

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

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25 **Abstract**

26

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

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50 **Introduction**

51

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

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

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

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

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

97 *p*-diphenol:O₂ 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

117 **Materials and Methods**

118

119 **Bacterial strains, media and culture conditions.** *C. jejuni* strain NCTC 11168 was routinely
120 cultured at 37 °C under microaerobic conditions (10 % [vol/vol] O₂, 5 % [vol/vol] CO₂ and 85%
121 [vol/vol] N₂ in a MACS growth cabinet (Don Whitley Scientific Ltd., Shipley, United Kingdom)
122 on Columbia agar containing 5 % (vol/vol) lysed horse blood and 10 µg ml⁻¹ each of
123 amphotericin B and vancomycin. Liquid cultures of *C. jejuni* were routinely grown
124 microaerobically at 200 rpm, either in Mueller-Hinton broth (Oxoid Ltd, UK) supplemented with
125 20 mM L-serine (MH-S) or in the defined medium MEM-α (Invitrogen Ltd. Cat. number 41061-
126 029, containing glutamine and deoxyribonucleotides but no phenol red), containing the above
127 antibiotics and 45 µM FeSO₄, 20 mM serine and 20 mM pyruvate. To select for the *C. jejuni*
128 *Cj1516* mutant, kanamycin was added to media at a final concentration of 30 µg ml⁻¹ and to
129 select for the *Cj1161c* mutant chloramphenicol was added to media to a final concentration of 30
130 µg ml⁻¹. *E. coli* DH5α was cultured in Luria-Bertani (LB) broth or agar supplemented with
131 appropriate antibiotics at 37 °C. For growth experiments, *C. jejuni* overnight starter cultures were
132 prepared in MH-S and washed three times, before inoculation into MEM-α. Growth was
133 monitored at 600 nm using an Amersham Pharmacia Biotech Ultrospec 2000 spectrophotometer.

134

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

140

141 **Over-expression and purification of Cj1516.** For the over-expression of the *Cj1516* gene
142 product, primers (forward, 5'-ATCAGCTAGCAATAGAAGAAATTTTTTA- 3'; and reverse
143 5'- TAGCGGATCCTTATTCTTTACTTCTAA -3', *NheI* site underlined, *BamHI* site in bold
144 italics) were designed to amplify the complete *Cj1516* gene from *C. jejuni* NCTC 11168
145 chromosomal DNA by PCR using a proofreading DNA polymerase enzyme (Pwo, Roche Ltd.,
146 United Kingdom). The PCR fragment was then cloned by blunt end ligation into pGEM3ZF (-)
147 (Promega Ltd., United Kingdom) to create pGEM1516. The gene was excised from pGEM1516
148 by digestion with *NheI* and *BamHI* and cloned into similarly digested pET21a(+) (Novagen Ltd.,
149 United Kingdom) to give pMCO1516. Automated DNA sequencing (Lark Technologies Inc,
150 Saffron Walden, UK) showed that the sequence of the *Cj1516* gene in pMCO1516 was correct.
151 pMCO1516 was transformed into *E. coli* BL21 (DE3) cells which were grown aerobically at 25
152 °C in LB medium containing ampicillin (50 µg ml⁻¹) and 1mM copper sulphate (CuSO₄) to an
153 optical density at 600 nm of 0.6 before 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) was
154 added. Induced cells were then grown for a further 16 h before harvesting by centrifugation (30
155 min, 4 °C, 3500 x g). Cell pellets were resuspended in 10 mM Tris-HCl pH 8.0 and disrupted by
156 sonication with an MSE Soniprep 150 (Sanyo, UK) using 6 x 20 second bursts of ultrasound
157 (amplitude ~ 15 microns peak to peak) with 30-second intervals between bursts. Cell debris and
158 soluble matter were separated by centrifugation at 12,000 x g for 20 minutes at 4 °C. The
159 fractions were kept on ice until used or stored at -20 °C. The supernatant was recovered as cell
160 free extract and fractionated on a DEAE sepharose Fast flow column (GE Healthcare, UK) by
161 ion-exchange chromatography. The protein was eluted from the resin by a gradient from 0-500
162 mM NaCl in 10 mM Tris-HCl, pH 7.5. Fractions were pooled and adjusted to 1M ammonium
163 sulphate and further fractionated by hydrophobic interaction chromatography using a 10 ml
164 Phenyl Sepharose (Sigma) column. A salt gradient of 1 M to 0 M (NH₄)₂SO₄ was used to elute

165 the proteins. Recombinant Cj1516 elution from the chromatography columns was detected by
166 monitoring of the A₆₁₀ due to type 1(blue) copper content. The final purification step utilised the
167 apparent thermal stability of multicopper oxidases and involved heat treatment of the samples as
168 previously described (30). Briefly, pooled fractions collected from the hydrophobic interaction
169 step were incubated at 70 °C for 5 min before being centrifuged to remove denatured proteins.
170 Phenoxidase activity was monitored before and after heat treatment to ensure activity of the
171 recombinant protein was not lost.

172

173 **Construction of mutants.** A *feoB* mutant was constructed by Mariner transposon insertion into
174 the *Cj1398* gene and was kindly provided by Dr A. Grant, Cambridge Veterinary School,
175 University of Cambridge, UK. *Cj1516* and *Cj1161c* mutants were constructed by insertion of
176 kanamycin and chloramphenicol resistance cassettes, respectively, into each gene in the same
177 transcriptional orientation. The *Cj1516* gene was amplified using the following specific primers:
178 Cj1516For: (5'–CAAAGTCCGCTACAAGTACAAC–3'), Cj1516rev: (5' –
179 CCGATCTTGAAACACGACATAGA – 3'). The resulting 1.59 kb fragment containing the
180 coding region of the gene was cloned into pGEM 3Zf (-) vector (Promega, UK). Transformants
181 were recovered by selection on plates containing ampicillin (50 µg ml⁻¹). The kanamycin
182 resistance cassette derived by PCR from plasmid pJMK30 was cloned into the unique restriction
183 site *Swa*I in the centre of *Cj1516* to produce plasmid p1516kan. For construction of a *Cj1161c*
184 mutant strain, primers *Cj1161c*F: (5' – ATGCATGGAAGAATTGCGTAT – 3') and *Cj1161c*R:
185 (5' – ATGCTCTTAAAGAATTAAGCACTACA – 3') were used to amplify a 2.085 kb
186 fragment containing the entire coding region of *Cj1161c*, this fragment was cloned into pGEM
187 T-Easy vector to produce plasmid pGEM1161c. The chloramphenicol resistance cassette derived

188 from pAV35 (64) was cloned into the unique *SwaI* 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 $\mu\text{g ml}^{-1}$ final concentration) or chloramphenicol (30 $\mu\text{g ml}^{-1}$ 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*-phenylenediamine of 14.7 $\text{mM}^{-1} \text{cm}^{-1}$ at 487 nm. Sigmaplot 8.0 (SPSS inc. USA)

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

213
214 **Ferroxidase assays and kinetics.** Ferroxidase assays were performed on purified recombinant
215 Cj1516 protein. The 1ml assay volume contained 50 mM sodium acetate buffer pH 5.7 and 0.8
216 μ M Cj1516 protein. The assay was started by the addition of ammonium ferrous sulphate to final
217 concentrations of 0.01 – 0.3 mM and rates recorded at 315nm as above. All assays were
218 performed at 37 °C in matching quartz cuvettes. Specific activities were calculated using an
219 extinction coefficient for Fe(III) of $2.2 \text{ mM}^{-1} \text{ cm}^{-1}$ at 315 nm. Sigmaplot 8.0 (SPSS inc. USA)
220 was used to calculate V_{max} and K_m values as above.

221
222 **Siderophore oxidase assays and kinetics.** Oxidation assays of an analogue of the bacterial
223 siderophore anthrachelin were performed on pure recombinant Cj1516 protein. Each assay
224 contained 50 mM sodium acetate buffer pH 5.7 and 0.8 μ M of pure Cj1516 protein. The assay
225 was started by the addition of 3,4- dihydroxybenzoate (3,4-DHB) to a concentration range of 0-8
226 mM for assays containing excess copper and 0-60 mM without excess copper and rates recorded
227 at 487nm using a Shimadzu UV-2401 dual wavelength scanning spectrophotometer as above.
228 All assays were performed at 37 °C. Specific activities were calculated using an extinction
229 coefficient for 3,4-DHB of $2.3 \text{ mM}^{-1} \text{ cm}^{-1}$ at 400nm. Sigmaplot 8.0 (SPSS inc. USA) was used
230 for the calculation of V_{max} and K_m values as above.

231
232 **Measurement of metal-ion oxidation-linked oxygen respiration.** Metal-ion oxidation by pure
233 Cj1516 protein was determined by measuring the change in dissolved oxygen concentration in a
234 Clark-type polarographic oxygen electrode (Rank brothers Ltd, Bottisham, Cambridge, UK)

235 comprising a water-jacketed perspex chamber that was stirred magnetically, linked to a chart
236 recorder and calibrated using air saturated 25 mM phosphate buffer (pH 7.5). 100 % saturation
237 was assumed to be $220\mu\text{M O}_2$. A zero oxygen baseline was determined by the addition of
238 Sodium dithionite. The cell suspension was maintained at $37\text{ }^\circ\text{C}$ and stirred at a constant rate.
239 Substrates were added by injection through a fine central pore in the airtight plug. Substrates
240 used were manganese chloride, ammonium ferrous sulphate and a caged copper form of copper
241 (I) which consisted of the compound tetrakis (acetonitrile) copper (I) hexafluorophosphate
242 (Sigma-Aldrich, UK) dissolved in argon-sparged 5% acetonitrile. $1.3\ \mu\text{M}$ of pure Cj1516 was
243 used in each cuprous oxidase assay and $0.8\ \mu\text{M}$ Cj1516 used for manganese and ferrous iron
244 assays. Rates were expressed in $\mu\text{mol O}_2$ utilised $\text{min}^{-1}\ \text{mg protein}^{-1}$. For analysis of cuprous
245 oxidase kinetics, the means of three assay measurements at various substrate concentrations were
246 used. The hyperbolic curve fitting algorithms of GraphPad Prism 5.0 for Mac (GraphPad
247 Software, San Diego, California USA) were used to analyse the data and calculate K_m and V_{max}
248 values based on the Michaelis-Menten equation.

249
250 **Spectroscopy.** UV-visible light spectra were collected by using a Shimadzu UV-2401
251 spectrophotometer (Shimadzu Ltd). Copper sites were detected by the addition of $0.024\ \mu\text{g}$ pure
252 Cj1516 protein to a 1ml cuvette containing 10 mM Tris-HCl pH 7.5. A wavelength range of 500-
253 700 nm was used to detect the characteristic peak produced by T1 copper centres at $\sim 600\ \text{nm}$. A
254 wavelength range of 300 nm to 700 nm were use to detect the T3 copper centre at $\sim 330\ \text{nm}$. All
255 spectra were obtained at 37°C . Electron paramagnetic resonance (EPR) spectra were recorded
256 with a Bruker (Billerica, Mass.) EMX spectrometer (X-band 9.38 GHz) equipped with an
257 ER4112HV liquid helium flow cryostat system. Spectra were recorded at a temperature of 30 K,

258 6 mT modulation amplitude and 0.2 mW microwave power. The protein used in analysis was as
259 isolated.

260

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

266

267 **Copper tolerance growth experiments.** Triplicate 10 ml cultures of each strain (wild type
268 NCTC 11168, *Cj1516*, *Cj1161c* and *Cj1516/1161c* double mutant) were grown in minimal
269 media (MEM- α) containing copper sulphate in the concentration range 0 – 1 mM. Cultures were
270 incubated from a starting OD₆₀₀ of 0.1 to stationary phase, microaerobically at 37 °C with
271 shaking. The final OD₆₀₀ was recorded using an Amersham Pharmacia Ultrospec 2000
272 spectrophotometer.

273

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

285 **Results**

286

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

306

307 **Overexpression and purification of *Cj1516*.** The *Cj1516* gene in the NCTC 11168 strain of *C.*
308 *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

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

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

347 T2 (or normal) copper centres do not produce intense features within the visible
348 absorption spectrum, but along with T1 sites, exhibit characteristic features that can be observed
349 by electron paramagnetic resonance (EPR) spectroscopy, owing to the open shell configuration
350 of electrons in oxidized (cupric) atoms providing an unpaired electron in the outer shell.
351 Conversely, whilst being visible at 330 nm in optical spectroscopy, T3 centres are EPR “silent”,
352 due to the coupling of the two copper atoms via a bridging ligand and thus, the loss of unpaired
353 ferromagnetically active electrons (58). Figure 3, spectrum A shows the results of the EPR
354 spectroscopy performed on as-purified Cj1516. The protein exhibited EPR features typical of
355 multicopper proteins, displaying a spectrum with narrow hyperfine splitting ($g_{\perp} \sim 2.05$, $g_{\parallel} \sim$
356 2.209 and $A_{\parallel} \sim 76.79 \times 10^{-4} \text{ cm}^{-1}$) for the T1 centre. Underlying features characteristic of a T2

357 centre are also evident at lower field and display an approximate hyperfine splitting of $A_{\parallel} =$
358 $156.11 \times 10^{-4} \text{ cm}^{-1}$. Figure 3 spectrum B shows the effects of the addition of ferrous iron to the
359 sample. Rapid reduction of each centre to Cu(I), with the concomitant oxidation of ferrous (Fe
360 II) to ferric (Fe III) iron, resulted in the loss of the T1 and T2 signals. These observations are
361 consistent with the ferroxidase activity of the protein described below.

362

363 **Spectrophotometric analysis of substrate specificity and kinetics of Cj1516, and the effect**
364 **of excess copper.** Cj1516 oxidized a number of phenolic compounds such as *p*-
365 phenylenediamine, N,N,N,N- tetramethyl-*p*-phenylenediamine (TMPD) (data not shown) and
366 3,4- DHB (anthrachelin). The V_{max} and K_m values for *p*-PD and 3,4-DHB after the addition of 1
367 mM CuSO_4 were markedly different to those in the absence of excess copper, suggesting an
368 enhancement in activity as seen in other MCOs (30, 51) (Table 1). Of the three phenol
369 compounds studied, the higher affinity and high V_{max} values for 3,4-DHB imply this is a
370 favoured substrate. Ferroxidase activity measured using the optical method previously described
371 (30) was also observed and greatly enhanced by the addition of excess copper; a five-fold
372 increase in V_{max} and a two-fold decrease in K_m value were observed (Table 1). Oxidation of
373 phenolic compounds did not take place in the absence of enzyme, even with excess copper
374 present (data not shown) suggesting that free copper does not take part in a redox cycle, in
375 agreement with others (30).

376

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

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

392

393 **Ferrous iron acquisition is not affected in a *Cj1516* mutant.** A mutant in *Cj1516* was
394 constructed by the insertion of a kanamycin resistance cassette into a unique *SwaI* site within the
395 cloned gene (Fig. 1B). After electroporation into wild-type cells, several antibiotic resistant
396 colonies were selected and a PCR with gene specific primers showed that the mutant
397 construction had been successful (data not shown). Intact cells and periplasmic protein fractions
398 of this mutant completely lacked *p*-PD oxidase activity. The kanamycin resistance cassette used
399 was inserted with the same polarity as the *Cj1516* gene and therefore should not interfere with
400 downstream transcription. However, the genes downstream of *Cj1516* are predicted to encode
401 the proteins Moad, MoadE and MoadA2 (Fig. 1B), all of which are essential for the synthesis of
402 the molybdopterin cofactor (Moco) of molybdoenzymes. As it is now known that copper is
403 needed for the correct biosynthesis of this cofactor (31), we wanted to ensure that mutation of
404 *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 $\sim 2.5 \mu\text{moles MV oxidised min}^{-1}$
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.

429

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

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

456

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

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

474

475

476

477 **Discussion**

478

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

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

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

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

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

532 A second gene *Cj1161c*, has also been shown to encode a protein with striking similarity
533 to a specific group of Cu(I) exporting proteins belonging to the P-type ATPase family. It is likely
534 that this gene encodes a P_{1B-1}-ATPase copper transporting protein, similar to CopA, which has a
535 central role in copper homeostasis in *E. coli* (42, 48). Mutations in both *Cj1516* and *Cj1161c*
536 genes resulted in a similar degree of increased sensitivity to excess copper in growth studies
537 compared to the wild type strain and a double mutant was also hypersensitive to elevated copper
538 levels under microaerobic conditions (Fig 6). The role of CopA has been well documented in
539 recent years in a number of bacteria (4, 37, 48) and has been found to export Cu(I) ions from the
540 cytoplasm to periplasm. It has also been proposed that a multicopper oxidase protein is then
541 involved in further detoxification of the Cu(I) ions by oxidation to Cu(II), a less toxic and less
542 membrane permeable form of copper in the periplasmic compartment (24). With an apparent
543 lack of additional copper management proteins in the organism, Cj1161 is likely to provide the
544 bacterium with an efficient copper (I) export system, with the multicopper oxidase Cj1516
545 providing periplasmic protection by oxidation to Cu(II). Thus, the evidence suggests that Cj1516
546 and Cj1161 operate together as part of a copper homeostasis system in the organism in a
547 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 these 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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592

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594

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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)
<i>p</i>-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 $\mu\text{mol substrate oxidised min}^{-1} \text{mg protein}^{-1}$, 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.

788

789

790 **Figure Legends**

791

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

800

801 **Figure 2. Over-expression and purification of Cj1516. (A) left panel:** SDS-PAGE
802 demonstrates expression of a ~56 kDa protein indicated by a black arrow in the BL21
803 λ DE3/pET1516 strain after induction with IPTG (lanes 4-7; 1,3,5 and 16h post-induction).
804 Expression appears to be maximal after 16 h growth (lane 7). This protein was absent in the
805 same strain without induction (lane 3) and absent from the control strain BL21 λ DE3/pET21a
806 after overnight growth and addition of IPTG (lane 2). Lane 1 contains pre-stained molecular
807 weight markers (BioRad UK). Cells were grown at 25°C with shaking. 1mM IPTG was used for
808 induction. **Right panel:** SDS-PAGE analysis of Cj1516 purification steps. CE, crude extract;
809 DEAE, ion-exchange column fraction; HIC, hydrophobic interaction column fraction; HT, heat
810 treatment step. **(B)** Optical absorbance spectroscopy of Cj1516. Absorbance was scanned at 300
811 to 700 nm with 0.6 mg ml⁻¹ protein in 50 mM Na-acetate pH 5.7. The spectrum shows the T1
812 copper site signal at ~600 nm and the T3 copper site signal at 330 nm. An unidentified peak at
813 420 nm is also apparent.

814

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

821

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

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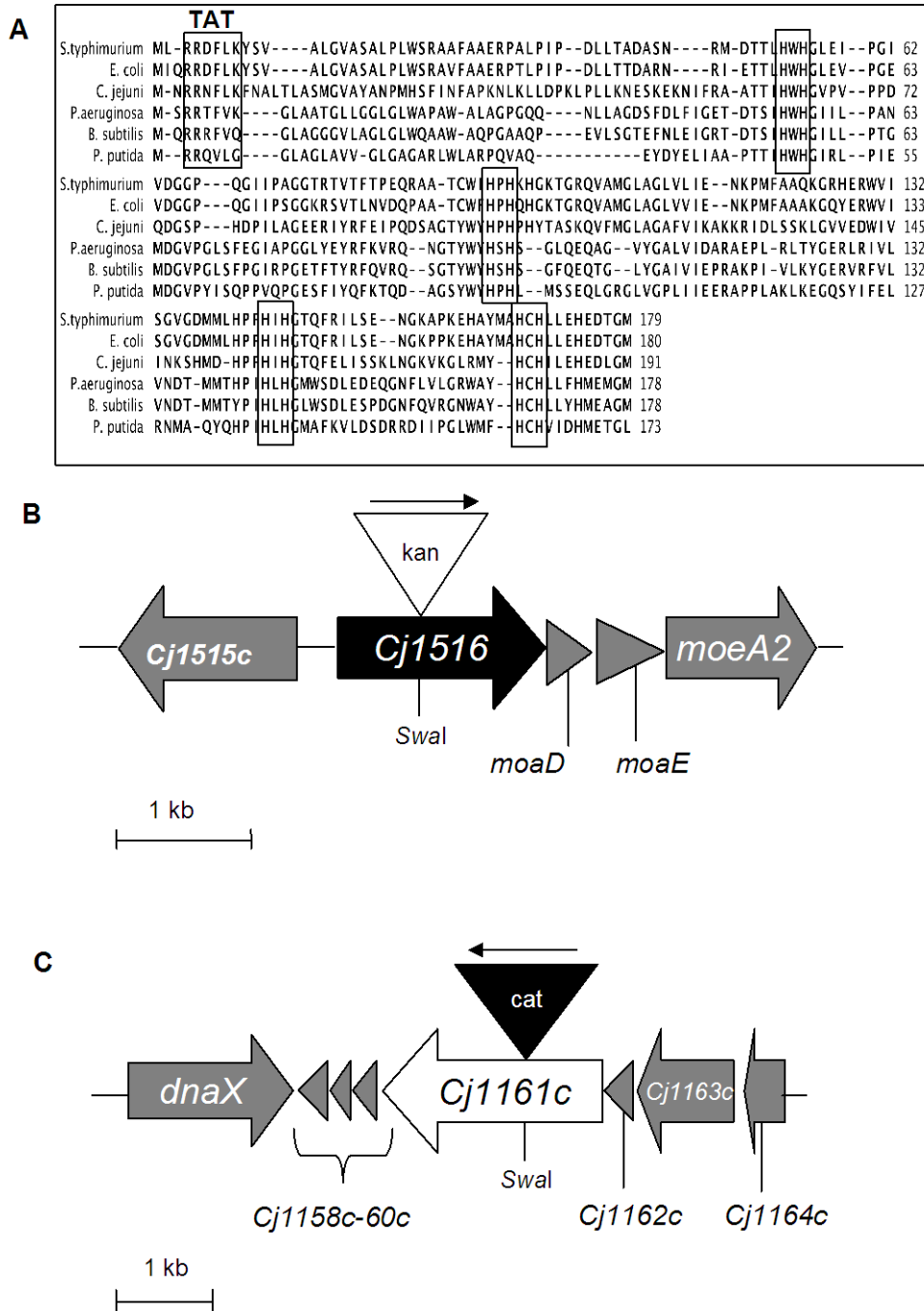
830 **Figure 5. Growth of wild-type and mutant strains under iron limited and iron replete**
831 **conditions.** Cultures were grown in minimal essential medium (MEM α) in the absence of added
832 iron (white bars) and the presence of 45 μ M ferrous iron (black bars) as described in Materials
833 and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

835

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

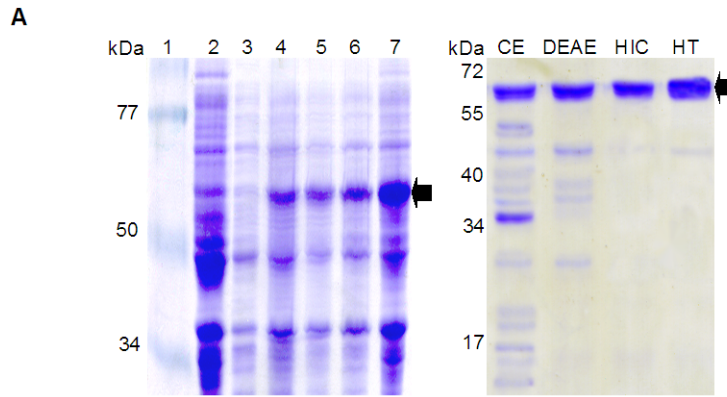
838 Medium (MEM α) containing varying concentrations of copper as described in Materials and
839 Methods. WT; black bars, *Cj1516*; white bars, *Cj1161c*; dark grey bars, *Cj1516/1161c* double
840 mutant; hatched bars. Data are the means and standard deviations of the final optical densities
841 reached after 16 h growth of three biological replicate cultures.

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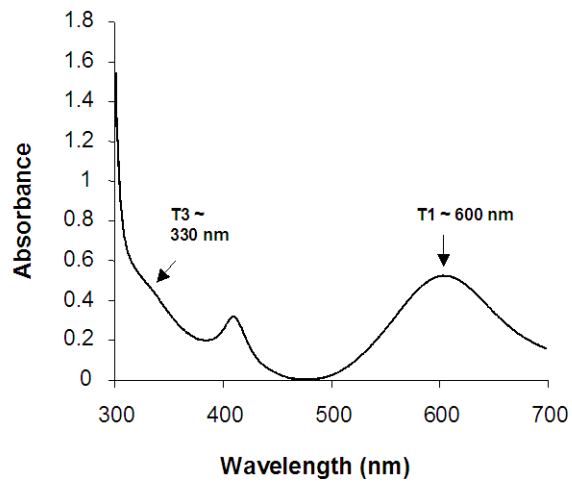


847 **Figure 2**

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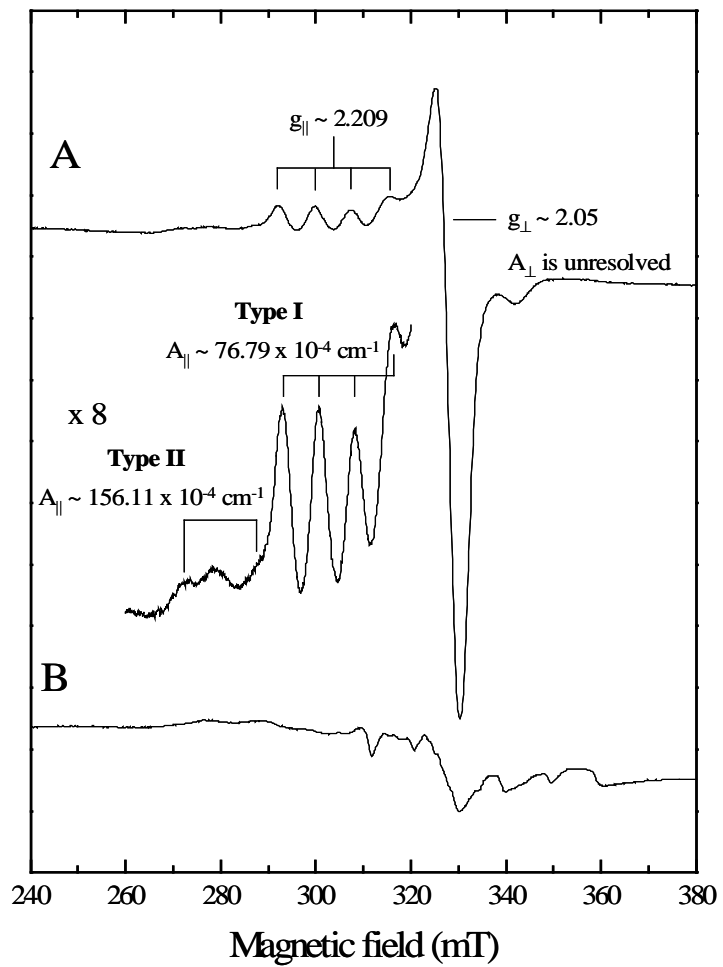


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851 **Figure 3**

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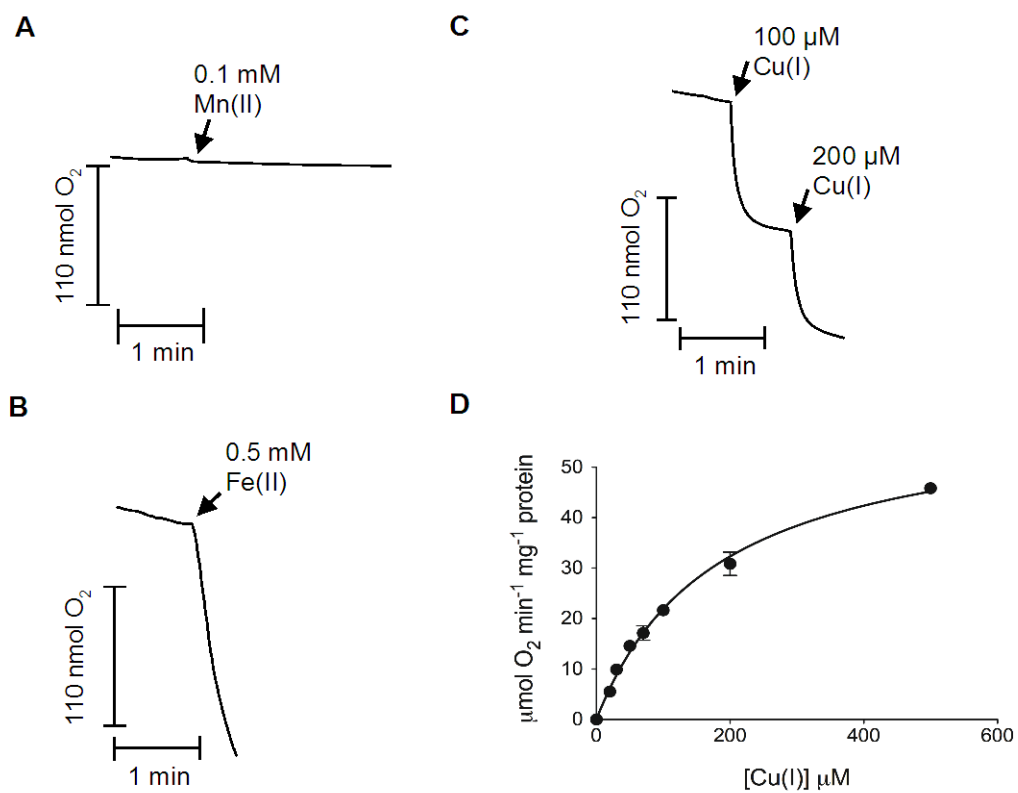


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855 **Figure 4**

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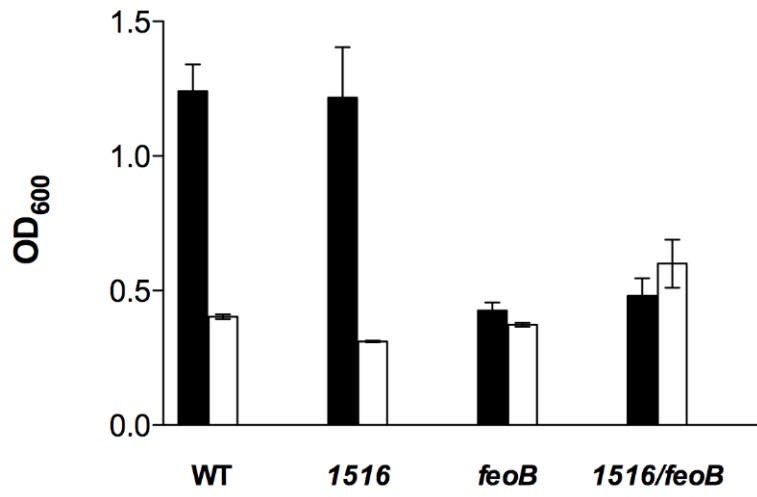


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859 **Figure 5**

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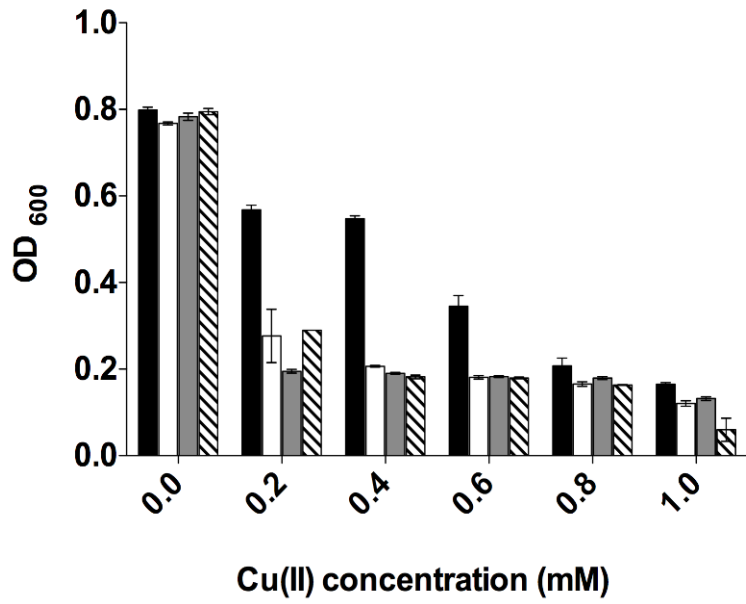


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863 **Figure 6.**

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