. SOR activity was linked to the Cj0005 protein by the partial purification of the enzyme using anion-exchange chromatography. The fractions containing the highest SOR activity correlated with the presence of Cj0005, which was detected using the St. novella SorA antibody. However, the correlation was not exact, with the fraction of highest activity not being the same as the fraction with the highest concentration of Cj0005. A possible explanation would be that a functioning Cj0004 protein must be present for the detection of the SOR activity using the horse heart cytochrome c reduction method. The Cj0004 protein could be eluted at a different time to the Cj0005 protein and would result in a fraction which has the highest concentration of Cj0005 but does not have the full SOR activity. Unfortunately, the Cj0004 protein could not be positively identified on haem stains of column fractions.

This study provides more evidence for the highly diverse and branched structure of the respiratory chains of this pathogen, and it is now clear that many of the alternative electron transport pathways employ periplasmic molybdenum dehydrogenases or reductases. These include formate dehydrogenase, nitrate reductase and DMSO/TMAO reductase, in addition to the sulphite oxidase (Kelly 2001; Myers & Kelly, 2005; Sellars et al., 2002). The one remaining uncharacterized periplasmic molybdenum enzyme encoded by Cj0379 is related to YedY in E. coli (Loschi et al., 2004) and is distantly related to Cj0005, but is not a sulphite oxidase (J.D. Myers & D. J. Kelly, unpublished). Fig. 7(a) shows how the SOR system connects with these other electron-transport chains in C. jejuni, and Fig. 7(b) shows how electrons from sulphite are transferred to oxygen via the periplasmic SOR and the membrane bound cb-type cytochrome c oxidase. It is clear that the ability to use such a wide range of electron donors and electron acceptors allows considerable respiratory chain flexibility, particularly under conditions of severe oxygen limitation, and this could be a key factor in the organism being able to survive and grow in a number of different niches, possibly including human and animal hosts.

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