A multicopper oxidase (Cj1516) and a CopA homologue (Cj1161) are major components of the copper homeostasis system of *Campylobacter jejuni*.

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Abstract

Metal-ion homeostasis mechanisms in the food-borne human pathogen *Campylobacter jejuni* are poorly understood. The *Cj1516* gene product is homologous to the multicopper oxidase (MCO) CueO, which is known to contribute to copper tolerance in *E. coli*. Here, we show by optical absorbance and electron paramagnetic resonance (EPR) spectroscopy that purified recombinant Cj1516 contains both T1 and tri-nuclear copper centres, which are characteristic of multicopper oxidases. Inductively coupled plasma mass spectrometry (ICP-MS) revealed the protein contained ~ 6 copper atoms per polypeptide. The presence of an N-terminal "twin arginine" signal sequence suggests a periplasmic location for Cj1516, which was confirmed by the presence of *p*-phenylenediamine (*p*-PD) oxidase activity in cellular periplasmic fractions. Kinetic studies showed that the pure protein exhibited *p*-PD, ferroxidase and cuprous oxidase activity and was able to oxidise an analogue of the bacterial siderophore anthrachelin (3,4- dihydroxybenzoate), although no iron uptake impairment was observed in a *Cj1516* mutant. However, this mutant was more sensitive to increased copper levels in minimal media, suggesting a role for Cj1516 in copper tolerance. A mutation in a second gene, *Cj1161c,* encoding a putative CopA homologue was also found to result in hypersensitivity to copper and a *Cj1516/Cj1161c* double mutant was found to be as copper sensitive as either single mutant. These observations and the apparent lack of alternative copper tolerance systems such as the *cus* system suggest that Cj1516 and Cj1161 are major proteins involved in copper homeostasis in *C. jejuni*.

Introduction

Although *Campylobacter jejuni* is part of the normal commensal flora of many bird species, it is pathogenic in humans and ingestion of contaminated poultry is a common route for infection. Consequently, *C. jejuni* is one of the most important causes of human enteric disease worldwide and continues to be a major public health and economic burden (23). Acute symptoms of *C. jejuni* infection in humans include diarrhoea, fever and abdominal pain but complications can include reactive arthritis and neurological sequelae such as the Miller-Fisher and the Guillaine-Barré syndromes (55). Despite the importance of *C. jejuni* as a food-borne pathogen and the sequencing of the genomes of a number of strains (19, 27, 40), there are many aspects of the biology of this bacterium that remain poorly defined, particularly stress responses and homeostatic mechanisms. The molecular mechanisms of pathogenesis of *C. jejuni* are still not completely understood, although a number of virulence factors have been identified that include motility and chemotaxis, adhesion to and invasion of host cells and toxin production. Iron acquisition is also an important virulence factor and in recent years this area has been studied extensively in *C. jejuni* (36, 47, 63). However, the acquisition, metabolism and homeostasis of other key metals in *C. jejuni*, such as copper and zinc, have been largely overlooked.

Metal homeostasis is extremely important in biological systems and metals such as copper, iron and zinc are essential for almost all bacterial growth. These metals are usually present in trace amounts in the environment, but play important roles in electron transport and redox reactions as cofactors of many enzymes such as cytochrome *c* oxidase (44) and superoxide dismutase (41). However, in excess they can be toxic and thus require specific systems to cope with metal induced stress. Toxicity occurs via a number of mechanisms and includes metal

atoms binding to thiol groups and disrupting protein function (38, 46, 56, 61), displacement of metal cofactors in proteins by competition and the generation of reactive oxygen species through Fenton-like reactions (59).

In *Escherichia coli*, as many as three distinct systems for copper tolerance have been identified and include the *cop*/*cue* and *cus* systems (38), encoded on the chromosome and the plasmid encoded *pco* system (11). The *cus* system consists of three proteins (CusCBA), which span the periplasm and outer membrane and CusF, a periplasmic binding protein. This system is involved in the efflux of excess copper under mainly anaerobic situations (22). The plasmid-encoded system *pco* is present in some strains of *E. coli* (33) and other organisms such as *Pseudomonas syringae* pv. *Tomato* (5). The system usually consists of seven genes encoding a multicopper oxidase, a periplasmic copper binding protein, three other proteins thought to form a membrane transporter and two genes encoding a two-component regulatory system (5, 11). The *cop/cue* system consists of CopA which has been described as the central component of copper homeostasis in *E. coli*, required for intrinsic copper resistance in both aerobic and anaerobic conditions (49) and CueO, a multicopper oxidase operating in the periplasm. Homologues of this system appear to be widespread in bacteria.

Multicopper oxidases (MCOs) are a diverse family of metalloenzymes widely distributed among eukaryotes. They are copper containing proteins characterised by distinctive structural, spectroscopic and enzymatic properties (58). The currently well-defined MCOs are Fet3 from *Sacharomyces cerevisiae* and Human ceruloplasmin, both of which have defined roles in iron acquisition (3, 15, 26). Extensive knowledge about the structure and roles of MCOs in eukaryotes contrasts with the situation in prokaryotes, where the widespread existence of MCOs in bacterial genomes (where they are often annotated as laccases) has only recently begun to be recognised (1). Almost all laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) exhibit

97 *p*-diphenol:O_2 oxidoreductase activity and are especially common in plants and fungi, but a link
98 between bacterial MCOs and transition metal metabolism is emerging from studies that suggest
99 their involvement in a range of important metal acquisition/homeostasis systems including those
100 for copper, manganese and iron. As mentioned above, in *E. coli* the MCO CueO has been
101 proposed to be involved in the removal of excess copper from the cell as part of a copper efflux
102 system consisting of CueO and CopA, under the control of a MerR-like regulatory element CueR
103 (24, 25, 38, 39). Manganese oxidation has been suggested as the physiological role for CumA,
104 an MCO present in *P. putida* (10). Compelling evidence has been presented that shows an MCO
105 in *P. aeruginosa,* with similarity to Fet3 and CueO, to be involved in the acquisition of ferrous
106 iron (29). Mutant strains lacking this protein were unable to grow aerobically with Fe(II) as the
107 sole iron source and iron uptake analysis showed the mutant was impaired in Fe(II) uptake, but
108 unaffected for Fe(III) uptake (29). Thus, it is clear that the physiological roles of prokaryotic
109 MCOs are diverse and cannot be determined by sequence homologies alone.
110 In this paper we have identified a periplasmic MCO in *C. jejuni* that possesses
111 phenoloxidase, ferroxidase and cuprous oxidase activity. From biochemical and mutant
112 phenotype data we propose that the major physiological role of this enzyme is the oxidation of
113 copper in the periplasm. However, by acting together with a homologue of the copper (I)
114 exporting class of P-type ATPases (CopA), these two proteins can remove and detoxify copper
115 from the cytoplasm and appear to form the major copper homeostasis system in *C. jejuni*.
116

Materials and Methods

Bacterial strains, media and culture conditions. *C. jejuni* strain NCTC 11168 was routinely cultured at 37 ºC under microaerobic conditions (10 % [vol/vol] O_2, 5 % [vol/vol] CO_2 and 85% [vol/vol] N_2 in a MACS growth cabinet (Don Whitley Scientific Ltd., Shipley, United Kingdom) on Columbia agar containing 5 % (vol/vol) lysed horse blood and 10 µg ml^{-1} each of amphotericin B and vancomycin. Liquid cultures of *C. jejuni* were routinely grown microaerobically at 200 rpm, either in Mueller-Hinton broth (Oxoid Ltd, UK) supplemented with 20 mM L-serine (MH-S) or in the defined medium MEM-α (Invitrogen Ltd. Cat. number 41061-029, containing glutamine and deoxyribonucleotides but no phenol red), containing the above antibiotics and 45 µM $FeSO_4$, 20 mM serine and 20 mM pyruvate. To select for the *C. jejuni Cj1516* mutant, kanamycin was added to media at a final concentration of 30 µg ml^{-1} and to select for the *Cj1161c* mutant chloramphenicol was added to media to a final concentration of 30 µg ml^{-1}. *E. coli* DH5α was cultured in Luria-Bertani (LB) broth or agar supplemented with appropriate antibiotics at 37 ºC. For growth experiments, *C. jejuni* overnight starter cultures were prepared in MH-S and washed three times, before inoculation into MEM-α. Growth was monitored at 600 nm using an Amersham Pharmacia Biotech Ultrospec 2000 spectrophotometer.

DNA isolation and manipulation. Plasmid DNA was isolated by using the Qiagen miniprep kit (Qiagen Ltd., Crawley, United Kingdom). *C. jejuni* chromosomal DNA was extracted by using the Wizard Genomic DNA purification kit (Promega, Madison, USA). Standard techniques were employed for the cloning, transformation, preparation, and restriction analysis of plasmid DNA from *E. coli* (52).

Over-expression and purification of Cj1516. For the over-expression of the *Cj1516* gene product, primers (forward, 5'-ATCAGCTAGCAATAGAAGAAATTTTTTA- 3'; and reverse 5'- TAGC***GGATCC***TTATTCCTTTACTTCTAA -3', *Nhe*I site underlined, *BamH*I site in bold italics) were designed to amplify the complete *Cj1516* gene from *C. jejuni* NCTC 11168 chromosomal DNA by PCR using a proofreading DNA polymerase enzyme (Pwo, Roche Ltd., United Kingdom). The PCR fragment was then cloned by blunt end ligation into pGEM3ZF (-) (Promega Ltd., United Kingdom) to create pGEM1516. The gene was excised from pGEM1516 by digestion with *Nhe*I and *Bam*HI and cloned into similarly digested pET21a(+) (Novagen Ltd., United Kingdom) to give pMCO1516. Automated DNA sequencing (Lark Technologies Inc, Saffron Walden, UK) showed that the sequence of the *Cj1516* gene in pMCO1516 was correct. pMCO1516 was transformed into *E. coli* BL21 (DE3) cells which were grown aerobically at 25 ºC in LB medium containing ampicillin (50 µg ml^{-1}) and 1mM copper sulphate ($CuSO_4$) to an optical density at 600 nm of 0.6 before 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added. Induced cells were then grown for a further 16 h before harvesting by centrifugation (30 min, 4 ºC, 3500 x g). Cell pellets were resuspended in 10 mM Tris-HCl pH 8.0 and disrupted by sonication with an MSE Soniprep 150 (Sanyo, UK) using 6 x 20 second bursts of ultrasound (amplitude ~ 15 microns peak to peak) with 30-second intervals between bursts. Cell debris and soluble matter were separated by centrifugation at 12,000 x g for 20 minutes at 4 °C. The fractions were kept on ice until used or stored at -20 °C. The supernatant was recovered as cell free extract and fractionated on a DEAE sepharose Fast flow column (GE Healthcare, UK) by ion-exchange chromatography. The protein was eluted from the resin by a gradient from 0-500 mM NaCl in 10 mM Tris-HCl, pH 7.5. Fractions were pooled and adjusted to 1M ammonium sulphate and further fractionated by hydrophobic interaction chromatography using a 10 ml Phenyl Sepharose (Sigma) column. A salt gradient of 1 M to 0 M $(NH_4)_2SO_4$ was used to elute

the proteins. Recombinant Cj1516 elution from the chromatography columns was detected by monitoring of the A_{610} due to type 1(blue) copper content. The final purification step utilised the apparent thermal stability of multicopper oxidases and involved heat treatment of the samples as previously described (30). Briefly, pooled fractions collected from the hydrophobic interaction step were incubated at 70 °C for 5 min before being centrifuged to remove denatured proteins. Phenoloxidase activity was monitored before and after heat treatment to ensure activity of the recombinant protein was not lost.

Construction of mutants. A *feoB* mutant was constructed by Mariner transposon insertion into the *Cj1398* gene and was kindly provided by Dr A. Grant, Cambridge Veterinary School, University of Cambridge, UK. *Cj1516* and *Cj1161c* mutants were constructed by insertion of kanamycin and chloramphenicol resistance cassettes, respectively, into each gene in the same transcriptional orientation. The *Cj1516* gene was amplified using the following specific primers: Cj1516For: (5'–CAAAGTCCGCTACAAGTACAAC–3'), Cj1516rev: (5' – CCGATCTTGAAACACGACATAGA – 3'). The resulting 1.59 kb fragment containing the coding region of the gene was cloned into pGEM 3Zf (-) vector (Promega, UK). Transformants were recovered by selection on plates containing ampicillin (50 μg ml^{-1}). The kanamycin resistance cassette derived by PCR from plasmid pJMK30 was cloned into the unique restriction site *Swa*I in the centre of *Cj1516* to produce plasmid p1516kan. For construction of a *Cj1161c* mutant strain, primers *Cj1161c*F: (5' – ATGCATGGAAGAATTGCGTAT – 3') and *Cj1161c*R: (5' – ATGCTCTTAAAGAATTAAGCACTACA – 3') were used to amplify a 2.085 kb fragment containing the entire coding region of *Cj1161c*, this fragment was cloned into pGEM T-Easy vector to produce plasmid pGEM1161c. The chloramphenicol resistance cassette derived

188 from pAV35 (64) was cloned into the unique *Swa*I restriction site in *Cj1161c* gene in
189 pGEM1161c to produce p1161cCat.

190 The p1516kan and p1161cCat plasmids were transformed by electroporation into *C.*
191 *jejuni* NCTC 11168 and transformants selected using Columbia blood agar plates supplemented
192 with either kanamycin (30 μg ml $^{-1}$ final concentration) or chloramphenicol (30 μg ml^{-1} final
193 concentration). Correct insertion of the antibiotic resistance cassettes into the target genes was
194 confirmed by PCR. Specific primers used to amplify *Cj1516* and *Cj1161c* (see above) were used
195 to confirm the allelic exchange by double crossover in each mutant. This was demonstrated by
196 an increase in PCR product size of 0.8 kb or 1.4 kb for the chloramphenicol and kanamycin
197 cassette insertions, respectively. *Cj1516* mutant strain was designated SJH400 and the *Cj1161c*
198 mutant strain was designated AH100. A double mutant was created by electroporation of AH100
199 with the p1516kan plasmid and selecting on Columbia agar blood plates containing both
200 kanamycin and chloramphenicol.

201

202 **Phenoloxidase assays and kinetics**. Phenoloxidase assays were carried out on purified
203 recombinant Cj1516 protein or cellular periplasmic fractions prepared by the osmotic shock
204 method described previously (35). The 1 ml assay volume consisted of 50 mM sodium acetate
205 buffer pH 5.7 containing 0.8 μM of pure Cj1516 protein or 50 μg periplasmic protein. The assay
206 was started by the addition of *p*-phenylenediamine to final concentrations between 0-8 mM for
207 assays containing excess copper and 0-60 mM without excess copper and rates recorded at 487
208 nm using a Shimadzu UV-2401 dual wavelength scanning spectrophotometer (Shimadzu Ltd).
209 All assays were performed at 37 °C. Specific activities were calculated using an extinction
210 coefficient for *p*-phenylenediamne of 14.7 mM^{-1} cm^{-1} at 487 nm. Sigmaplot 8.0 (SPPS inc. USA)

was used for calculation of V_{max} and K_m values, data were averaged from at least three separate assays and the hyperbolic curve fitting algorithms of Sigmaplot used to analyse the data.

Ferroxidase assays and kinetics. Ferroxidase assays were performed on purified recombinant Cj1516 protein. The 1ml assay volume contained 50 mM sodium acetate buffer pH 5.7 and 0.8 µM Cj1516 protein. The assay was started by the addition of ammonium ferrous sulphate to final concentrations of 0.01 – 0.3 mM and rates recorded at 315nm as above. All assays were performed at 37 °C in matching quartz cuvettes. Specific activities were calculated using an extinction coefficient for Fe(III) of 2.2 mM^{-1} cm^{-1} at 315 nm. Sigmaplot 8.0 (SPSS inc. USA) was used to calculate V_{max} and K_m values as above.

Siderophore oxidase assays and kinetics. Oxidation assays of an analogue of the bacterial siderophore anthrachelin were performed on pure recombinant Cj1516 protein. Each assay contained 50 mM sodium acetate buffer pH 5.7 and 0.8 µM of pure Cj1516 protein. The assay was started by the addition of 3,4- dihydroxybenzoate (3,4-DHB) to a concentration range of 0-8 mM for assays containing excess copper and 0-60 mM without excess copper and rates recorded at 487nm using a Shimadzu UV-2401 dual wavelength scanning spectrophotometer as above. All assays were performed at 37 °C. Specific activities were calculated using an extinction coefficient for 3,4-DHB of 2.3 mM^{-1} cm^{-1} at 400nm. Sigmaplot 8.0 (SPSS inc. USA) was used for the calculation of V_{max} and K_m values as above.

Measurement of metal-ion oxidation-linked oxygen respiration. Metal-ion oxidation by pure Cj1516 protein was determined by measuring the change in dissolved oxygen concentration in a Clark-type polargraphic oxygen electrode (Rank brothers Ltd, Bottisham, Cambridge, UK)

comprising a water-jacketed perspex chamber that was stirred magnetically, linked to a chart recorder and calibrated using air saturated 25 mM phosphate buffer (pH 7.5). 100 % saturation was assumed to be 220 µM O_2. 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 were added by injection through a fine central pore in the airtight plug. Substrates used were manganese chloride, ammonium ferrous sulphate and a caged copper form of copper (I) which consisted of the compound tetrakis (acetonitrile) copper (I) hexaflourophosphate (Sigma-Aldrich, UK) dissolved in argon-sparged 5% acetonitrile. 1.3 µM of pure Cj1516 was used in each cuprous oxidase assay and 0.8 µM Cj1516 used for manganese and ferrous iron assays. Rates were expressed in µmol O_2 utilised min^{-1} mg $protein^{-1}$. For analysis of cuprous oxidase kinetics, the means of three assay measurements at various substrate concentrations were used. The hyperbolic curve fitting algorithms of GraphPad Prism 5.0 for Mac (GraphPad Software, San Diego, California USA) were used to analyse the data and calculate K_m and V_{max} values based on the Michaelis-Menten equation.

Spectroscopy. UV-visible light spectra were collected by using a Shimadzu UV-2401 spectrophotometer (Shimadzu Ltd). Copper sites were detected by the addition of 0.024 µg pure Cj1516 protein to a 1ml cuvette containing 10 mM Tris-HCl pH 7.5. A wavelength range of 500-700 nm was used to detect the characteristic peak produced by T1 copper centres at ~600 nm. A wavelength range of 300 nm to 700 nm were use to detect the T3 copper centre at ~330 nm. All spectra were obtained at 37°C. Electron paramagnetic resonance (EPR) spectra were recorded with a Bruker (Billerica, Mass.) EMX spectrometer (X-band 9.38 GHz) equipped with an ER4112HV liquid helium flow cryostat system. Spectra were recorded at a temperature of 30 K,

6 mT modulation amplitude and 0.2 mW microwave power. The protein used in analysis was as isolated.

Protein and copper content determinations. The concentration of protein was determined by the Bradford method (9) using bovine serum albumin as the standard. Copper content was determined by inductively coupled plasma mass spectrometry (ICP-MS) using an Agilent 4500 spectrometer (Agilent systems, USA) operated by the University of Sheffield Centre for Chemical Instrumentation and Analytical Services.

Copper tolerance growth experiments. Triplicate 10 ml cultures of each strain (wild type NCTC 11168, *Cj1516*, *Cj1161*c and *Cj1516*/*1161*c double mutant) were grown in minimal media (MEM-α) containing copper sulphate in the concentration range 0 – 1 mM. Cultures were incubated from a starting OD_{600} of 0.1 to stationary phase, microaerobically at 37 °C with shaking. The final OD_{600} was recorded using an Amersham Pharmacia Ultrospec 2000 spectrophotometer.

Iron-limited growth experiments. Ferrous iron restricted and replete experiments were performed with wild-type, *Cj1516*, *feoB* and *Cj1516/feoB* mutants. Starter cultures were grown at 37 °C microaerobically to late exponential phase and then washed in chelex-treated MH-S to remove excess iron. The washed cells were then used to inoculate 200 ml of chelex treated MH-S broth. Iron replete cultures were supplemented with FBP (ferrous sulphate, sodium metabisulphite and sodium pyruvate) (14). FBP was added as an iron source and oxidative stress protectant. The final concentration of iron in the cultures was 45 μM. Cultures were incubated to

281 stationary phase microaerobically at 37 °C with shaking. Growth was monitored by measuring
282 the optical density at 600 nm every hour using an Amersham Pharmacia Ultrospec 2000
283 spectrophotometer. Experiments were repeated three times with independent cultures.
284

Results

Identification of a *C. jejuni* periplasmic multicopper oxidase. *Cj1516* in the genome sequence of strain NCTC 11168 is described as encoding a periplasmic oxidoreductase with an unassigned function. The full length deduced protein is ~ 60 kDa in size and has 38% identity with the multicopper oxidase CueO of *E. coli.* Multicopper oxidases are characterised by three different Cu(II) centres which couple four, one electron oxidation reactions to the four electron reduction of oxygen to water. These are a T1 centre comprising a single Cu atom liganded to two histidines and a cysteine and a T2/T3 trinuclear site liganded to six histidines (58). These copper centres have been observed in the crystal structure of CueO of *E. coli* (50). Figure 1 shows the protein sequence alignment of Cj1516 compared to sequences of known MCOs, some of which have defined or suggested roles in iron acquisition and copper tolerance. Clearly, Cj1516 possesses all the amino acid residues critical for the formation of the T1 and trinuclear centres. Further sequence analysis using the TatP (7) and SignalP (6) web servers suggest the protein is secreted to the periplasm via the Tat system (8), due to the presence of a typical Tat signal motif (Fig. 1) and cleavage is predicted to remove 20 amino acids during export. The mature protein is expected to be ~ 56 kDa . The presence of the Cj1516 protein in the periplasm of *C. jejuni* was shown by assaying the characteristic phenoloxidase activity with the chromogenic substrate *p*-phenylenediamine (*p*-PD). Rates of ~800 nmol p-PD oxidised min mg protein were found with periplasmic fractions of wild-type cells, whereas a *Cj1516* mutant completely lacked this activity (see below).

Overexpression and purification of Cj1516. The *Cj1516* gene in the NCTC 11168 strain of *C. jejuni* was PCR amplified and cloned into the expression vector pET21a(+), such that the

309 recombinant protein would be expressed from the T7 promoter with the original *C. jejuni* signal
310 sequence and without any tags. Induction of *E. coli* BL21(DE3) (pMCO1516) with IPTG at 37
311 °C resulted in only insoluble protein. However induction at 25 °C resulted in the overproduction
312 of a soluble protein (Fig. 2A), that was purified to homogeneity from cell-free extracts using a
313 combination of ion-exchange, hydrophobic interaction chromatography and heat treatment. SDS-
314 PAGE analysis showed the protein migrated as a single band with a molecular mass of ~ 56 kDa
315 (Fig. 2A, right panel). The eluted recombinant protein was monitored at each step by
316 phenoloxidase activity with *p*-phenylenediamine and by identifying fractions that were slightly
317 blue in colour probably due to the presence of fully oxidised T1 copper centres, a phenomenon
318 observed previously (30). Optical spectroscopy of these blue fractions confirmed the presence of
319 the protein (Fig. 2B).
320 The predicted molecular mass of the processed protein was calculated to be 56.6 kDa. N-
321 terminal amino-acid sequencing revealed a sequence of YANPMH which is identical to residues
322 21-26 of the deduced complete sequence and consistent with correct cleavage after the AYA
323 signal peptidase recognition site, predicted using the signal sequence web servers SignalP 3.0 (6)
324 and TatP 1.0 (7).
325
326 **Protein copper content.** Copper content in the protein was determined using inductively
327 coupled plasma mass spectrometry and the Bradford assay to determine protein concentration.
328 The copper content was found to be 6.4 atoms per polypeptide chain.
329
330 **Optical absorbance spectroscopy and electron paramagnetic resonance spectroscopy show**
331 **that Cj1516 is a multicopper oxidase**. Multicopper oxidases are ideal proteins for studies using
332 spectroscopic techniques, having a number of distinctive features that are used for classification

and characterisation (58). Consequently two spectroscopic techniques were employed to determine the presence and type of copper centres present in Cj1516. Copper sites have historically been divided into three classes, reflecting the geometric and electronic structures of the active site (58). They are; type 1 (T1) or blue copper comprising a single Cu atom liganded to two histidines, type 2 (T2) and type 3 (T3) sites, which form the trinuclear centre liganded by six histidines (58).

Optical absorbance spectroscopy performed on the as purified 56 kDa protein produced a characteristic peak at ~ 600 nm (Fig. 2B). The blue colour of the pure protein further established this feature. T3 binuclear copper centres produce a characteristic peak at 330 nm in the visible region. Figure 2B shows a full spectral scan of purified Cj1516 protein in which a peak can be seen at 330 nm, this peak is masked somewhat by the large peak appearing at 280 nm corresponding to the large amount of protein present. Nevertheless, the shoulder of the 330 nm T3 peak is visible and indicated in Fig. 2B. A strong 420 nm absorbance was also observed. This has not been reported for other multicopper oxidases and its origin is unknown.

T2 (or normal) copper centres do not produce intense features within the visible absorption spectrum, but along with T1 sites, exhibit characteristic features that can be observed by electron paramagnetic resonance (EPR) spectroscopy, owing to the open shell configuration of electrons in oxidized (cupric) atoms providing an unpaired electron in the outer shell. Conversely, whilst being visible at 330 nm in optical spectroscopy, T3 centres are EPR "silent", due to the coupling of the two copper atoms via a bridging ligand and thus, the loss of unpaired ferromagnetically active electrons (58). Figure 3, spectrum A shows the results of the EPR spectroscopy performed on as-purified Cj1516. The protein exhibited EPR features typical of multicopper proteins, displaying a spectrum with narrow hyperfine splitting ($g_{\perp} \sim 2.05$, $g_{\parallel} \sim 2.209$ and $A_{\parallel} \sim 76.79 \times 10^{-4}$ cm^{-1}) for the T1 centre. Underlying features characteristic of a T2

centre are also evident at lower field and display an approximate hyperfine splitting of $A_{\parallel}$= 156.11 x 10^{-4} cm^{-1}. Figure 3 spectrum B shows the effects of the addition of ferrous iron to the sample. Rapid reduction of each centre to Cu(I), with the concomitant oxidation of ferrous (Fe II) to ferric (Fe III) iron, resulted in the loss of the T1 and T2 signals. These observations are consistent with the ferroxidase activity of the protein described below.

Spectrophotometric analysis of substrate specificity and kinetics of Cj1516, and the effect of excess copper. Cj1516 oxidized a number of phenolic compounds such as *p*-phenylenediamine, N,N,N,N- tetramethyl-*p*-phenylenediamne (TMPD) (data not shown) and 3,4- DHB (anthrachelin). The V_{max} and K_m values for *p*-PD and 3,4-DHB after the addition of 1 mM $CuSO_4$ were markedly different to those in the absence of excess copper, suggesting an enhancement in activity as seen in other MCOs (30, 51) (Table 1). Of the three phenol compounds studied, the higher affinity and high V_{max} values for 3,4-DHB imply this is a favoured substrate. Ferroxidase activity measured using the optical method previously described (30) was also observed and greatly enhanced by the addition of excess copper; a five-fold increase in V_{max} and a two-fold decrease in K_m value were observed (Table 1). Oxidation of phenolic compounds did not take place in the absence of enzyme, even with excess copper present (data not shown) suggesting that free copper does not take part in a redox cycle, in agreement with others (30).

Cj1516 exhibits iron and copper dependent oxygen uptake. Multicopper oxidases are oxygen dependent enzymes and as such can be assayed by measuring the substrate-linked uptake of oxygen using a Clark-type oxygen electrode. Manganese was tested as a possible substrate but no uptake of oxygen was observed (Fig. 4A). However, consistent with the data in Table 1,

Figure 4B shows that significant oxygen consumption occurred when Fe(II) in the form of ammonium ferrous sulphate was used as substrate. Cuprous oxidase activity was also measured with the oxygen electrode, using the caged copper (I) substrate previously described (51) to minimise interference from chemical oxidation of the unstable copper (I). High rates of oxygen uptake upon addition of the compound demonstrated that Cj1516 is capable of oxidising cuprous copper (Fig. 4C). In the absence of enzyme, negligible background rates were observed at the caged copper concentrations used (Data not shown). The concentration dependence of the cuprous oxidase activity followed Michaelis-Menten kinetics as shown in Fig 4D. A K_m of 180 µM was calculated for cuprous copper, this is similar to that previously measured for CueO (54) and in the same region as that of Fe(II) for Cj1516 (Table 1). The V_{max} was the highest of any of the substrates tested (Table1).

Ferrous iron acquisition is not affected in a *Cj1516* mutant. A mutant in *Cj1516* was constructed by the insertion of a kanamycin resistance cassette into a unique *Swa*I site within the cloned gene (Fig. 1B). After electroporation into wild-type cells, several antibiotic resistant colonies were selected and a PCR with gene specific primers showed that the mutant construction had been successful (data not shown). Intact cells and periplasmic protein fractions of this mutant completely lacked *p*-PD oxidase activity. The kanamycin resistance cassette used was inserted with the same polarity as the *Cj1516* gene and therefore should not interfere with downstream transcription. However, the genes downstream of *Cj1516* are predicted to encode the proteins MoaD, MoaE and MoeA2 (Fig. 1B), all of which are essential for the synthesis of the molybdopterin cofactor (Moco) of molybdoenzymes. As it is now known that copper is needed for the correct biosynthesis of this cofactor (31), we wanted to ensure that mutation of Cj1516 did not interfere with Moco synthesis. *C. jejuni* expresses a number of Moco containing

405 proteins that function as part of the electron transport pathway (35, 45, 53), including
406 trimethylamine-*N*-oxide (TMAO) reductase (Cj0264). We found that TMAO reductase activity
407 using the methyl violgen assay described previously (53) was comparable in the *Cj1516* mutant
408 to that of the wild-type parent strain, both giving high rates of ~ 2.5 µmoles MV oxidised min^{-1}
409 mg^{-1} protein in intact cells, indicating that molybdenum cofactor synthesis is not affected in the
410 mutant.

411 In order to determine any effects on iron acquisition in a *Cj1516* mutant, iron limited
412 growth experiments were carried out. However, since *C. jejuni* NCTC 11168 possesses the well-
413 known FeoB ferrous iron transporting protein, a double *feoB* and *Cj1516* mutant strain was also
414 constructed for use in these experiments. The *feoB/Cj1516* double mutant was constructed by
415 transforming p1516kan into a *feoB* mutant. The *feoB* mutant was created by a chloramphenicol
416 resistance Mariner transposon insertion into the FeoB encoding gene *Cj1398* (kindly provided by
417 Dr A. Grant, Cambridge, UK). Colonies resistant to both chloramphenicol and kanamycin were
418 selected and a PCR with gene specific primers showed that double mutant construction was
419 successful.

420 The ability of the mutants to acquire ferrous iron was determined. Growth experiments
421 were carried out in which the *Cj1516*, *feoB* and *feoB*/*Cj1516* double mutant and wild type
422 strains were grown in liquid culture in the presence and absence of a ferrous iron source. None
423 of these strains grew significantly in the absence of ferrous iron (Fig. 5) but after 16 hours
424 microaerobic growth in the presence of ferrous iron both the WT and 1516 strains had grown to
425 an OD600 of ~ 1.2. However, the *feoB* and double mutant strains were unable to grow even with
426 a ferrous iron source present, highlighting the importance of FeoB as an iron acquisition protein,
427 as described in a recent study (36). The *Cj1516* mutant clearly showed no iron acquisition
428 related phenotype in this growth assay.

Bioinformatic evidence suggests *Cj1161c* encodes a copper exporting P_{1B}-type ATPase. The genome sequence of *C. jejuni* contains other genes encoding proteins with homology to well known copper management proteins (40). In addition to the putative multicopper oxidase Cj1516, two genes (*Cj1161c* and *Cj1155c*) encode Cop-like proteins. Cop proteins are members of the large P-type ATPase family, which couple the hydrolysis of ATP to the transport of a substrate (2, 57). More specifically they belong to the heavy metal-transporter sub-group P_{1B}. P_{1B}-ATPases have a distinct structure compared to other P-type ATPases, characterised by a reduced number of transmembrane (TM) helices, having typically eight compared to 10 or more in P2- or P3-ATPases (34, 62). Within this sub-group the presence of conserved amino acid residues in TM helices 6,7 and 8 further classify the proteins into groups based on the type of metal ion transported (2, 12, 57). Analysis of the protein sequence of *Cj1155c* of *C. jejuni* revealed that this protein contains a modified version of the highly conserved signature phosporylation site motif DKTGT found in all P-type ATPases (2). However it does contain the CPC motif as well as an N-terminal CxxC motif found in copper transporting ATPases. The annotation and location of the gene within an apparent operon encoding homologues of the cytochrome *c* oxidase maturation protein cluster CcoGHIS found in many bacteria (31), suggests it is involved in the assembly of the copper containing terminal oxidase encoded by the genes *Cj1487c* - *Cj1490c* in the *C. jejuni* NCTC 11168 genome (40). Analysis of the amino acid sequence of Cj1161 showed this protein to be a more likely candidate as a Cop-like P-ATPase copper exporter. The TMHMM v2.0 prediction programme suggested a total of eight transmembrane helices with two cytoplasmic loops, which probably accommodate the phosphorylation site (data not shown). The protein also contains the DKTGT signature, the CPC motif and N-terminal metal binding domain motif CxxC. In addition, the protein also contains

amino acids in TM helices 6, 7 and 8 proposed to participate in determining metal selectivity. All of these are defining features of proteins in the P_{1B-1}-ATPase group which are involved in the export of Cu(I) from cytoplasm to periplasm (2).

Mutations in either *Cj1516* or *Cj1161c* lead to a copper sensitive phenotype. A mutant in *Cj1161c* was constructed by the insertion of a chloramphenicol resistance cassette into a unique *Swa*I site within the cloned gene (Fig. 1C). After electroporation into wild-type cells, several chloramphenicol resistant colonies were selected and a PCR with gene specific primers showed that the mutant construction had been successful (data not shown). To fully explore the hypothesis that both the proteins encoded by the genes *Cj1516* and *Cj1161c* are involved in copper homeostasis, a double mutant strain was created by transforming the p1516kan plasmid into the *Cj1161c* mutant AH100. Several colonies resistant to both kanamycin and chloramphenicol were selected and a PCR of the genomic DNA of these colonies with gene specific primers showed that mutant construction was successful.

Copper sensitivity growth curves performed in minimal essential medium, with the mutants *Cj1516*, *Cj1161c* and the double mutant, showed that all three strains were more sensitive to increased copper levels when compared to the parent strain NCTC 11168 (Fig. 6). After 16 hours microaerobic growth all three mutants showed significant sensitivity to increased copper in the medium, as demonstrated by the lower overall OD600 measurements at copper concentrations above zero (Fig. 6). In contrast, the wild type strain showed resistance up to a copper concentration of 0.6 mM.

Discussion

The acquisition, utilisation and management of transition metals is crucially important in pathogenic bacteria and contributes to their survival in the host and external environments. In *C. jejuni*, these processes have only been studied in detail for iron. However, the importance of other metals is obvious, not least copper, which is required as a cofactor for proteins such as the major electron transport terminal oxidase complex, cytochrome *c* oxidase. Copper is also now known to be required in the biosynthesis of the molybdopterin cofactor (32), yet it is also extremely toxic in excess and requires strict management. In this study, we have demonstrated that *C. jejuni* possesses mechanisms for dealing with excess copper and that the removal of these mechanisms render the organism susceptible to the toxic effects of this transition metal. We have determined the function of an unknown protein, Cj1516 by biochemical characterisation, mutagenesis and phenotypic analysis and shown by mutation that a second gene encoding a probable Cop-like protein is also involved in copper management.

The data presented demonstrates that Cj1516 is a protein which binds copper atoms in the specific copper centre formations characteristic of MCOs (58). These have been demonstrated in a number of bacterial proteins, for instance, the related protein CueO in *E. coli* (24). Bioinformatic analysis (Fig 1) revealed the presence of the critical copper ion binding residues present in other MCOs. There are also at least two MxxM motifs (Fig. 1) that could also act as copper binding sites. Inductively-coupled plasma mass spectrometry analysis shows Cj1516 to contain approx. 6 copper atoms per molecule, which is similar to *E. coli* CueO, whose copper content has been quoted as being 4, with two more atoms present, one of which is a labile "regulatory" copper and a sixth surface copper (50, 51). In spectroscopic studies, the protein displayed optical and electron paramagnetic resonance spectra consistent with the presence of

type 1, type 2 and type 3 copper centres. A strong optical A_{610} maximum indicated a type 1 copper centre and a shoulder at A_{330} indicated a type 3 copper centre (Fig. 2). The narrow hyper-fine splitting observed in the EPR spectra were indicative of a type 2 centre and further proof of a type 1 centre. Upon the addition of ferrous iron to the pure protein sample, both type 1 and 2 signals were lost presumably due to reduction of each centre, thus providing initial evidence of ferroxidase capabilities.

Despite the similarities between eukaryotic and prokaryotic MCOs, only a few bacterial proteins, such as CueO (30) and an MCO from *P. aeruginosa* (29) have been shown to exhibit the same phenoloxidase and ferroxidase activities as seen in eukaryotic enzymes such as Fet3p and Human ceruloplasmin. Biochemical characterisation clearly showed that Cj1516 exhibits both these activities. In *P. aeruginosa,* a mutation in the MCO encoding gene led to the loss of ferrous iron acquisition in the organism under aerobic conditions (29). A model similar to that in *S. cerevisiae*, in which an MCO (Fet3) oxidises iron for uptake by an integral membrane permease (Ftr1) (3), was proposed for *P. aeruginosa* (29) and also for the magnetotactic bacterium MV-1 (16). In this organism it is anticipated that an additional gene product with homology to p19, a periplasmic Fur regulated protein in *C. jejuni* is involved, along with a multicopper oxidase and a permease-like protein, in iron acquisition (16). In *C. jejuni* the Fur-regulated periplasmic protein p19 (Cj1659) is part of a large gene cluster also containing an iron permease (Cj1658). A similar gene arrangement is also found in an iron uptake pathogeneicity island in the gamma proteobacterium *Yersinia pestis* (13). These observations led us to the possibility that Cj1516 was likely to be involved in iron metabolism and the data presented here shows that the enzyme is clearly able to oxidise Fe(II) with reasonable kinetics. In addition, a previous global transcriptomic study showed that *Cj1516* gene expression is induced 3-fold under conditions of iron-limitation (28). However, we did not observe a phenotype relating to

ferrous iron acquisition in growth experiments involving a *Cj1516* null mutant or in a *Cj1516/feoB* double mutant. These data suggest that the ferroxidase activity of the protein may not be physiologically relevant, at least under the growth conditions used. Manganese oxidation is also a feature of some bacterial MCOs (20, 21), yet the purified Cj1516 protein did not exhibit manganese-linked oxygen uptake. However, Cj1516 exhibited high rates of cuprous oxidase activity, and a *Cj1516* mutant was more copper sensitive than the wild-type parent strain, indicative of a physiological role in copper detoxification.

A second gene *Cj1161c,* has also been shown to encode a protein with striking similarity to a specific group of Cu(I) exporting proteins belonging to the P-type ATPase family. It is likely that this gene encodes a P_{1B-1}- ATPase copper transporting protein, similar to CopA, which has a central role in copper homeostasis in *E. coli* (42, 48). Mutations in both *Cj1516* and *Cj1161c* genes resulted in a similar degree of increased sensitivity to excess copper in growth studies compared to the wild type strain and a double mutant was also hypersensitive to elevated copper levels under microaerobic conditions (Fig 6). The role of CopA has been well documented in recent years in a number of bacteria (4, 37, 48) and has been found to export Cu(I) ions from the cytoplasm to periplasm. It has also been proposed that a multicopper oxidase protein is then involved in further detoxification of the Cu(I) ions by oxidation to Cu(II), a less toxic and less membrane permeable form of copper in the periplasmic compartment (24). With an apparent lack of additional copper management proteins in the organism, Cj1161 is likely to provide the bacterium with an efficient copper (I) export system, with the multicopper oxidase Cj1516 providing periplasmic protection by oxidation to Cu(II). Thus, the evidence suggests that Cj1516 and Cj1161 operate together as part of a copper homeostasis system in the organism in a microaerobic environment. Both proteins are also likely to be important for copper tolerance

548 when oxygen is limiting, since *C. jejuni* lacks the Cus copper efflux system found in other
549 bacteria and which operates in anaerobic conditions (25, 38).

550 The exact mechanism by which bacterial multicopper oxidases confer copper resistance
551 is yet to be established. However, the most widely held view is that the MCOs oxidise the toxic
552 Cu(I) to the much less toxic Cu(II). *E. coli* CueO is also capable of oxidising catecholate
553 siderophores, and the resulting pigments may then sequester copper. *Campylobacter jejuni* does
554 not synthesise its own siderophores (18, 43), it does however, utilise siderophores produced by
555 other organisms (17). Consistent with this, Cj1516 is able to oxidise both Cu(I) and the
556 catecholate siderophore analogue 3,4-DHB, which may also have a role in copper tolerance.

557 The Cue/Cop system described in *E. coli* (24, 42, 48), is regulated by CueR in the
558 cytoplasm, although none of the genes are in the same operon. CueR is a MerR-like
559 transcriptional regulator with a helix-turn-helix motif, which is induced by copper (60). We have
560 not so far investigated the regulation of the Cop/Cue homologues in *C. jejuni* and we could not
561 find an obvious homologue of CueR in the genome of *C. jejuni* 11168, although there is an
562 example of a MerR-like protein, encoded by *Cj1563c* and it is possible that this protein could
563 fulfil the regulatory role. Recently it has been proposed that genes involved in molybdenum
564 cofactor biosynthesis in *E. coli* are regulated by excess copper via CueR (32, 65). Interestingly,
565 as noted above, several genes involved in molybdenum cofactor biosynthesis, *moaD*, *moaE* and
566 *moeA2* are located downstream of the MCO encoding *Cj1516* in *C. jejuni* NCTC 11168 (Fig.
567 1B). It should also be noted that *Cj1516* is conserved in other sequenced *C. jejuni* strains (e.g.
568 81-176, RM1221 and 81116) and Moco biosynthesis genes are also located in similar positions
569 in these strains.

570 In addition to *Cj1161c*, in the same region a number of unusual and unknown genes are
571 present which may also be involved in the putative Cop system and indeed may form an operon

572 which includes *Cj1161c* (Fig. 1B). Genes *Cj1162-1164c* encode proteins which are all predicted
573 to possess at least one CxxC motif each. *Cj1163c* contains a histidine rich N-terminal domain. It
574 is also predicted to possess six transmembrane helices and as such is similar in structure to the P-
575 type ATPases. However it lacks the highly conserved and essential ATP binding motif DKTGT.
576 Both *Cj1162c* and *Cj1164c* encode small hypothetical proteins (64 amino acids and 87 amino
577 acids, respectively), each containing a CxxC motif. In the other sequenced strains of *C. jejuni*, a
578 very similar gene arrangement exists as found in the reference strain NCTC 11168. No data
579 exist with respect to these three genes, however, given their location they merit further
580 investigation for a copper related role.

581 In conclusion, we have identified and characterised two gene products involved in the
582 homeostasis of copper in *C. jejuni*. The genes *Cj1516* and *Cj1161c* encode a multicopper
583 oxidase and a copper transporting P-type ATPase, respectively. Our studies have shown that the
584 removal of theses genes renders the organism more sensitive to copper and we were unable to
585 identify any other genes associated with the known alternative systems of copper management in
586 the organism. The regulation of these copper homeostasis genes in *C. jejuni* requires further
587 investigation.

588

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780

781 **Table 1**. Kinetic parameters for Cj1516 enzyme activities.

Substrate	Plus 1 mM Copper (II) sulphate		Without added Copper	
	V_{max}	K_m (mM)	V_{max}	K_m (mM)
***p*-PD**	3.04 ± 0.14	0.27 ± 0.05	1.99 ± 0.13	22 ± 3.10
3,4 - DHB	11.60 ± 3.64	0.16 ± 0.03	ND	ND
Fe (II) [a]	19.60 ± 2.62	0.19 ± 0.01	3.82 ± 0.57	0.40 ± 0.20
Cu (I) [b]	61.49 ± 2.03	0.18 ± 0.01	ND	ND

782

783 The *p*-PD, 3,4-DHB and Fe(II) activities were determined spectrophotometrically as described in
784 Materials and Methods. The Cu(I) activity was determined by oxygen uptake assays as plotted in
785 Fig. 4D. The V_{max} values are given in µmol substrate oxidised min^{-1} mg $protein^{-1}$, and all data are
786 the mean values and standard deviations for three replicate titrations. ND; not determined.
787 [a]; Fe (II) was added as ferrous ammonium sulphate. [b] Cu(I) added as caged copper complex.

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Figure Legends

Figure 1. **A**. **Sequence alignment of Cj1516 with related MCOs**. The twin arginine signal motif (TAT) and the 4 pairs of histidine residues involved in copper ligand formation are in boxes. Alignments were performed using CLC Workbench and ClustalX. **B**. Gene context and mutagenesis strategy of *Cj1516*. Note the presence of Moco biosynthesis genes downstream of *Cj1516*. **C.** Gene context and mutagenesis strategy of *Cj1161c*. The genes upstream of *Cj1161c* may also have a role in copper homeostasis as discussed in the text. Arrows above the kanamycin (kan) and chloramphenicol (cat) cassettes used for mutagenesis indicate the direction of transcription of the resistance gene promoter.

Figure 2. Over-expression and purification of Cj1516. (A) left panel: SDS-PAGE demonstrates expression of a ~56 kDa protein indicated by a black arrow in the BL21 λ DE3/pET1516 strain after induction with IPTG (lanes 4-7; 1,3,5 and 16h post-induction). Expression appears to be maximal after 16 h growth (lane 7). This protein was absent in the same strain without induction (lane 3) and absent from the control strain BL21 λ DE3/pET21a after overnight growth and addition of IPTG (lane 2). Lane 1 contains pre-stained molecular weight markers (BioRad UK). Cells were grown at 25°C with shaking. 1mM IPTG was used for induction. **Right panel:** SDS-PAGE analysis of Cj1516 purification steps. CE, crude extract; DEAE, ion-exchange column fraction; HIC, hydrophobic interaction column fraction; HT, heat treatment step. **(B)** Optical absorbance spectroscopy of Cj1516. Absorbance was scanned at 300 to 700 nm with 0.6 mg ml^{-1} protein in 50 mM Na-acetate pH 5.7. The spectrum shows the T1 copper site signal at ~600 nm and the T3 copper site signal at 330 nm. An unidentified peak at 420 nm is also apparent.

Figure 3. EPR spectrum of Cj1516 T1 and T2 copper centres. A Bruker EMX spectrometer (X-band 9.38 GHz) was used to analyse the copper centre active sites of the multicopper oxidase Cj1516. Line **A** shows the spectrum recorded for Cj1516 as isolated, with the type 1 copper centre hyper-fine splitting displayed. Type 2 copper centre hyper-fine splitting is shown in the 8x amplified signal. Line **B** shows the spectrum for Cj1516 after addition of Fe(II) in the form of 1 mM ammonium ferrous sulphate. The protein was in 50 mM Na-acetate, pH 5.0 for both spectra.

Figure 4. Substrate-linked oxygen consumption of purified Cj1516. Pure Cj1516 protein was assayed for oxidase activities using a Clark-type oxygen electrode as described in Materials and Methods. The substrates used were **A**, manganese (II) chloride, **B**, ferrous ammonium sulphate, **C**, caged copper (I). In **D**, the dependence of the rate of oxygen consumption on the caged copper (I) concentration is plotted, and the data from three independent titrations fitted to the Michaelis-Menten equation (black line). The kinetic parameters from this titration are given in Table 1.

Figure 5. Growth of wild-type and mutant strains under iron limited and iron replete conditions. Cultures were grown in minimal essential medium (MEM α) in the absence of added iron (white bars) and the presence of 45 μM ferrous iron (black bars) as described in Materials and Methods. Data are the means and standard deviations of the final optical densities reached after 16 h growth of three biological replicate cultures.

Figure 6. The effect of copper on the growth of *C. jejuni* wild-type and mutant strains. WT and mutant cultures were grown microaerobically to stationary phase in Minimal Essential

838 Medium (MEM α) containing varying concentrations of copper as described in Materials and
839 Methods. WT; black bars, Cj*1516*; white bars, Cj*1161c*; dark grey bars, Cj*1516/1161c* double
840 mutant; hatched bars. Data are the means and standard deviations of the final optical densities
841 reached after 16 h growth of three biological replicate cultures.
842

843 **Figure 1.**

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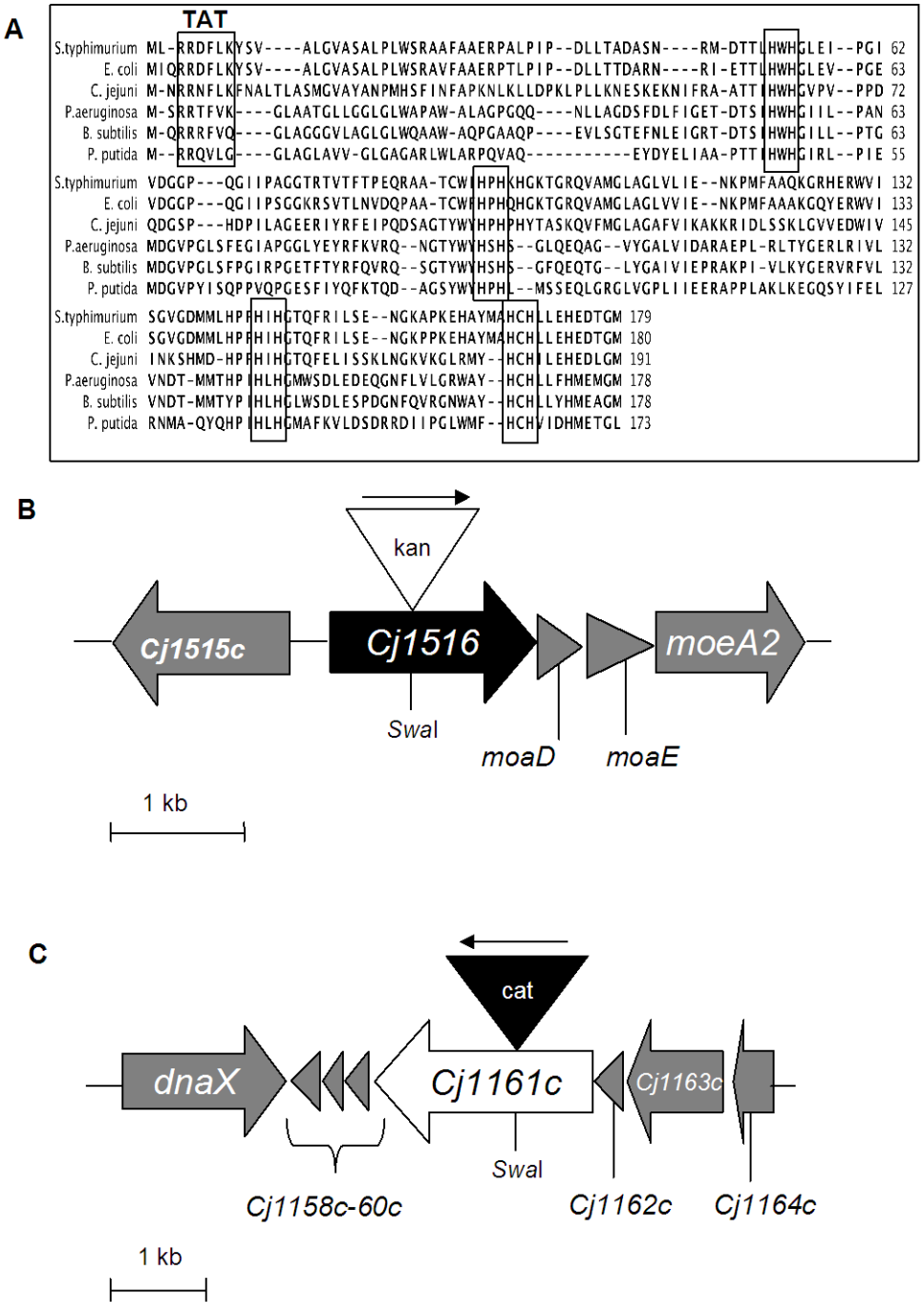

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847 **Figure 2**

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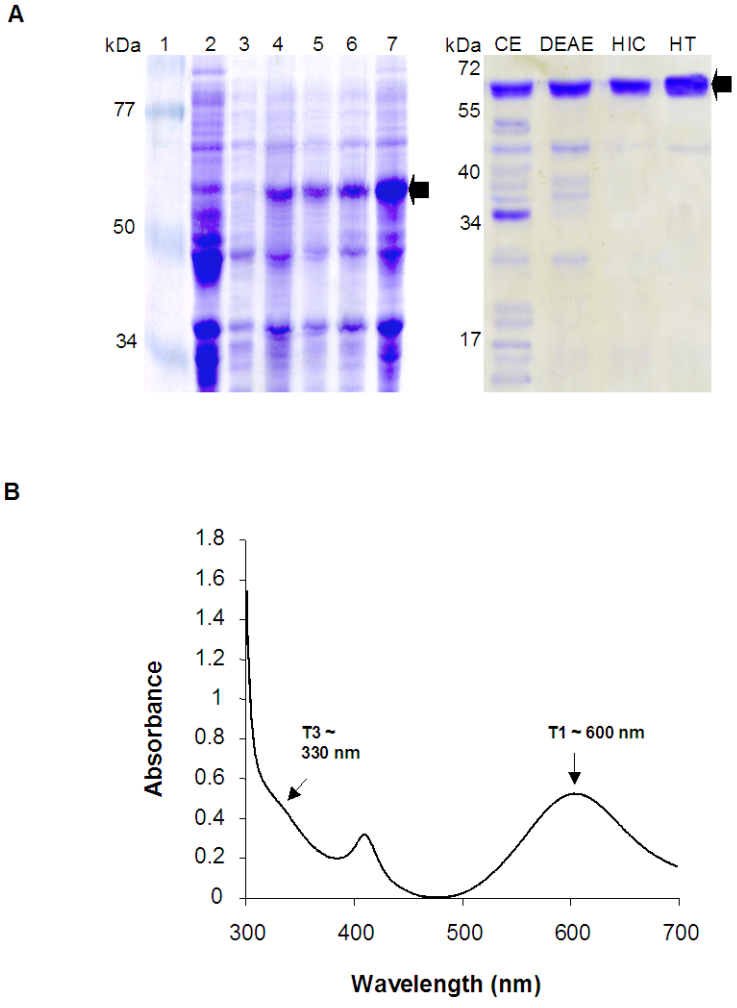

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851 **Figure 3**

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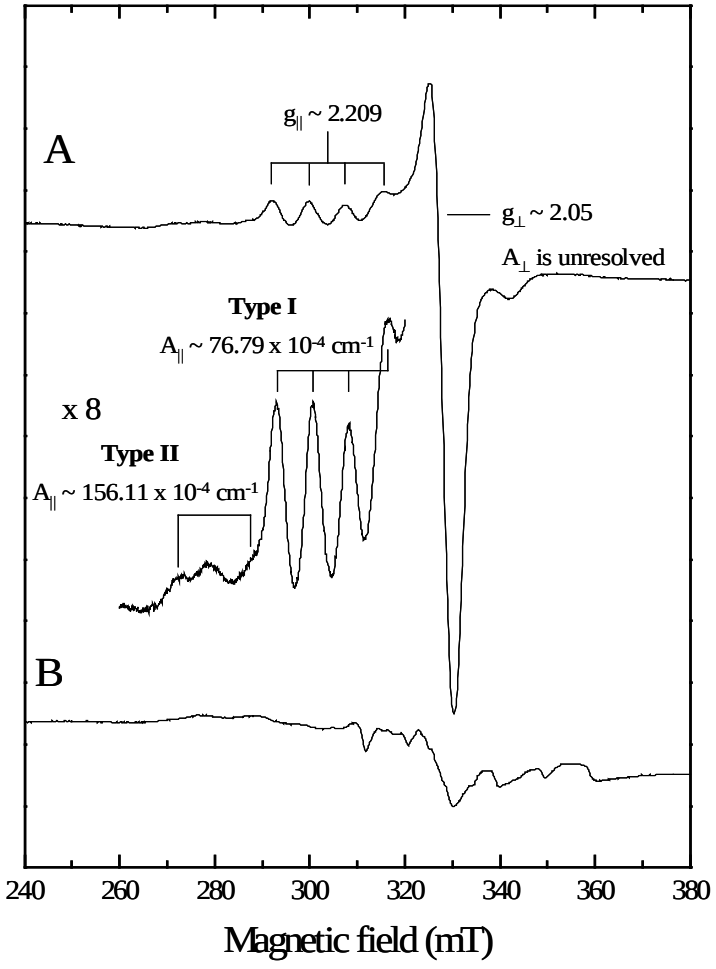

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Figure 4

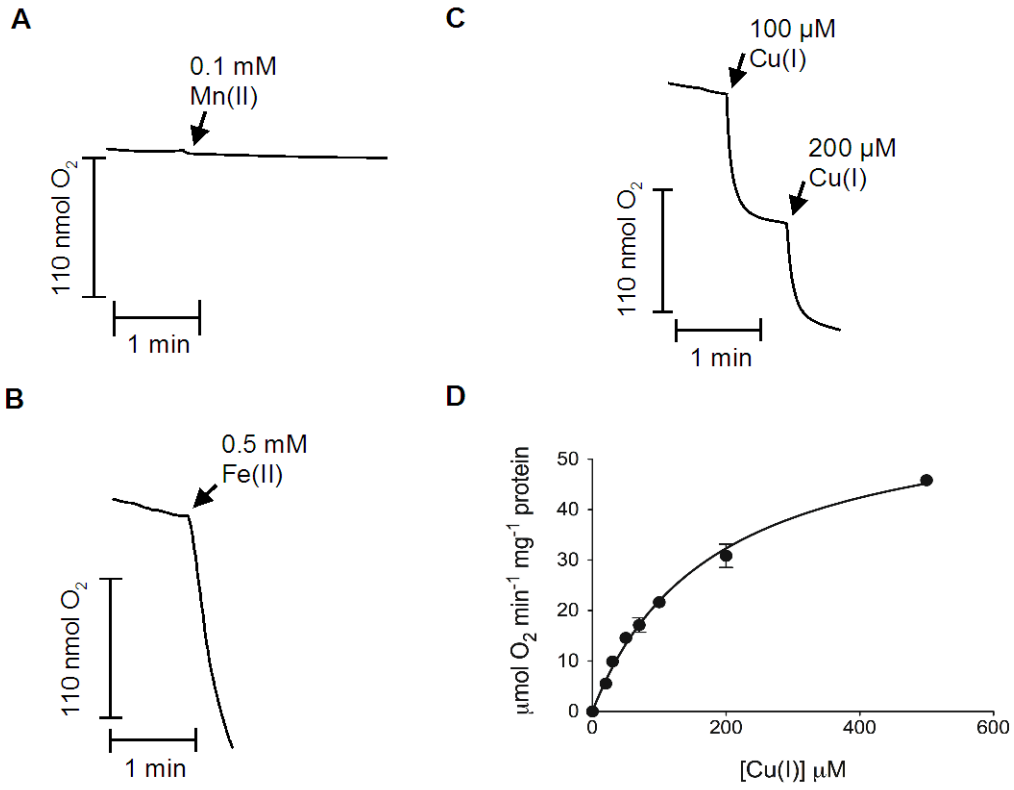
A
0.1 mM
Mn(II)
110 nmol O2
1 min
B
0.5 mM
Fe(II)
110 nmol O2
1 min
C
100 µM
Cu(I)
200 µM
Cu(I)
110 nmol O2
1 min
D
µmol O2 min-1 mg-1 protein
0
10
20
30
40
50
0
200
400
600
[Cu(I)] µM

Figure 5

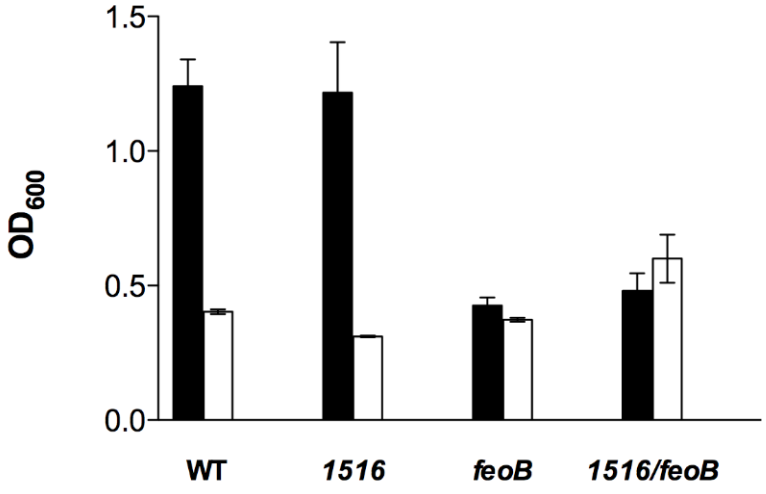

Figure 6.