Roles of the twin-arginine translocase and associated chaperones in the biogenesis of the electron transport chains of the human pathogen *Campylobacter jejuni*

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The zoonotic pathogen *Campylobacter jejuni* NCTC 11168 uses a complex set of electron transport chains to ensure growth with a variety of electron donors and alternative electron acceptors, some of which are known to be important for host colonization. Many of the key redox proteins essential for electron transfer in this bacterium have N-terminal twin-arginine translocase (TAT) signal sequences that ensure their transport across the cytoplasmic membrane in a folded state. By comparisons of 2D gels of periplasmic extracts, gene fusions and specific enzyme assays in wild-type, *tatC* mutant and complemented strains, we experimentally verified the TAT dependence of 10 proteins with an N-terminal twin-arginine motif. NrfH, which has a TAT-like motif (LRRKILK), was functional in nitrite reduction in a *tatC* mutant, and was correctly rejected as a TAT substrate by the TATFIND and TatP prediction programs. However, the hydrogenase subunit HydA is also rejected by TATFIND, but was shown to be TAT-dependent experimentally. The YedY homologue Cj0379 is the only TAT translocated molybdoenzyme of unknown function in *C. jejuni*; we show that a *cj0379c* mutant is deficient in chicken colonization and has a nitrosative stress phenotype, suggestive of a possible role for Cj0379 in the reduction of reactive nitrogen species in the periplasm. Only two potential TAT chaperones, NapD and Cj1514, are encoded in the genome. Surprisingly, despite homology to TorD, Cj1514 was shown to be specifically required for the activity of formate dehydrogenase, not trimethylamine *N*-oxide reductase, and was designated FdhM.

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Abbreviations: BHI, brain heart infusion; BHI–FCS, BHI–fetal calf serum; GADH, gluconate dehydrogenase; ICDH, isocitrate dehydrogenase; LB, Luria–Bertani; pmf, proton-motive force; MetSO, L-methionine sulfoxide; MHS, Mueller–Hinton broth supplemented with 20 mM L-serine; MTSO, methylene sulfoxide; NO, nitric oxide; *p*-PD, *p*-phenylenediamine; REMP, redox enzyme maturation protein; SNP, sodium nitroprusside; SPF, specific pathogen free; TAT, twin-arginine translocase; TMAO, trimethylamine *N*-oxide; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; TMSO, tetramethylene sulfoxide.

Two supplementary tables, showing primers used in this study and predicted substrates of the TAT system, and a supplementary figure, showing 2D gel electrophoresis of a selection of periplasmic proteins, are available with the online version of this paper.

INTRODUCTION

The Gram-negative epsilonproteobacterium *Campylobacter jejuni* is prevalent in the gut microbiota of many bird and animal species but is also an important human pathogen; ingestion of contaminated poultry is a common route of infection (Wagenaar *et al.*, 2008). Consequently, *C. jejuni* is one of the most common causes of human enteric disease worldwide and is a major public health and economic burden (Jacobs-Reitsma *et al.*, 2008). *C. jejuni* is micro-aerophilic and most strains are unable to grow well under conditions of full aeration or strict anaerobiosis (Sellars *et al.*, 2002; Attack *et al.*, 2008).

Elucidation of the major pathways of electron transport in *C. jejuni* has been achieved via a multi-faceted approach involving experimental testing of predictions from the genome sequences (for review see Kelly, 2008). For a small genome (1.7 Mb) pathogen, a remarkably complex and

branched electron transport system is present (see Fig. 1), which allows energy conservation and growth under a wide range of environmental conditions. Inorganic and organic compounds, including hydrogen, sulphite, formate, lactate and gluconate can be utilized as electron donors (Myers & Kelly, 2005; Pajaniappan *et al.*, 2008; Weerakoon *et al.*, 2009; Thomas *et al.*, 2010), and pathways to a number of alternative electron acceptors have been identified, permitting growth and energy conservation under extreme oxygen limitation, as might occur in the gut. Examples include fumarate (Weingarten *et al.*, 2009; Guccione *et al.*, 2010), nitrate and nitrite (Pittman *et al.*, 2007), trimethylamine *N*-oxide (TMAO) and DMSO (Sellars *et al.*, 2002; Weingarten *et al.*, 2008). When *C. jejuni* is growing microaerobically with sufficient oxygen, electrons are transferred from menaquinone ($E_{m,7} = -75$ mV) to one of two terminal oxidases, directly to the non-electrogenic

quinol oxidase CioAB or via the proton-translocating cytochrome *bc₁* complex and cytochrome *c* to the *cb*-oxidase (Jackson *et al.*, 2007).

A significant number of the *C. jejuni* electron transport enzymes are known or predicted to be localized in the periplasm (Kelly, 2008). The twin-arginine translocase (TAT) provides a unique periplasmic transport pathway that utilizes the proton motive force to move fully folded proteins across the cytoplasmic membrane (Berks, 1996; Berks *et al.*, 2003). In bacteria, the TAT pathway can be involved in many cellular functions including respiration, cell envelope biogenesis, biofilm formation and metal homeostasis systems (Stanley *et al.*, 2001; Ochsner *et al.*, 2002; Ize *et al.*, 2004; Joshi *et al.*, 2010). In pathogens, the pleiotropic effects resulting from mutation of the TAT system can cause defects in virulence and host colonization

Fig. 1. Effect of *tatC* mutation on the electron transport pathways in *C. jejuni* NCTC11168. The experimentally established or highly likely routes of electron transport are shown for the wild-type (a) and a *tatC* mutant (b). This study has experimentally demonstrated that a *tatC* mutant cannot utilize formate, hydrogen, sulphite or gluconate as electron donors and is unable to use TMAO or nitrate as an alternative electron acceptor to oxygen. In addition, MfrA, MfrB, Cj0379 and the multi-copper oxidase CueO are absent in the periplasm of a *tatC* mutant. Note that NrfH is not translocated by the TAT machinery despite having a TAT-like signal sequence. The dashed lines in (b) represent routes of electron transport expected to be lost in a *tatC* mutant due to the suspected mislocalization of the PetA Rieske protein, which is part of the cytochrome *bc₁* complex. Figure modified and updated from a figure by Kelly (2008).

(Ochsner *et al.*, 2002; De Buck *et al.*, 2008; Rajashekara *et al.*, 2009; Mickael *et al.*, 2010), although these are often hard to ascribe to the mislocalization of a particular TAT substrate. Proteins bearing conserved twin-arginine residues in an N-terminal signal sequence motif S/TRRxFLK (Berks, 1996) are targeted to a membrane-integrated translocase complex consisting of three proteins: TatABC. TatB and C are believed to recognize the substrate, which is subsequently exported across the membrane via a pore formed by multiple TatA monomers (Gohlke *et al.*, 2005; Palmer *et al.*, 2005). *C. jejuni* contains homologues of *tatA* (*cj1176c*), *tatB* (*cj0579c*) and *tatC* (*cj0578c*) (Parkhill *et al.*, 2000). Substrates for the TAT pathway include electron transport enzymes with complex redox cofactors which must be inserted in the cytoplasm, multi-subunit complexes which require assembly prior to export and proteins whose folding is incompatible with the Sec translocation system (Santini *et al.*, 1998; Rodrigue *et al.*, 1999; Bronstein *et al.*, 2004).

For some complex bacterial TAT substrates, there is a preceding level of quality control present early on in their assembly. Regulation of transport is achieved by employment of dedicated cytoplasmic chaperone proteins that act as a 'proof reading' mechanism to coordinate protein folding and cofactor insertion with export (Sargent *et al.*, 2002; Palmer *et al.*, 2005). The substrates that require the action of these chaperones almost always contain a redox cofactor and TAT chaperones belong to a superfamily often referred to as redox enzyme maturation proteins (REMPs) (Turner *et al.*, 2004). They are thought to function by binding to the signal peptide of cofactor-less immature apoproteins, hence blocking recognition by TatC and preventing premature export while they aid protein folding, cofactor biosynthesis and loading and docking of any partner subunits (Palmer *et al.*, 2005; Genest *et al.*, 2008, 2009; Li *et al.*, 2010). Genes encoding REMPs are normally located in operons encoding the structural genes of the redox enzyme and chaperone protein function is specific to the redox enzyme in that particular operon; however, this is not always the case (Turner *et al.*, 2004; Chan *et al.*, 2009). The best studied Tat chaperones are DmsD (Oresnik *et al.*, 2001; Ray *et al.*, 2003) and TorD (Pommier *et al.*, 1998; Ilbert *et al.*, 2003; Jack *et al.*, 2004), which act as REMPs for the DMSO and TMAO reductases, respectively, and NapD (Maillard *et al.*, 2007), which functions with the Nap-type nitrate reductase. There is also evidence that general cytoplasmic chaperones, such as DnaK and SlyD, may have roles as Tat-signal-binding chaperones (Oresnik *et al.*, 2001; Graubner *et al.*, 2007). DnaK has been shown to have an important role in Tat targeting of the *Escherichia coli* multi-copper oxidase CueO (Graubner *et al.*, 2007), while SlyD may participate in the assembly of Ni-Fe hydrogenases in *E. coli* (Zhang *et al.*, 2005) and urease in *Helicobacter pylori* (Stingl *et al.*, 2008).

Only two proteins, alkaline phosphatase (Cj0145) and the nitrate reductase NapA subunit have thus far been shown to be TAT substrates in *C. jejuni* (strain 81116; van Mourik

et al., 2008). A recent study of the TAT system in *C. jejuni* 81-176 (Rajashekara *et al.*, 2009) has shown that a *tatC* null mutant exhibits an increased sensitivity to antimicrobial compounds and has defects in biofilm formation, motility and flagellation. The ability of this mutant to survive under osmotic shock and oxidative and nutrient stress was also reduced, as was its capability to colonize chickens. However, the colonization defect cannot be solely ascribed to the loss of the TAT system, as changes in motility/flagellation can also result in reduced virulence and colonization (Nachamkin *et al.*, 1993; Hendrixson & DiRita, 2004) and it is possible that at least some of the diverse phenotypes observed in the mutant are due to indirect secondary effects on global cellular processes, rather than specific absences of TAT substrates in the periplasm (Bronstein *et al.*, 2004; Rajashekara *et al.*, 2009).

In this study we have identified, by proteomics and activity measurements, the majority of the proteins that are predicted to be exported via the TAT pathway in the NCTC11168 wild-type strain, an isogenic *tatC* mutant and a *tatC/tatC*⁺ complemented strain. This has allowed a mechanistic explanation of specific growth phenotypes resulting from loss of the TAT system. The results clearly reveal the key role played by the TAT system in the assembly of the electron transport chains, particularly regarding export of the novel tungstoenzyme formate dehydrogenase and the molybdo-enzymes sulphite oxidase, nitrate reductase, TMAO/DMSO reductase and Cj0379 (see Fig. 1). The last protein is the only TAT-transported molybdoenzyme in *C. jejuni* with an unknown function; based on mutant studies, we suggest a possible role in periplasmic nitrosative stress resistance. Finally, the role and specificity of a homologue of the TorD chaperone has been investigated and shown to be essential only for formate dehydrogenase activity.

METHODS

Bacterial strains, media and culture conditions. *C. jejuni* strains NCTC11168 and the hypermotile variant 11168H (Karlyshev *et al.*, 2002) were routinely cultured at 37 °C under microaerobic conditions [10% (v/v) O₂, 5% (v/v) CO₂ and 85% (v/v) N₂] in a MACS growth cabinet (Don Whitley Scientific) on Columbia agar containing 5% (v/v) lysed horse blood and 10 µg ml⁻¹ each of amphotericin B and vancomycin. Where appropriate, kanamycin or chloramphenicol was added at a final concentration of 30 µg ml⁻¹. Liquid cultures of *C. jejuni* for enzyme assays were routinely grown in Mueller-Hinton broth (Oxoid) supplemented with 20 mM L-serine (MHS) under standard microaerobic conditions (gas concentrations as above), with 50–100 ml medium contained in 250 ml conical flasks with continuous orbital shaking at 180 r.p.m. Liquid cultures of *C. jejuni* for growth curves under oxygen-limited conditions were grown in brain heart infusion (BHI) broth supplemented with 5% (v/v) fetal calf serum (BHI-FCS). The gas atmosphere was the same as described for standard microaerobic conditions but the diffusion of oxygen was restricted by using 500 ml medium contained in a 500 ml conical flask with no shaking, as described previously (Sellars *et al.*, 2002; Guccione *et al.*, 2010). Electron acceptors were added from filter-sterilized stock solutions to a final concentration of 20 mM. Cultures were maintained in the MACS-VA500 incubator and growth was

monitored by measuring OD₆₀₀. Growth curves shown are representative of single experiments but all growth experiments were repeated three times with similar results. *E. coli* DH5 α was cultured in Luria–Bertani (LB) broth or agar supplemented with appropriate antibiotics at 37 °C.

DNA isolation, manipulation and construction of mutants. *C. jejuni* NCTC11168 chromosomal DNA was extracted by using the wizard genomic DNA purification kit (Promega). Standard techniques were employed for the cloning, transformation, preparation and restriction analysis of plasmid DNA from *E. coli* (Sambrook *et al.*, 1989). Mutants were constructed by insertion of an antibiotic resistance gene in the same transcriptional orientation as the gene of interest. Accuzyme (BioLine) proof reading DNA polymerase was used for all PCRs and the primers used are detailed in Supplementary Table S1, available with the online version of this paper. The *tatC* gene (*cj0578c*) was amplified from genomic DNA using the specific primers *tatC*-F and *tatC*-R. The resulting 899 bp fragment was cloned into pGEM3Zf(–) (Promega). The kanamycin resistance (*kan*) cassette from *Campylobacter coli* (Wang & Taylor, 1990), derived by PCR from plasmid pJMK30 (van Vliet *et al.*, 1998) using primers *kan*-F and *kan*-R, was cloned into the unique restriction site *Bsa*BI in *tatC* to produce plasmid *ptatCkan*. Correct insertion of the antibiotic resistance cassette into the target gene was confirmed by PCR. For inactivation of *cj0379c*, PCR primers *cj0379c*-F and *cj0379c*-R were used to amplify a 1.4 kb product containing the *cj0379c* ORF, which was cloned into pGEMT-easy (Promega) to form *pcj0379*. A 1.7 kb *Sma*I fragment containing the *kan* resistance gene from pJMK30 was then cloned into a unique *Kpn*I site in *cj0379c* to form *pcj0379kan*. The *cj1514c* (*torD* homologue) gene and 400 bp upstream flanking DNA was amplified using primers *torD*-F and *torD*-R. The 1.1 kb PCR product was cloned into the *Eco*RI site of pGEM3Zf(–) and transformants were recovered as above. The *kan* cassette from pJMK30 was blunt end cloned into the unique *Eco*RV site in *cj1514* to produce plasmid *ptorDkan*. Plasmids *ptatCkan*, *pcj0379kan* and *ptorDkan* were transformed by electroporation into *C. jejuni* NCTC11168 and transformants were selected using Columbia blood agar plates supplemented with kanamycin (30 μ g ml^{–1} final concentration). *pcj0379kan* was also transformed into NCTC11168H for chicken colonization studies (see below). Gene-specific primers were used to confirm allelic exchange by double crossover in each of the mutants.

Construction of complemented strains. For construction of a complemented *tatC* strain, the *tatC* gene plus the upstream ribosome-binding site was amplified by PCR using primers *tatC*-F and *tatC*-R and cloned into the *Xba*I site of the vector pRRC (Karlyshev & Wren, 2005) that integrates into one of the three rRNA loci in *C. jejuni*, to produce *pCtatC*. This was then transformed into the *tatC* mutant strain of *C. jejuni* 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 *tatC*-F and *C-seq*-R. For complementation of *torD*, the ORF plus upstream promoter and ribosome-binding site was amplified by PCR using the primers *torD*comp-F and *torD*comp-R. The resulting fragment was digested with *Bsm*BI and cloned into pC46 (Gaskin *et al.*, 2007) to create the vector *pCtorD*. This was transformed into the *torD* mutant strain as above. This complementation construct integrates at the *cj0046* pseudogene locus and the presence of both a wild-type and mutated copy of *torD* was confirmed by PCR with *torD*-gene-specific primers.

Construction of TAT peptide–TorA fusion strains. The TorA-encoding gene *cj0264c* was amplified by PCR so that the N-terminal signal sequence of the protein was removed. This fragment was digested with *Xba*I and cloned into similarly digested pRRC vector

(Karlyshev & Wren, 2005) to produce pSJHX. PCR products encoding the signal peptide of the putative translocated protein Cj0414 and the *torA* signal peptide (positive control), were digested with *Age*I and *Nar*I and cloned into similarly digested pSJHX to produce in-frame signal peptide–*torA* fusions. The plasmid pSJHX carries a chloramphenicol resistance gene with its own promoter, which acts as the selection marker and drives expression of the signal peptide–*torA* fusion *in vivo*. The fusion plasmids were electroporated into *C. jejuni* strain SJH100, which carries an insertional mutation in the chromosomal *torA* gene (Sellars *et al.*, 2002), and the *tatC* mutant.

Enzyme assays. All assays were performed at 37 °C using a Shimadzu UV-2401PC spectrophotometer. Methyl/benzyl-viologen-linked reductase assays were carried out with intact cells or periplasmic extracts in a 1 ml assay mixture contained in a screw top glass cuvette with a silicone seal to prevent gas exchange (Guccione *et al.*, 2010). All buffers and solutions were made anaerobic by sparging with oxygen-free nitrogen. The final assay mixture contained 25 mM sodium phosphate buffer, pH 7, (whole cells) or 10 mM Tris/HCl buffer, pH 7.5, (periplasm) with 1 mM methyl viologen (TMAO and nitrate reduction) or benzyl viologen (fumarate reduction) and 5 mM electron acceptor (TMAO, sodium nitrate or sodium fumarate). Cells or periplasm were added to the buffer plus viologen mixture, sparged with oxygen-free nitrogen, and aliquots of freshly prepared sodium dithionite solution were injected into the cuvette until a steady absorbance at 585 or 578 nm was achieved (for methyl and benzyl viologen, respectively). The assay was initiated by the injection of an anaerobic solution of electron acceptor and the absorbance decrease was recorded. For hydrogenase activity, the buffer/methyl viologen assay mixture was saturated with hydrogen gas and the assay was initiated by the addition of cells. Formate dehydrogenase assays were carried out as for reductase assays but the sparged cell/buffer/benzyl viologen mixture was not reduced with dithionite prior to the assay being started by the addition of an anaerobic solution of 10 mM sodium formate. Specific activities for all substrates were calculated using an absorption coefficient for methyl viologen of 11.8 mM^{–1} cm^{–1} at 585 nm and for benzyl viologen of 8.6 mM^{–1} cm^{–1} at 578 nm. Phenoloxidase assays were carried out on whole cells as described previously (Hall *et al.*, 2008). The 1 ml assay volume consisted of 25 mM sodium acetate buffer pH 5.7 and 100 μ l of cells. Assays were started by addition of the chromogenic substrate *p*-phenylenediamine (*p*-PD) to a final concentration of 10 mM and rates were recorded at 487 nm. Specific activities were calculated using an absorption coefficient for *p*-PD of 14.7 mM^{–1} cm^{–1} at 487 nm (Hall *et al.*, 2008). Protein concentrations of cell suspensions were determined using the method described by Markwell *et al.* (1978).

Disc diffusion assays and dose–response curves. For disc diffusion assays, 100 ml BHI–FCS liquid cultures were grown microaerobically with shaking for 16 h at 37 °C. The final OD₆₀₀ was adjusted to 0.1 by the addition of fresh BHI broth and the suspension was added to molten Mueller–Hinton agar at 50 °C, and plates were poured. Inhibition zones surrounding 8 mm diameter, sterile filter discs containing 5 μ l 100 mM sodium nitroprusside were measured after 2 days microaerobic incubation at 37 °C (12 replicate plates per strain). For dose–response curves, 100 ml BHI–FCS cultures of the strains to be tested were grown for 3 h after inoculation to an OD₆₀₀ 0.1 with an overnight starter culture. The OD₆₀₀ of the cultures were equalized to 0.1 by dilution with fresh sterile BHI. Three replicates of 150 μ l each culture were added to wells in a sterile 96-well microtest plate (Costar). Sodium nitroprusside (0–20 mM) was added to each of the replicate cultures to make the final volume 200 μ l. The microtest plates were then incubated in a microaerobic atmosphere at 37 °C for 1 h. After incubation, the cultures were serially diluted with sterile BHI and plated onto

Mueller–Hinton agar plates. The agar plates were incubated in a microaerobic atmosphere at 37 °C for 3–4 days before counting the c.f.u.

Measurement of respiration rates and nitric oxide (NO) consumption. Substrate-dependent oxygen respiration rates were determined as described previously (Myers & Kelly, 2005; Thomas *et al.*, 2010), 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). The cell suspension was maintained at 37 °C and stirred at a constant rate. Substrates [0.5 mM sodium sulphite, 10 mM organic acids or 0.25 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) plus 1 mM ascorbic acid] were added by injection through a fine central pore in the airtight plug. Consumption of NO by intact cells of the wild-type and *cj0379c* mutant was measured as described by Pittman *et al.* (2007) in a similar oxygen electrode as above, but with the reaction-chamber plug adapted with a hole to allow the additional use of a NO-specific electrode connected to an ISO-NO Mark II nitric oxide meter (World Precision Instruments). Both electrodes were linked to a chart recorder and the oxygen electrode was calibrated using air-saturated water as above. The NO electrode was calibrated by additions from a saturated solution of NO gas in anaerobic water (1.8 mM). Sodium formate (5 mM final concentration) was added to the cell suspension as an electron donor. The reaction chamber was allowed to become anaerobic by the consumption of oxygen by the cells, after which 4.5 µM NO solution was injected and the resulting response was recorded by the NO electrode. The NO electrode trace was measured for peak height and length of time for 50% degradation to occur.

Determination of nitrite reduction in cell suspensions. Cells were harvested from 150 ml overnight MHS cultures by centrifugation, washed twice, and finally resuspended in 3 ml 25 mM phosphate buffer (pH 7.5) to approximately 6 mg total cell protein ml⁻¹. This was added directly to the chamber of a Clark-type oxygen electrode, which was set up as above. Cells were provided with 20 mM sodium succinate as an electron donor and allowed to respire until a zero oxygen baseline was reached. After 5 min, 3 mM sodium nitrite was added to the chamber as the electron acceptor, 50 µl samples were taken at time intervals, immediately microcentrifuged and the supernatants were stored at -20 °C. Diluted culture supernatants (50 µl) were added to 850 µl of 1% (w/v) sulphanilamide dissolved in 1 M HCl and 100 µl 0.02% (w/v) naphthylethylenediamine (Pittman *et al.*, 2007). After 15 min, the absorbance at 540 nm was measured, and nitrite concentrations were determined by reference to a standard curve.

Cellular fractionation of *C. jejuni*. For 2D gel proteomic analysis, an osmotic shock method developed previously for *C. jejuni* was used (Myers & Kelly, 2005; Guccione *et al.* 2010). For rapid determination of selected enzyme activities, periplasm and cytoplasm were isolated by polymyxin B treatment of whole cells, as described previously (Sommerlad & Hendrixson, 2007; Attack *et al.*, 2008). Contamination of the periplasm by cytoplasmic proteins was assayed using cytochrome *c* content as the periplasmic marker and isocitrate dehydrogenase (ICDH) activity as the cytoplasmic marker, using the assays described by Myers & Kelly (2005). These assays showed that with either fractionation method, the periplasm contained about 10% of the total ICDH activity.

1D and 2D gel electrophoresis, immunoblotting and proteomics. Proteins were separated by standard SDS-PAGE using the Mini-PROTEAN 3 apparatus (Bio-Rad) on 10% (w/v) acrylamide gels, and immunoblotting with anti-MfrA antibodies, carried out exactly as described previously (Guccione *et al.*, 2010). Periplasmic

fractions were analysed by 2D gel electrophoresis using the protocol described in our previous work (Leon-Kempis *et al.*, 2006; Guccione *et al.*, 2010). Proteins were stained with SYPRO Ruby (Bio-Rad) and the gels were imaged using the Pharos FX+ Molecular Imager with Quantity One imaging software (Bio-Rad). Protein spots were excised from the gel using the ProPick excision robot (Genomic Solutions) and in-gel tryptic digestion was performed as described by Holmes *et al.* (2005). Tryptic digests were analysed using an Ultraflex MALDI-TOF instrument (Bruker). Proteins were identified by using the Protein Mass Fingerprint technique using the MASCOT search tool (Matrix Science; <http://matrixscience.com>). Mowse scores greater than 55 were statistically significant at *P*<0.05.

Chicken colonization studies. Chicken colonization studies were performed under licence at the Institute of Animal Health, Compton, Berkshire, UK. Specific pathogen free (SPF) Rhode Island Red chicks were obtained on the day of hatching from the Poultry Production unit, Institute for Animal Health, Compton, UK. All birds were inoculated orally with 0.1 ml *Campylobacter*-free adult gut flora preparation, produced by static culture of 1 g caecal contents obtained from a freshly killed 50-week-old SPF chicken in 10 ml LB broth for 23 h at 37 °C. Inoculated birds were housed and nourished on a vegetable-based diet (Special Diets Services) under appropriate SPF conditions until 3 weeks of age when they were used for the colonization trial. Groups of 24 3-week-old birds were inoculated orally with 0.1 ml culture containing 10⁷ c.f.u. of the desired *Campylobacter* strain. Unidirectional sterile swabs of the birds cloacal were taken and faecal secretion was tested by direct count and enrichment count as follows: cloacal swabs were taken at weekly intervals and were mixed into 1 ml modified Exeter broth (Baylis *et al.*, 2000). For direct counts, swabs were streaked onto *Campylobacter* blood-free selective agar (CCDA and CM739 containing selective supplement SR155; Oxoid) and colony counts were scored positive or negative after 2 days of incubation. Swabs were then placed back into the enrichment media (modified Exeter broth) and were incubated under microaerobic conditions at 37 °C. After 2 days, the enrichment cultures were checked for *Campylobacter* by plating on *Campylobacter* blood-free agar. These were scored as positive or negative results and referred to as enrichment counts. At 7 weeks post-inoculation, the birds were killed and the average post-mortem c.f.u. g⁻¹ was determined from the caecal contents counts of three and five birds from the groups inoculated with the 11168H and *cj0379cH* strains, respectively. Samples were diluted in PBS and were immediately plated on *Campylobacter* blood-free agar.

RESULTS

Predicted TAT substrate proteins in *C. jejuni* NCTC11168

Supplementary Table S2 shows the proteins identified as potential TAT pathway targets in the *C. jejuni* NCTC11168 genome based on the results from the prediction programs TATFIND v1.4 (Rose *et al.*, 2002; Dilks *et al.*, 2003) and TatP v1.0 (Bendtsen *et al.*, 2005) when run on all the translated proteins from the complete genome sequence. The results are largely in agreement with those of Rajashekara *et al.* (2009) in *C. jejuni* strain 81-176, except that we excluded Cj0692 which has a very poor TAT motif (LRRRFKK) more than 40 residues from the N terminus. Despite negative predictions from both TATFIND and TatP (seemingly due to lack of a cleavage site), we considered the electron transfer proteins NapH and NrffH as examples of

possible uncleaved, membrane-bound TAT substrates due to the presence of an appropriately placed arg-arg (RR) containing N-terminal motif. This would be similar to the iron-sulphur cluster containing Rieske protein (Cj1186), a subunit of the cytochrome *bc₁* complex that is predicted to be anchored to the membrane on the periplasmic side by an uncleaved TAT signal peptide. The prediction programs disagreed in the case of two proteins, alkaline phosphatase (Cj0145) and hydrogenase (Cj1267); the former has been shown experimentally to be a TAT substrate (van Mourik *et al.*, 2008). Although TAT substrates containing a twin-arginine motif are most common, proteins with a lys-arg (KR) rather than RR pair have been identified (Hinsley *et al.*, 2001). A further four proteins with a KR motif are present in strain NCTC11168, including a MurF (UDP-*N*-acetylmuramoyl-L-alanyl-D-glutamyl-*meso*-2,6-diaminopimeloyl-D-Ala-D-Ala synthetase) homologue, a protease and a lipoprotein. These were not investigated further in this study as they are not predicted to be involved in electron transport. Fig. 1(a) highlights those predicted TAT substrate proteins that play a crucial role in the electron transport pathways of *C. jejuni*. The TAT substrate predictions suggest that *C. jejuni* relies heavily on the TAT system for the efficient biogenesis and assembly of respiratory pathways and that the respiratory capabilities of a strain lacking this pathway are likely to be severely compromised.

Proteomic analysis of a *tatC* mutant

In order to obtain an initial view of the effects of the loss of the TAT system on the periplasmic protein complement of *C. jejuni*, periplasmic fractions from wild-type and *tatC* mutant cells grown under microaerobic conditions to exponential and stationary phase were separated by 2D gel electrophoresis followed by SYPRO Ruby staining (Fig. 2). Mass spectrometry analysis of spot picks (Mowse scores >55) positively identified a selection of proteins (see Fig. 2 and Supplementary Fig. S1). Periplasmic proteins without a TAT motif (Supplementary Fig. S1) could always be detected in both wild-type and mutant samples, although there was some variation in their abundance on the gels, despite equal protein loadings. However, a number of possible TAT-transported substrates were consistently less abundant or completely absent in the *tatC* mutant periplasm compared with the wild-type. The clearest example in exponential phase samples was HydB, the large subunit of the hydrogenase (compare Fig. 2a and b). This does not have its own TAT sequence but is likely to be translocated along with the TAT signal-containing small subunit, HydA, which was also identified on the gel run with wild-type proteins (Fig. 2a) but was absent on the gel of the *tatC* mutant (Fig. 2b). Both subunits of the gluconate dehydrogenase Cj0414 and Cj0415 were clearly identified as being absent in the *tatC* mutant periplasm; only Cj0414 has a TAT signal sequence, suggesting co-translocation of Cj0415. The periplasmic subunits of the Mfr fumarate reductase (MfrA and MfrB) are highly

induced by oxygen limitation (Guccione *et al.*, 2010) and were only identified in wild-type stationary phase periplasm (Fig. 2c). Of the periplasmic molybdoenzymes in *C. jejuni* NCTC11168, three (NapA, TorA and Cj0379) were identified as being present in the wild-type and absent in the *tatC* mutant periplasm (Fig. 2a and c), consistent with their translocation in a TAT-dependent manner.

A *tatC* mutant has both aerobic and oxygen-limited growth defects

Fig. 3 shows the growth characteristics of the wild-type strain, the *tatC* mutant and the mutant complemented with the wild-type *tatC* gene (a *tatC/tatC*⁺ merodiploid). Under microaerobic conditions in complex media, there was a clear reduction in the growth rate of the mutant and also in the final cell yield achieved. This indicates that the *tatC* mutation reduces the energetic efficiency of aerobic growth. Under oxygen-limited conditions with the alternative electron acceptors TMAO (Fig. 3b) or nitrate (Fig. 3c) the mutant showed a severe growth defect that would be consistent with a failure of export of the molybdoenzyme subunits of the TMAO reductase (TorA, Cj0264) and nitrate reductase (NapA) observed in Fig. 2. In each case, the mutant growth phenotypes were restored to the wild-type characteristics in the *tatC/tatC*⁺ merodiploid complemented strain.

Verification of the TAT dependence of specific electron transport proteins

To confirm that the predicted TAT substrates are periplasmically localized via their TAT signal sequences, a combination of direct enzyme activity and respiration rate measurements were carried out in the isogenic set of wild-type, *tatC* and *tatC/tatC*⁺ strains. Table 1 shows that the wild-type and complemented strains were able to aerobically respire sulphite at an approximately equal rate, but sulphite-dependent oxygen respiration was undetectable in the *tatC* mutant. That this loss of activity was due to mislocalization of the sulphite oxidase subunit SorA and not due to a disturbance of electron flux to cytochrome *c* oxidase, was confirmed by rates of oxygen consumption with TMPD/ascorbic acid (which donates electrons directly at the level of periplasmic cytochrome *c* into the *cb*-type cytochrome *c* oxidase) being similar in both the mutant and parent strain [~ 200 nmol O₂ min⁻¹ (mg protein)⁻¹].

To assay TMAO/DMSO reductase, periplasmic fumarate reductase (Mfr; mis-annotated as succinate dehydrogenase, see Guccione *et al.*, 2010; Juhnke *et al.*, 2009) and nitrate reductase, reduced methyl or benzyl viologen was utilized as the electron donor, which couples with the active site subunit of each complex that contains the TAT recognition sequence (i.e. TorA, MfrA and NapA, respectively). TMAO and nitrate were able to act as electron acceptors in intact wild-type and *tatC/tatC*⁺ cells but activity was totally lost in the *tatC* mutant (Table 1). To

Fig. 2. Proteomic analysis reveals the absence of specific proteins in the periplasm of a *tatC* mutant. Periplasmic protein (approx. 200 µg) from microaerobically grown wild-type (a, c) and *tatC* mutant (b, d) was separated by 2D gel electrophoresis as described in Methods. Selected proteins were identified from spot picks after trypsin digestion and mass spectrometric analysis. Cells were harvested in exponential phase (OD_{600} 0.6, ~ 7 h growth) (a, b) or in stationary phase (OD_{600} 1.0, ~ 16 h growth) (c, d).

Fig. 3. Microaerobic and oxygen-limited growth of wild-type, *tatC* and *tatC/tatC⁺* strains. (a) Microaerobic (oxygen sufficient) growth in 50 ml volumes of un-supplemented BHI-FCS media in 250 ml conical flasks shaken at 180 r.p.m. in a gas atmosphere of 10% (v/v) oxygen, 5% (v/v) carbon dioxide and 85% (v/v) nitrogen. For oxygen-limited growth, BHI-FCS was supplemented with either 20 mM TMAO (b) or 20 mM sodium nitrate (c) contained within almost completely filled unshaken conical flasks. In the absence of an electron acceptor, none of the strains was able to grow under oxygen limitation (data not shown). All data are representative of three independent growth experiments.

specifically assay the periplasmic fumarate reductase activity of the MfrA subunit without interference from the cytoplasmic-facing FrdA, periplasmic extracts were used for viologen-linked assays (Fig. 4a) and immunoblots with anti-Mfr antibodies (Fig. 4b). The results clearly showed the TAT dependence of MfrA activity, the absence of mature MfrA in the *tatC* mutant periplasm and the

Table 1. Enzyme activities of predicted TAT substrates in wild-type, *tatC* and *tatC/tatC⁺* strains

The rates shown are the mean $\pm$ SD of at least three biological replicates.

Enzyme	Rate (nmol min ⁻¹ mg protein ⁻¹)		
	Wild-type	<i>tatC</i>	<i>tatC/tatC⁺</i>
Sulphite oxidase*	26 $\pm$ 5	<1	24 $\pm$ 7
TMAO reductase†	717 $\pm$ 43	<1	705 $\pm$ 18
Nitrate reductase†	1572 $\pm$ 453	<1	1482 $\pm$ 384
Hydrogenase‡	22 $\pm$ 3	<1	19 $\pm$ 6
Formate dehydrogenase‡	166 $\pm$ 4	5 $\pm$ 2	162 $\pm$ 9
Multicopper oxidase§	7 $\pm$ 1	1 $\pm$ 1	5 $\pm$ 2

*O₂ consumption was measured.

†Viologen oxidation was measured.

‡Viologen reduction was measured.

§*p*-PD oxidation was measured.

accumulation of the larger unprocessed form of MfrA in the cytoplasm of the *tatC* mutant.

Hydrogen and formate are key electron donors for *C. jejuni* that are likely to be used in the host gut environment

Fig. 4. MfrA is mislocalized in a *tatC* mutant. (a) Fumarate reductase activity in wild-type, *tatC* mutant and *tatC/tatC⁺* periplasmic fractions. Reduced benzyl viologen was utilized as an artificial electron donor. Data are means $\pm$ SD. (b) Western blot analysis of wild-type and *tatC* cell-free extracts (top) and periplasm (bottom) with anti-MfrA antibody. Approximately 20 μ g protein was loaded in each lane.

(Weerakoon *et al.*, 2009). Hydrogenase enzyme assays performed with intact cells (Table 1) showed a complete lack of activity in the *tatC* mutant, consistent with the absence of the HydAB subunits in the periplasm (Fig. 2). Similarly, viologen-linked formate dehydrogenase activity was markedly *tatC*-dependent (Table 1) and similar results were obtained when formate-dependent oxygen respiration was assayed (data not shown).

It has previously been shown that the multicopper oxidase (CueO) of *C. jejuni*, encoded by *cj1516*, is located in the periplasm (Hall *et al.*, 2008). To determine the dependence of periplasmic localization on the TAT system, the characteristic phenoloxidase activity of this protein was assessed in the wild-type, *tatC* mutant and complemented strain (Table 1). Although the absolute rates were quite low in these experiments, a clear TAT dependence was observed, with a sevenfold difference in activities between wild-type and mutant cells. The complemented strain showed a restoration in activity to similar levels to that of the wild-type parent.

Cj0414/0415 have been shown to constitute a temperature-regulated gluconate dehydrogenase (GADH) involved in respiration-dependent energy conservation in *C. jejuni* strain 81-176 (Pajaniappan *et al.*, 2008). GADH activity in strain 81-176 was demonstrated by a dye-linked enzyme assay, and by respiration with D-gluconate as the electron donor. In both cases activity was approximately threefold higher after growth at 42 °C compared with that at 37 °C (Pajaniappan *et al.*, 2008). We carried out the same assays at both growth temperatures with strain NCTC11168 but could not detect any gluconate-dependent dye reduction or gluconate respiration, yet the protein subunits were present in the wild-type but not *tatC* mutant periplasm (Fig. 2). In order to further confirm that the predicted TAT signal sequence of *cj0414* was functional in protein translocation, we fused this region in-frame with the easily assayable *torA* (*cj0264c*) gene, lacking its own TAT signal sequence, using the pRR plasmid system (Fig. 5a). The fusion plasmid was introduced into a *torA* null mutant background so that any viologen-linked TMAO reduction in intact cells must be due to correct periplasmic translocation of the chimeric protein. TMAO reductase activity was indeed present in this strain, was clearly dependent on the presence of the Cj0414 signal sequence (Fig. 5b), but was abolished when the fusion plasmid was introduced into a *tatC* mutant background (Fig. 5b).

Finally, NrfH (Cj1358) is a membrane-bound tetra-haem NapC-like cytochrome *c* and is the sole electron donor to the periplasmic penta-haem nitrite reductase NrfA (Pittman *et al.*, 2007). NrfH is predicted to be membrane-anchored by what could be an uncleaved TAT-like signal sequence with the rest of the protein extending into the periplasm. However, both the TATFIND and TatP programs reject NrfH as a true TAT substrate. To determine if NrfH assembly into the cytoplasmic membrane is TAT-dependent, its activity as an electron donor to NrfA was measured by comparing rates of

succinate-dependent nitrite reduction in intact cell suspensions of wild-type and *tatC* mutant strains incubated under anaerobic conditions. As controls, *nrfH* and *nrfA* null mutants were shown not to reduce nitrite in this assay, as expected (Fig. 6a). As shown in Fig. 6(a, b), the rate of nitrite reduction in the *tatC* mutant compared to its wild-type parent was very similar, indicating that NrfH is not a TAT substrate.

A *cj0379c* mutant has a nitrosative stress phenotype

While the majority of the functions of the TAT substrates listed in Supplementary Table S2 are known, Cj0379 is the only TAT-translocated molybdoenzyme in *C. jejuni* whose function remains to be determined. It is homologous to the YedY protein in *E. coli*, which has some reductase activity with DMSO, tetramethylene sulfoxide (TMSO), L-methionine sulfoxide (MetSO) and TMAO (Loschi *et al.*, 2004). However, the kinetic parameters with all these substrates were very poor. In order to test the possibility that Cj0379 has a physiological role as an N- or S-oxide reductase, growth experiments were carried out with wild-type and isogenic *cj0379c* mutant strains under oxygen-limited conditions with TMAO, DMSO, TMSO, MetSO or methylene sulfoxide (MTSO) as electron acceptors. Growth of both strains on all of these compounds was similar (data not shown) and similar rates of electron-acceptor-dependent oxidation of reduced methyl viologen were found with all these substrates, most likely catalysed by the broad specificity TMAO reductase Cj0264. In addition, no differences in sulphite oxidase, or viologen-linked nitrate or nitrite reductase activity between wild-type and *cj0379c* strains could be detected (data not shown).

One possible role we considered for Cj0379 was in detoxification. Nitrosative stress caused by various reactive nitrogen species is a major feature of host-pathogen interactions, and *C. jejuni* is already known to use the cytoplasmically located globin Cgb (Elvers *et al.*, 2004) and the periplasmic nitrite reductase NrfA (Pittman *et al.*, 2007) to reduce NO. Nitric oxide in solution has a short half-life and a variety of NO donors and nitrosating agents can be used to apply nitrosative stress to bacterial cells. Disc diffusion assays were employed to determine the response of the *cj0379c* mutant to sodium nitroprusside (SNP), a nitrosating agent that predominantly donates the nitrosonium cation (NO⁺) to cellular targets. Fig. 7(a) shows the mean diameter of SNP inhibition zones of either wild-type, *cj0379c* or *torA* mutants (an additional control for the specificity of phenotypes observed for the *cj0379c* mutant). The *cj0379c* mutant was found to have a significantly higher sensitivity ($P=0.008$) to SNP than the wild-type and the *torA* strains. The response to a different source of nitrosative stress, an NO releaser (spermine NONOate) was also investigated by disc diffusion assays and similar results were obtained (data not shown). These strains were also tested for sensitivity to superoxide to determine

Fig. 5. The Cj0414 TAT signal sequence is able to translocate the TorA reporter protein across the cytoplasmic membrane. (a) Strategy for TAT signal sequence fusion. The *torA* (*cj0264c*) gene lacking its own TAT signal sequence was cloned into the *XbaI* site of the pRRC vector (Karlyshev & Wren, 2005) generating the plasmid pSJHX. Signal peptides amplified by PCR were digested with *AgeI* and *NarI* and cloned into similarly digested pSJHX to produce in-frame signal peptide-*torA* fusions. These fusion plasmids were used to transform *C. jejuni* SJH100 carrying an insertionally inactivated *torA* gene (Sellars *et al.*, 2002), and the *tatC* mutant. (b) TMAO reductase activity of wild-type and fusion strains measured using reduced methyl viologen as an artificial electron donor. The subscript numbering following the gene name in the fusion strains corresponds to the amino acid sequence in the wild-type *cj0414* and *torA* genes. The gene fusions were transformed into either the SJH100 *torA* mutant or the *tatC* mutant background as indicated. Data shown are means $\pm$ SD from three biological replicates.

whether the phenotype was specific to nitrosative stress or was an indirect effect resulting in general oxidative stress sensitivity. Paraquat (methyl viologen) was used as a superoxide-generating agent in disc diffusion assays; the results showed no significant difference in the diameter of growth-inhibited zones between wild-type and the *cj0379c* mutant (data not shown).

Further experiments were carried out in liquid cultures to examine the effect of SNP-induced nitrosative stress on cell viability. Fig. 7(b) shows a dose-response curve of the viability of wild-type, *cj0379c* and *torA* strains after a 1 h exposure to increasing concentrations of SNP. At a concentration of 10 mM SNP, there was a 1-log difference in killing of *cj0379c* compared with the wild-type and *torA* strains. At 15 mM SNP, *cj0379c* was greater than 6-logs less viable than wild-type and *torA*. All strains were completely killed at concentrations above 20 mM SNP. However, SNP can release cyanide in addition to donating NO⁺ ions to cellular targets. Control experiments with up to 20 mM sodium cyanide showed less than a 1-log decrease in viability and no significant difference between the strains (data not shown).

Finally, direct consumption of NO in anaerobic whole cell suspensions was investigated to determine if the inactivation of *cj0379c* reduced the ability of *C. jejuni* to degrade exogenous NO added as an aqueous solution. This can be conveniently measured using an NO selective electrode, but often occurs in a complex nonlinear manner (Pittman *et al.*, 2007), as degradation of NO to nitrite and other nitrogen species can proceed both enzymically and by chemical/non-biological routes (Hughes 1999; Watmough *et al.*, 1999). In the experiment shown in Fig. 7(c), the time taken for 50% of the NO to be degraded when a pulse of NO saturated anaerobic water is injected into the reaction chamber was measured. The NO half-degradation time was increased by about 15% in *cj0379c* compared with the wild-type parent strain, and the difference in half-degradation time was shown to be significant ($P=0.035$) in these experiments. There was also an increase in the average peak-height of the electrode response in the mutant cell suspensions immediately after injection of the NO solution (Fig. 7c), which may also be expected if these cells have a reduced ability to degrade NO, but this was not significant at $P>0.05$.

Fig. 6. A *C. jejuni* *tatC* mutant retains the ability to utilize nitrite as an electron acceptor. (a) Nitrite utilization in intact wild-type ($\blacktriangle$), *nrfA* ($\bullet$) and *nrfH* ($\square$) mutant cells. (b) Corresponding rate of nitrite utilization in a *tatC* mutant. Nitrite utilization was used as an indicator of electron transfer from succinate to nitrite, via NrfH. The decrease in nitrite concentration in the supernatant was determined by using the assay described by Pittman *et al.* (2007) following an initial addition of 3 mM nitrite to a suspension of anaerobic cells (6 mg ml⁻¹ cell protein in each assay, 20 mM succinate as electron donor) in an oxygen electrode. Data shown are means $\pm$ SD of at least two biological replicates.

A *cj0379c* mutant is defective in chicken colonization

The effect of disrupting the *cj0379c* gene in colonization of *C. jejuni* was tested in a chicken model. The *cj0379c* mutation was introduced into *C. jejuni* NCTC11168H, a hyper-motile variant previously described by Karlyshev *et al.* (2002) by the mutagenesis strategy described above using the *pcj0379kan* plasmid construct. The *cj0379cH* mutant was confirmed to be motile and to grow at the same rate as the parent strain. The colonization model used has been described previously (Jones *et al.*, 2004). It involved the inoculation of 2-week-old Light Sussex chickens possessing an acquired natural gut flora with the desired strains, and testing the birds over a 7 week period for evidence of colonization. The hyper-motile wild-type strain used was previously adapted for colonization (Jones *et al.*, 2004) and was found to have a high and persistent level of colonization over the course of this study; Fig. 7(d) shows it was detected in 100 % of the birds from the first week of the study through to the 7th week, after which a post-mortem examination showed the birds contained a

mean of 2.6×10^9 c.f.u. (g caecal contents)⁻¹. Fig. 7(d) also shows that the *cj0379cH* mutant strain was not detectable by the direct count method throughout the 7-week study, and only 10 % colonization was detected by the enrichment method in the first week which reduced to 5 % in weeks 2 and 3, and then colonization was undetectable by enrichment for the rest of the study. A post-mortem examination of the caecal contents revealed that the *cj0379cH* strain was below detectable limits of <100 c.f.u. g⁻¹.

The TorD homologue Cj1514 has a specific role in the biogenesis of formate dehydrogenase, but is not required for TMAO reductase activity

The sole *torD* homologue of *C. jejuni*, *cj1514c*, is paradoxically located upstream of the formate dehydrogenase operon, *fdhABCD* (Parkhill *et al.*, 2000; Turner *et al.*, 2004), and overlaps *cj1513c*, which is a small gene of unknown function predicted to be TAT-transported and translationally coupled to the downstream *fdhA* gene (see Fig. 8a, b). In order to ascertain whether the *C. jejuni* *torD* homologue acts specifically or has a more general chaperone activity, a *cj1514c* knockout mutant was constructed and assayed (Fig. 8c, d) for its ability to oxidize formate and sulphite, and reduce TMAO and nitrate, the substrates of the four assayable molybdo/tungsto-enzymes in *C. jejuni* NCTC 11168 (Smart *et al.*, 2009; Taveirne *et al.*, 2009). Both wild-type and *cj1514c* mutant cells were able to catalyse viologen-dependent nitrate reduction, and respire sulphite at similar rates (Fig. 8d). TMAO reductase activity was actually slightly increased in the mutant strain, possibly indicating a negative effect of Cj1514 on the export or maturation of TorA. However, formate dehydrogenase activity was completely abolished in the *cj1514c* mutant when assayed by either formate-dependent viologen reduction or formate respiration (Fig. 8c). In order to confirm that this phenotype was due to inactivation of *cj1514c* and not any polar effect on the downstream *cj1513c* and *fdh* operon, a complemented merodiploid strain was generated. Activity in this strain was restored to ~65 % of the wild-type rate in both the formate dehydrogenase assays described above (Fig. 8c). We thus propose that the *cj1514c* gene should be designated *fdhM* (for formate dehydrogenase maturation).

DISCUSSION

One of the key features of *C. jejuni* that is essential to understanding its capacity for both host colonization and survival in the environment is the diversity of the electron transport chains that allow continued energy conservation under changing conditions of electron donor and electron acceptor availability. As with other bacteria, the active site subunits of many of the cofactor-containing primary dehydrogenases and terminal reductases are located in the periplasm and are predicted to be translocated by the TAT system (see Fig. 1). In this study we used a *tatC* knockout mutant to gain experimental evidence for the

Fig. 7. Nitrosative stress sensitivity and chicken colonization phenotype of a *cj0379c* mutant. (a) Disc diffusion assays with SNP. Inhibition zones for wild-type, *cj0379c* and *cj0264c* mutants were measured (12 replicates) after 2 days microaerobic incubation. The asterisk indicates a significant difference between the wild-type and *cj0379c* mutant ($P=0.008$) by Student's *t*-test. Error bars indicate SD. (b) Dose-response curve of SNP killing. Data shown are means $\pm$ SD from three replicate cultures. (c) NO consumption measured by an NO electrode as in Methods. The asterisk indicates a significant difference ($P=0.035$) in the NO degradation time of the *cj0379c* mutant compared with the wild-type. The difference in peak height (maximal electrode response after injection of NO) was not significant ($P=0.138$). Error bars indicate SD. (d) Chicken colonization of NCTC11168H hyper-motile strain and isogenic *cj0379cH* mutant. Groups of 24 3-week-old Rhode Island Red chickens were inoculated orally at 10^7 c.f.u. and cloacal swabs were taken weekly for direct (filled bars) and enrichment (hashed bars) counts as described in Methods.

TAT dependence of the localization of many of these predicted substrates. An initial proteomics approach using 2D gel separation revealed that some of the key predicted TAT substrates are clearly absent in the *tatC* periplasm, but variation in protein abundance, the membrane anchoring of some TAT substrates and the fact that not all TAT substrates will necessarily be expressed under the growth conditions or in the media used meant that this approach was unlikely to identify all the proteins of interest. It did, however, highlight the likely co-translocation of the HydB, MfrB and Cj0415 subunits along with the TAT motif containing HydA, MfrA and Cj0414 subunits, respectively, via a probable 'piggy-backing' mechanism (Rodrigue *et al.*, 1999; Berks *et al.*, 2005; Guccione *et al.*, 2010).

The pronounced microaerobic growth defect of the *tatC* mutant observed here was also previously reported with strains 81116 (van Mourik *et al.*, 2008) and 81-176 (Rajashekara *et al.*, 2009). The most likely explanation for this is disruption of electron flow to oxygen via the

cytochrome *bc₁* complex, as the membrane-bound Rieske iron-sulphur subunit is a well predicted TAT substrate and has been reported to be TAT translocated in other bacteria (Bachmann *et al.*, 2006; De Buck *et al.*, 2007). The bioenergetic consequences of a non-functioning *bc₁* complex would be a decreased growth yield, not only as this is a major site of proton-translocation and thus proton-motive force (pmf) generation but also because it feeds electrons to the *cb*-type cytochrome *c* oxidase which is also proton-translocating (Pitcher & Watmough, 2004). The only alternative electron transport pathway to oxygen in the *tatC* mutant is thus via the quinol oxidase CioAB (Jackson *et al.*, 2007) which is not energy conserving, leaving electrogenic primary dehydrogenases which are not TAT substrates as the only mechanism to generate a pmf. There seem to be few examples of these (Fig. 1b) but the unusual flavodoxin-dependent Nuo complex that has been shown to be involved in 2-oxoglutarate respiration (Weerakoon & Olson, 2008) may become especially important in the *tatC* mutant. On the other hand, the complete

Fig. 8. *Cj1514* is required for formate dehydrogenase activity. (a) The arrangement of the genes upstream of *fdhA*; *cj1514c* is a *torD* homologue and *cj1513c* is a TAT domain protein of unknown function. The positions of the TAT consensus sequence are shown by arrows and white boxes. (b) The sequence overlap between (left) the 3' end of *cj1514c* and 5' end of *cj1513c*; and (right) the 3' end of *cj1513c* and the 5' end of *fdhA*. The sequence numbers match the bold and underlined nucleotides. (c) Formate dehydrogenase activity of wild-type, *cj1514* and *cj1514/cj1514⁺* strains of *C. jejuni* measured both as viologen-linked activity and formate-dependent oxygen respiration. (d) The activity of the assayable molybdoenzymes in wild-type and *cj1514* mutant strains. TMAO reductase and nitrate reductase activity was determined using viologen dyes as artificial electron donors, and sulphite oxidase activity by oxygen uptake measurements. Data shown are means $\pm$ SD of three biological replicates. The slight increase in TMAO reductase activity in the *cj1514c* mutant is significant at $P < 0.05$.

lack of growth of the *tatC* mutant under oxygen-limited conditions on TMAO or nitrate as electron acceptors is easily explained by the absolute TAT dependence of the respective terminal reductases.

Previous studies of the TAT system in *C. jejuni* strain 81116 have confirmed that two predicted substrates are actually translocated in a TAT-dependent manner, namely alkaline phosphatase (Cj0145) and nitrate reductase (van Mourik *et al.*, 2008). It should be noted that TATFIND rejects alkaline phosphatase as a TAT substrate, underlining the need for experimental verification of each prediction. Here, we employed multiple enzyme assays to compare catalytic rates in the isogenic set of wild-type, *tatC* and *tatC/tatC⁺* complemented strains to enable verification of most of the additional predicted TAT substrates in strain 11168. The only enzyme that (for unknown reasons) we could not detect activity for in this strain was gluconate dehydrogenase (Cj0414/415), and apart from the 2D gel evidence discussed above, we were able to show by a gene fusion approach that the Cj0414 signal peptide is able to facilitate the transport of a heterologous TAT substrate (Fig. 5). The

majority of the TATFIND/TatP predictions were confirmed by the enzyme assay results shown in Table 1 and Figs 4 and 6. However, TATFIND rejects HydA as a TAT substrate, because the twin-arginine motif is more than 35 residues from the N terminus, yet the assay results in Table 1 (as well as the proteomics in Fig. 2) clearly show it is TAT dependent, as are all periplasmic NiFe uptake hydrogenases (Berks *et al.*, 2003). In some cases, previous studies had shown that some of the proteins we predicted (Supplementary Table S2) were localized to the periplasm, for example the sulphite oxidase catalytic subunit SorA was previously detected in the periplasm of *C. jejuni* by immunoblotting (Myers & Kelly, 2005), the MfrA subunit was detected on 2D gels and by activity assays and immunoblotting of periplasmic extracts (Guccione *et al.*, 2010) and the multicopper oxidase CueO by periplasmic enzyme assays (Hall *et al.*, 2008). The data obtained here confirm that their periplasmic location is dependent on the TAT system. In the case of NrfH, which has a convincing twin-arginine motif located at the extreme N terminus (similar to alkaline phosphatase) but was rejected by TATFIND and TatP, we were able to show by nitrite reduction assays

that the prediction is correct and that NrfH is not a TAT substrate. Unfortunately, similar assays based on nitrate reduction could not be used to determine the TAT dependence of NapG and NapH, two proteins involved in electron transport to the active site subunit NapA (Pittman *et al.*, 2007), as NapA is also a TAT substrate.

The most prominent TAT substrate with an unknown function in *C. jejuni* is the molybdoenzyme encoded by *cj0379c*. Cj0379 is a member of a distinct group of molybdoenzymes (the YedY family) that contain a simple molybdopterin (MPT) cofactor and which are present in a large number of diverse prokaryotes (Workun *et al.*, 2008). Most of the proteins homologous to Cj0379 are encoded in a two-gene operon, designated *yedYZ* in *E. coli*. YedZ is a membrane-bound *b*-type cytochrome that donates electrons to the periplasmic YedY (Brokx *et al.*, 2005), suggesting a similar function for Cj0378 in *C. jejuni*. Indeed, the crystal structure of YedY (Loschi *et al.*, 2004) shows the active site is similar to that of a reductase, although the overall fold is related to that of sulphite oxidase. All of the key residues in YedY that co-ordinate the MPT cofactor are conserved in Cj0379, including a single, absolutely conserved cysteine at residue 133, which provides an MoS ligand in YedY (Loschi *et al.*, 2004). Although YedY can catalyse reduction of N- and S-oxides, the kinetics are so poor that these are unlikely to be physiological substrates (Loschi *et al.*, 2004), and no evidence was found in this study that Cj0379 can reduce these electron acceptors. We did gain evidence, however, that the *cj0379c* mutant is more sensitive to killing by nitrosating and NO-releasing agents (with the nitrosating agent SNP producing the greatest effect), and has a decreased capacity to degrade NO. Superoxide sensitivity was the same as the wild-type, indicating that the increased sensitivity to reactive nitrogen species is a specific effect and not due to a general response to oxidative stress. The phenotypic differences we observed were small, but this may be expected in view of the fact that the wild-type and *cj0379c* mutant will both express Cgb and NrfA – by far the two major contributors to cellular NO defence (Elvers *et al.*, 2004; Pittman *et al.*, 2007). The data imply that Cj0379 may be able to reduce one or more reactive nitrogen species, either NO itself or a reaction product like peroxynitrite (ONOO⁻), but this can only be confirmed by studies with the purified enzyme. Nevertheless, the colonization defect of the mutant would suggest an important role for Cj0379 *in vivo*, either as part of an NO defence system or because it can reduce some unknown additional electron acceptor that is important for caecal growth.

Most periplasmic redox enzymes with complex cofactors have dedicated REMPs that partly serve to co-ordinate cofactor insertion in the cytoplasm with TAT translocation. In *C. jejuni*, only two such REMPs are encoded in the genome (Turner *et al.*, 2004), Cj1514 and Cj0785 (NapD). Surprisingly, despite being a member of the TorD family, we found that Cj1514 is not required for maturation of the TMAO reductase Cj0264, but is essential for the activity of

formate dehydrogenase, consistent with the location of the cognate gene upstream of FdhA. Polar effects of the mutation were not responsible for this phenotype as complementation with only the *cj1514c* gene at a distant locus restored formate dehydrogenase activity. In other bacteria, the formate dehydrogenase REMP has yet to be clearly identified, so the use of a TorD homologue in this role is unprecedented. It is proposed that Cj1514 should be designated FdhM (for maturation). Furthermore, *cj1514c* has a complex overlapping gene arrangement with a novel small gene (*cj1513c*) that also overlaps FdhA. Cj1513 is composed of a TAT signal sequence with a strongly predicted cleavage site that would give a mature peptide of just 33 residues (Fig. 8). The role of this novel protein is unknown but it seems to be specific to the epsilonproteobacteria. One intriguing possibility is that this protein is somehow related to the likely use of a tungsten-pterin cofactor in the *C. jejuni* formate dehydrogenase (Smart *et al.*, 2009; Taveirne *et al.*, 2009).

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