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An ATP-binding cassette-type cysteine transporter in *Campylobacter jejuni* inferred from the structure of an extracytoplasmic solute receptor protein

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Summary

Campylobacter jejuni* is a Gram-negative food-borne pathogen associated with gastroenteritis in humans as well as cases of the autoimmune disease Guillain–Barré syndrome. *C. jejuni* is asaccharolytic because it lacks an active glycolytic pathway for the use of sugars as a carbon source. This suggests an increased reliance on amino acids as nutrients and indeed the genome sequence of this organism indicates the presence of a number of amino acid uptake systems. Cj0982, also known as CjaA, is a putative extracytoplasmic solute receptor for one such uptake system as well as a major surface antigen and vaccine candidate. The crystal structure of Cj0982 reveals a two-domain protein with density in the enclosed cavity between the domains that clearly defines the presence of a bound cysteine ligand. Fluorescence titration experiments were used to demonstrate that Cj0982 binds cysteine tightly and specifically with a K_d of ~10^{-7} M consistent with a role as a receptor for a high-affinity transporter. These data imply that Cj0982 is the binding protein component of an ABC-type cysteine transporter system and that cysteine uptake is important in the physiology of *C. jejuni.

Introduction

Campylobacter jejuni is a Gram-negative, microaerophilic food-borne pathogen and a leading cause of acute bacterial gastroenteritis in humans (Bryant, 1983; Altekruse *et al*., 1999). It colonizes the intestinal tract of many warm-blooded animals (Svedhem and Kaijser, 1981) and is commensal in poultry, cattle and swine (Harris *et al*., 1986; Kazwala *et al*., 1990). The characteristics of the disease caused by *C. jejuni* in humans are diarrhoea, cramps, abdominal pains and fever, unfolding 2–5 days after exposure, usually via contaminated poultry products or drinking water (Palmer *et al*., 1983; Jones *et al*., 1990; Rosef *et al*., 2001). The illness typically lasts 1 week and in most cases recovery takes place without treatment, but in immunocompromized individuals, severe complications such as bacteraemia may occur (Vargas *et al*., 1992; Schonheyder *et al*., 1995). *C. jejuni* infections are also believed to be responsible for more than 30% of cases of the autoimmune disease Guillain–Barré syndrome, a form of neuromuscular paralysis (Kaldor and Speed, 1984). Globally increasing numbers of infections pose a significant socioeconomic problem demanding the development of efficient preventative strategies. Next to providing access to safe drinking water and promoting food hygiene, a solution to this problem can be found in vaccination of poultry.

An obstacle to vaccine production is hypervariability observed among genes encoding surface proteins in *C. jejuni.* CjaA, also termed OmpH1 (outer membrane protein H1) (Pawelec *et al*., 2000) and reclassified as Cj0982 following genome sequencing, is a conserved immunodominant protein in this organism and a promising vaccine candidate because the protein is expressed in multiple *Campylobacter* isolates from both chicken and human hosts (Pawelec *et al*., 1997; 2000; Martin and Mulks, 1999; Wyszynska *et al*., 2004). In sequence, Cj0982 closely resembles glutamine-binding protein from *Bacillus subtilis*; however, previous attempts to confirm the role of Cj0982 in glutamine transport were unsuccessful and its precise function remained undefined (Pawelec *et al*., 2000). Interestingly, antibodies raised against Cj0982 cross-react with a second immunodominant *C. jejuni* protein CjaC (Wyszynska *et al*., 2004). CjaC is also a component of a putative amino acid uptake system, in this case histidine.

The uptake and metabolism of amino acids is likely to be of special importance in *C. jejuni* as this bacterium is unable to utilize sugars as a source of carbon or energy (Kelly, 2001). Its glycolytic pathway is incomplete and its comparatively small genome contains few genes for the uptake or degradation of carbohydrates (Parkhill *et al*.,

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2000). Consistent with an important role of amino acids in energy production, it has been shown that omission of single amino acids from the medium of cultures of *C. jejuni* grown *in vitro* decreases cell yield (Velayudhan *et al*., 2004). As a result, induction in the chicken's gut of antibodies directed against proteins involved in amino acid uptake would be expected to reduce the pathogen's viability and capacity to colonize the intestinal tract. It has recently been shown that *C. jejuni* colonization of the chicken caecum is reduced in birds immunized with an avirulent strain of *Salmonella* expressing plasmid-borne *cjaA* heterologously (Wyszynska *et al*., 2004).

The signal peptide of Cj0982 is a potential substrate for both signal peptidases I and II suggesting the possibility of alternative post-translational processing giving rise to either a periplasmic or an outer membrane-bound location for the secreted protein (Pawelec *et al*., 1997). Its first 26 residues have the basic-hydrophobic-polar pattern of residues recognized by signal peptidase I. Cleavage at this site would give rise to a typical ATP binding cassette (ABC) periplasmic solute-binding protein. However it also contains the motif ^{17}LAAC^{20} recognized by signal peptidase II where cleavage would be accompanied by fatty acid acylation of the amino terminal cysteine and anchoring to the outer membrane (Pei and Blaser, 1993).

In the course of a structural genomics project (Structural Proteomics IN Europe), to elucidate structures of proteins from *C. jejuni*, extracytoplasmic solute receptors (ESRs), otherwise known as periplasmic binding proteins, of ABC transporters have been selected as a set of targets. While their solute-binding function can be straightforwardly assigned from sequence, their specificity cannot. This is attributed to the existence of paralogues with different substrate specificity as well as functional redundancy. These proteins usually copurify with their substrates, so that it should, in principle, be possible to assign specificity to transporter systems through X-ray analysis of crystals of their receptor proteins. The crystal structure determined here reveals (i) that Cj0982 has a classical extracytoplasmic solute receptor fold and (ii) that the ligand-binding pocket contains unambiguous density for a cysteine ligand. The implication that Cj0982 functions as a selective L-cysteine-binding protein has been confirmed using tyrosine-fluorescence spectroscopy. This is the first report of an ABC-type cysteine uptake system. These are rare in bacteria, in part because this amino acid is readily oxidized to the disulphide-linked cystine and taken up as this cross-linked species.

Results

Expression and purification

For expression, the sequence coding for Cj0982 was inserted into pET22b such that the coding sequence for its putative leader peptide was replaced by that of the *pelB* gene, and a sequence encoding hexa-histidine was fused in-frame at the 3′ end. pETCj0982 thus directs expression of a 292 residue preprotein consisting of 22 residues of the PelB leader peptide followed by the sequence MAS, residues 2–260 of Cj0982 and the C-terminal sequence LEHHHHHH. It was anticipated that the recombinant protein would be secreted to the periplasm as a 270 residue mature form.

Analysis of the recombinant protein liberated by osmotic shock treatment and fractionation on a Ni-NTA column by electrospray ionization (ESI) mass spectrometry revealed the presence of two major species with masses of 32 402 and 30 833 Da. The larger species would appear to be the 292-residue recombinant preprotein (calculated M_r = 32 403). The 30 833 species has a mass larger than expected for the mature recombinant in which the 22-residue PelB leader sequence has been removed, and rather suggests that seven residues from this leader sequence have been retained. The purified processed protein thus consists of residues 2–260 of Cj0982 with a 10-residue N-terminal fusion (AAQPAMAMAS) and an eight-residue C-terminal fusion (LEHHHHHH) giving a calculated mass of 30 833 Da.

Cj0982 has a two-domain α/β structure

The crystal structure of Cj0982 was solved to 2.0 Å resolution by molecular replacement using the co-ordinate set for *Escherichia coli* glutamine-binding protein (*Ec*GlnBP; PDB code 1wdn) as the search model. The refined model (Table 1) consists of residues 10–257 of molecule A, residues 7–257 of molecule B, a bound cysteine in each molecule, 324 waters and one glycerol molecule. Following least squares superposition of Cα atoms of residues 10–257 of chains A and B of Cj0982, the rmsΔ in Cα positions is 0.31 Å.

The Cj0982 polypeptide forms nine α-helices and 15 β-strands, assembled as two domains, each with a central five-stranded β-pleated sheet against which the α-helices are packed. The two domains are connected by two segments of the polypeptide (Fig. 1). In domain I, which consists of residues 7–108 and 203–257, the strand order is β5–β1–β6–β15–β7 with strand β15 running in an antiparallel direction to the other four strands. The topology of the β-sheet in domain II, which comprises residues 109–202, is similar with a strand order β11–β10–β12–β9–β13 with strand β9 antiparallel to the other four strands. The chain topology is characteristic of Type II ESRs (Fukami-Kobayashi *et al*., 1999). There are two additional β-sheets. One comprises residues 29–44 composing a three-stranded antiparallel β-sheet β2–β3–β4, which gives rise to the prominent protrusion on the surface of the molecule (Fig. 1A). The other is composed of the antiparallel pair of

Table 1. Data collection and refinement statistics.

Summary of data collection and refinement statistics[a]	
Beamline	ESRF 14-1
Wavelength (Å)	0.93400
Space group	*C*2
Cell dimensions	*a* = 93.9 Å, *b* = 88.8 Å, *c* = 72.1 Å, β= 107.8°
Resolution range (Å)	20.0–2.0
Completeness (%)	78.8
Number of unique reflections	30 084 (1158)
Redundancy	2.0 (1.9)
R_{merge}[b]	0.07 (0.53)
$I/\sigma(I)$	14.7 (1.9)
Refinement	
R_{factor}	18.0 (24.5)
R_{free}[c]	23.0 (32.2)
RmsΔ bonds (target value 0.022)	0.011 Å
RmsΔ angles (target value 1.966)	1.317°
Average isotropic B value (Å^2)	
Main chain	35.6
Side chain	36.5
Ligand	31.0
Water	39.4
Ramachandran plot (%)	
Allowed region	90.9
Additionally allowed region	8.4
Generously allowed region	0.7
Disallowed region	0.0

a. Numbers in parentheses refer to the highest resolution shell (2.03–2.00 Å).
b. $R_{merge} = \Sigma(|I - \langle I \rangle|)/\Sigma\langle I \rangle$, where I is the measured intensity of each reflection and $\langle I \rangle$ is the intensity averaged from multiple observations of symmetry-related reflections.
c. R_{free} is the R_{factor} calculated with 4.9% of the reflections chosen at random and omitted from refinement.

segments (β8 and β14) as the polypeptide passes from one domain to the other.

The ligand-binding pocket contains L-cysteine

Periplasmic solute-binding proteins frequently copurify with their ligands, a consequence of their moderate-to-high ligand affinity (K_d values in the range 0.1–10 μM) and their slow off-rates. In addition, the proteins are generally maintained at high concentrations during purification (Miller *et al*., 1983). The ligand-binding pocket normally resides between the two domains, with ligand binding being accompanied by domain closure according to a mechanism often likened to a Venus-fly trap (Newcomer *et al*., 1981). Examination of interdomain regions of the two Cj0982 molecules in the asymmetric unit of the crystal revealed the presence of significant electron density that could not be accounted for by the protein itself. These features appeared to define an amino acid in each ligand-binding pocket. Based on the shape and size of the side chain density, attempts were made initially to refine this peak as a serine. It soon became clear that a serine side chain could not account for the height of the observed electron density peak suggesting the presence of an atom with higher atomic number. As a result the side chain oxygen was replaced with sulphur and cysteine ligands were introduced into molecules A and B. Cysteine accounted for the electron density in the side chain pocket satisfying in addition geometric and X-ray criteria (Fig. 2A and B).

The observation of cysteine bound to Cj0982 accounts for the presence of a pair of peaks in the ESI-mass spectrum of the purified protein. The preprotein and the mature protein peaks are accompanied by satellite peaks with masses 125 and 122 Da above that of the main peak respectively. These peaks can be accounted for by the presence of a bound cysteine ligand (M_r = 125).

Cj0982 – cysteine interactions

As shown in Fig. 2C and Table 2, the cysteine ligand makes an extensive set of interactions with the surrounding protein including 12 hydrogen bonding/electrostatic interactions. The mode of binding is very similar for the two crystallographically independent molecules in the asymmetric unit. The interactions balance the charge of the ligand and fulfil its hydrogen bonding potential. The α-NH_3^+ group forms an ion pair with the carboxylate of Asp^{177} and further polar interactions with the side chain hydroxyl of Thr^{91} and the main chain carbonyl of Asn^{91}. The α-CO_2^- group on the cysteine ligand forms a pair of bidentate ionic interactions with the side chains of Arg^{74} and Arg^{96} with each of the carboxylate oxygens forming an additional interaction with the main chain amide >N-H groups of Thr^{91} and Thr^{139} respectively.

The side chain sulphur is in close proximity to the charged side chains of Lys^{30} and Arg^{74} (mean S···Nζ and S···NH1 distances of 3.3 Å and 3.4 Å respectively). There is a nearby water molecule (mean S···O distance 3.1 Å) that is itself hydrogen-bonded to the side chains of His^{176}, Asn^{159} and Asn^{135} (Table 2). The presence of two positively charged groups so close to the ligand's sulphydryl moiety

Table 2. Polar interactions between Cj0982 and bound cysteine.

		Distance (Å)	
Ligand group	Protein group	Mol A	Mol B
α-NH_3^+	Thr^{91} Oγ	2.8	2.8
α-NH_3^+	Asp^{177} OD2	2.8	2.7
α-NH_3^+	Asn^{89} O	2.8	2.9
α-CO_2^- OXT	Arg^{96} NH1	2.7	2.8
α-CO_2^- OXT	Thr^{91} >NH	2.8	3.0
α-CO_2^- OXT	Arg^{74} NH1	3.3	3.1
α-CO_2^- O	Arg^{74} NH2	3.0	3.0
α-CO_2^- O	Arg^{96} NH2	3.1	2.9
α-CO_2^- O	Thr^{139} >NH	2.9	3.0
-SH	Lys^{30} Nζ	3.1	3.4
-SH	Arg^{74} NH1	3.5	3.3
-SH	H_2O	3.2	3.0

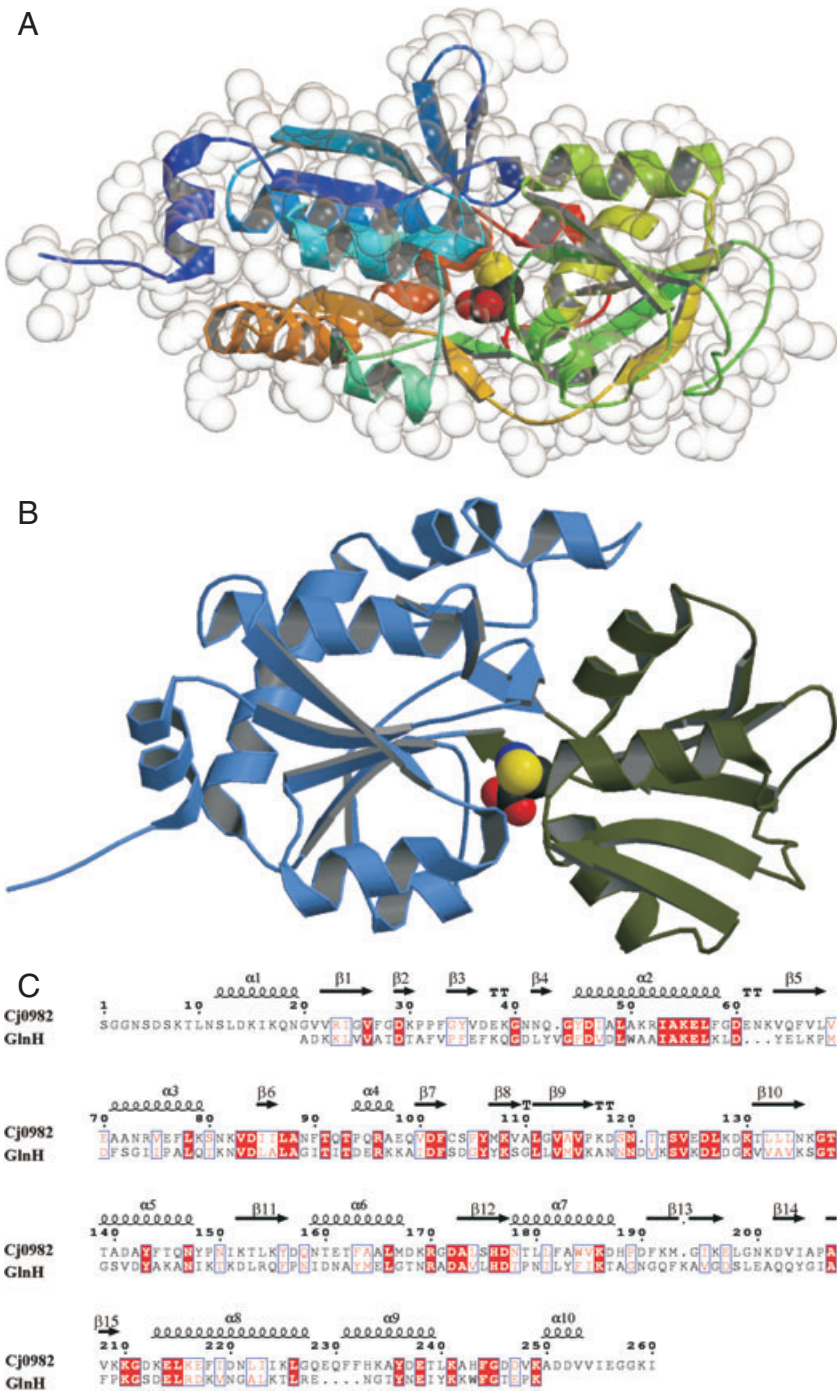

Fig. 1. A and B. Ribbon diagrams showing the course of the polypeptide backbone in Cj0982. In A the chain is colour-ramped from its amino terminus (blue) to its carboxyl terminus (red). In B the chain is coloured by domain and the cysteine ligand is shown in space-filling format. The structure figures were generated by Molscript (Kraulis, 1991) and rendered with Raster3D (Merritt and Bacon, 1997).
C. Alignment of the sequence of Cj0982 with that of glutamine-binding protein from *E. coli* . The secondary structure elements in Cj0982 are indicated above the alignment. Identical residues are highlighted with a red background while conserved residues are boxed.

suggests the possibility that the bound cysteine side chain may be ionized. Cysteine is bound as the thiolate form in another high-affinity cysteine-binding protein, cysteinyl-tRNA synthetase though in this system the thiolate is co-ordinated to a zinc ion (Newberry *et al.*, 2002). In serine acetyl transferase to which cysteine binds as a feedback inhibitor of its own biosynthesis, the sulphydryl of the cysteine ligand is stabilized by hydrogen bonding to a pair of imidazole groups and a main chain amide >N-H from the protein (Olsen *et al.*, 2004).

Cj0982 binds cysteine with high affinity

Ligand binding to Cj0982 was investigated by measuring changes in the intrinsic protein fluorescence upon addition of potential ligands. The protein contains a single tryp-

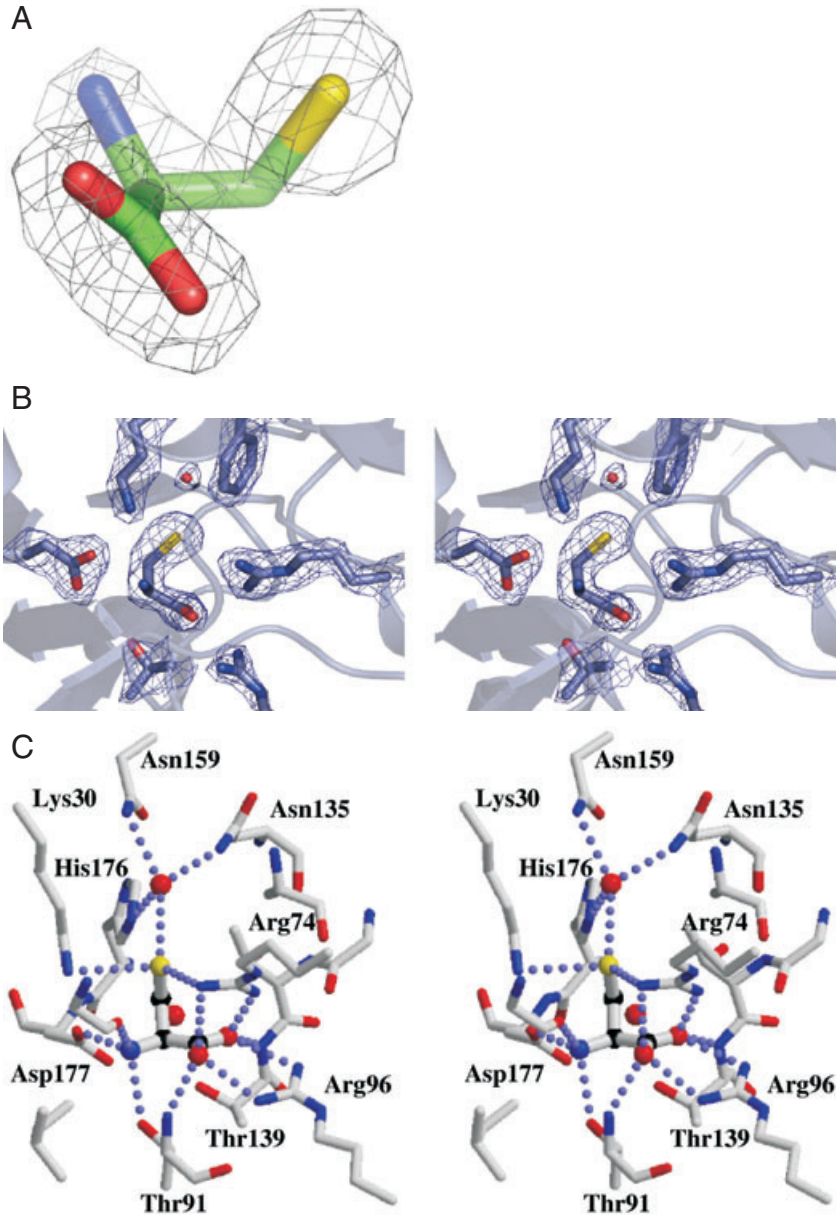

Fig. 2. A. $2F_o$-F_c electron density map displayed on the refined cysteine co-ordinates. The map was calculated using model phases before the cysteine was introduced.
B. Stereo view of the cysteine ligand (centre) and surrounding residues. The electron density is displayed at a contour of 1.5 σ. Residues shown are (clockwise from top left): Lys^{30}, Phe^{27}, Arg^{74}, Arg^{96}, Thr^{91} and Asp^{177}. This figure was drawn using PyMol (DeLano, 2002).
C. Stereo figure showing the bound cysteine (centre) and surrounding protein residues, which are labelled. Atoms are coloured according to element: carbon, grey; nitrogen, blue; oxygen, red; sulphur, yellow. Polar protein–ligand interactions are indicated by dashed lines.

tophan, which does not yield a strong signal when excited at 295 nm and hence the combined tyrosine and tryptophan fluorescence changes were measured by exciting at 280 nm. Under these conditions the protein has a maximal emission at 308 nm (Fig. 3A).

L-cysteine caused a 4% quench in the fluorescence of the protein (Fig. 3A). Initially, the oxidation of unbound L-cysteine to cystine, and subsequent dissociation of L-cysteine from the protein caused complications with the assay and hence for ligand titration experiments, 100 μM DTT was added to keep the sample reduced. L-cysteine bound to Cj0982 with a K_d of 0.14 ± 0.04 μM (Fig. 3B).

The addition of serine resulted in a larger fluorescence quench of around 6% and an excess of bound serine (200 μM) could be displaced by the addition of a small amount of L-cysteine (10 μM in Fig. 3C) indicating that L-cysteine binds with higher affinity. Subsequent titration of serine binding revealed a K_d of 32.2 ± 2.4 μM, over 200-fold lower affinity than for L-cysteine binding (Fig. 3D). We could not detect binding of any other related ligands for Cj0982, including alanine, glutamine, threonine or homocysteine. *S*-methyl-L-methionine was also investigated as a potential ligand for Cj0982 based on the observation of a m + 164 mass unit peak in the mass spectrum, but we did not observe any evidence of ligand binding.

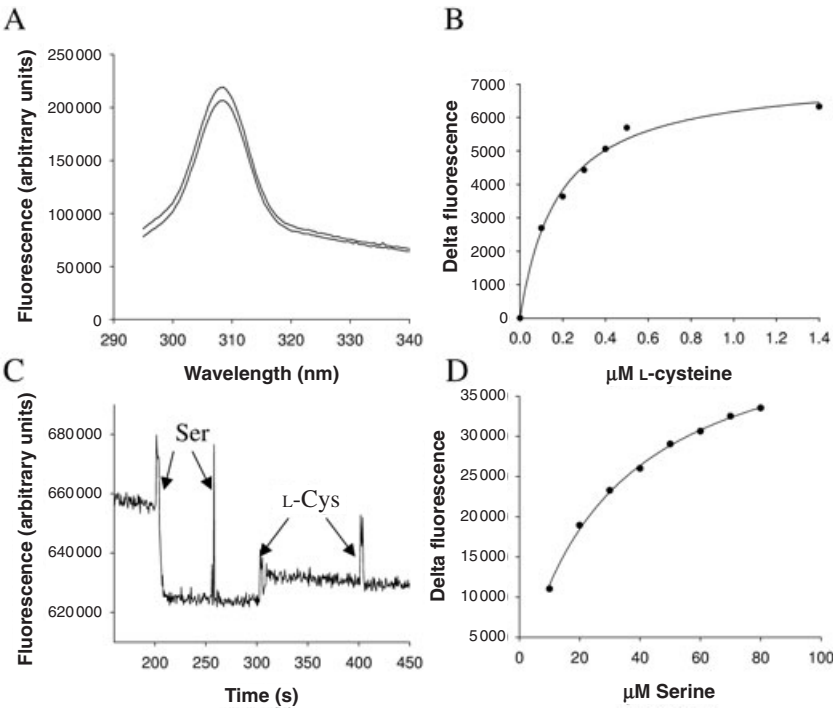

Fig. 3. Steady-state fluorescence analysis of ligand binding to Cj0982.
A. Fluorescence emission spectrum of 0.2 μM Cj0982 excited at 280 nm in the absence (upper spectrum) or presence (lower spectrum) of 100 μM L-cysteine.
B. Titration of 0.2 μM Cj0982 with L-cysteine. A representative set of data is displayed with a fit to a hyperbolic binding equation.
C. Displacement of L-serine with L-cysteine. Two additions of 100 μM L-serine to 0.2 μM Cj0982 were followed by two additions of 10 μM L-cysteine. The signal peaks are artefacts attributed to external light during sample addition.
D. Titration of 0.2 μM Cj0982 with L-serine. A representative set of data is displayed with a fit to a hyperbolic binding equation.

Discussion

Function from structure

The crystal structures of a large number of ESRs solved in the presence and absence of ligands have provided detailed insights into the mechanisms of ligand binding and the determinants of specificity (Wilkinson and Verschueren, 2003). These studies have shown that specificity is determined by a small number of residues contributed principally by loops that line the interdomain cavity where the bound solute resides. As a result it is difficult to predict specificity with precision from sequence alone. As solute-binding proteins invariably copurify with their ligands, and because they are generally 'crystallizable', crystal structure determination is an efficient route to define specificity more precisely. In one recent example, Tp32, a 32 kDa 'amino acid' binding protein from *Treponema pallidum* was shown by X-ray crystallography to be a methionine-binding protein (Deka *et al*., 2004) while a peptide-binding protein, AppA, from *B. subtilis* was shown by similar methods to contain a nonapeptide, a much larger ligand than expected (Levdikov *et al*., 2005). The finding of cysteine in the binding pocket of Cj0982 extends this series and implies that Cj0982 is part of a cysteine transporter.

Amino acid binding in related proteins

The observation of cysteine-bound Cj0982 brings to seven the number of periplasmic solute binding proteins solved in complex with a cognate amino acid ligand. The other structures reveal amino acid binding to Lysine-Arginine-Ornithine-binding protein (Oh *et al*., 1994a), histidine-binding protein (Oh *et al*., 1994b; Yao *et al*., 1994), glutamine-binding protein (Sun *et al*., 1998), glutamate/glutamine-binding protein (Takahashi *et al*., 2004), leucine-binding protein (Magnusson *et al*., 2004) and methionine bound to Tp32 (Deka *et al*., 2004). Cj0982 and glutamine-binding protein from *E. coli* (*Ec*GlnBP) have closely similar structures. Following alignment and superposition, 148 pairs of equivalent Cα atoms in Cj0982 and *Ec*GlnBP have root mean squared positional deviations of 1.15 Å (Fig. 4A). Based on this superposition, the ligands occupy a similar position within the proteins, yet the detailed modes of cysteine binding to Cj0982 and glutamine binding to *Ec*GlnBP are clearly different as shown in Fig. 4B and C. When the structures are superposed on the protein Cα atoms (Fig. 4B), it can be seen that the glutamine and cysteine ligand side chains explore their surroundings in quite different ways. The ligands' Cβ atoms are displaced by 2.0 Å from each other as a result of small shifts in the position and orientation of the main chain atoms of the ligand. When the structures are superimposed on the ligands' main chain atoms, it can be seen that there is a 120° difference in the χ1 angles of the side chain (Fig. 4C). Thus, even though the Cj0982 and GlnBP structures superpose closely and the ligand-binding pockets are similarly constructed, from the ligands' point of view these pockets are very different. This is illustrated in Fig. 4C for His^{176} of Cj0982 and His^{156} in *Ec*GlnBP. The

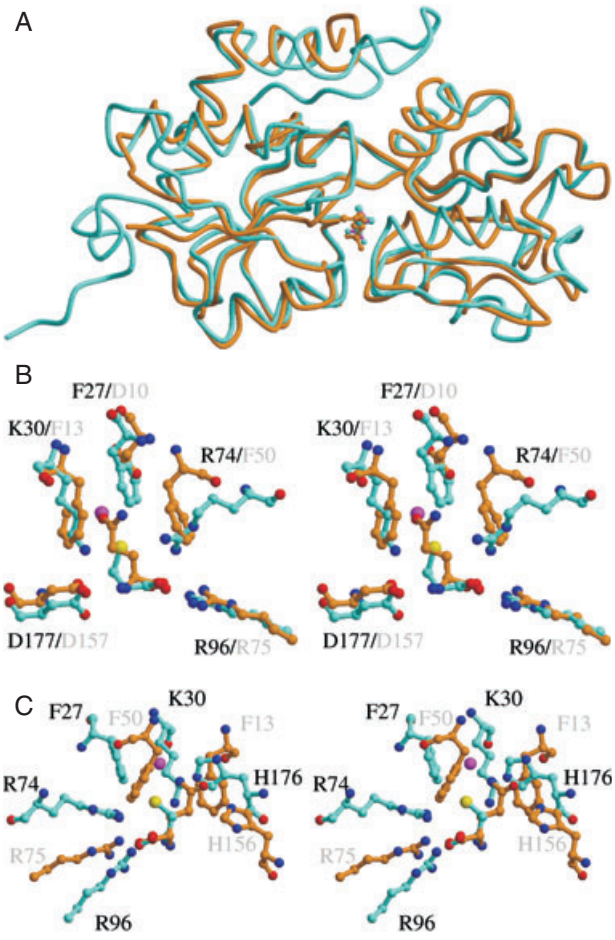

Fig. 4. A. Superposition of *Ec*GlnBP (orange) and Cj0982 (cyan).
B. Comparison of the ligand-binding pocket based on an overlay of proteins' Cα atoms.
C. Comparison of the ligand-binding pocket based on an overlay of the ligands main chain atoms.

equivalence of these residues in the sequence alignment in Fig. 1C is not matched by an equivalent juxtaposition with respect to the bound amino acid in Fig. 4C. Figure 4C also illustrates the distinct orientations of residues Arg^{74} and Arg^{96} in Cj0982 and Arg^{75} in *Ec*GlnBP with respect to the ligands' carboxylate.

Cysteine transport in bacteria

The structure of Cj0982 suggests a function as an extracellular receptor for an ABC-type cysteine uptake system. Extracellular solute-binding protein-dependent amino acid uptake systems are common in bacteria and a number of ABC transporters with specificities for single amino acids or groups of structurally related amino acids have been characterized (Hosie *et al*., 2001; Hosie and Poole, 2001). In *E. coli* and *Salmonella typhimurium*, for example, these include systems for His, Gln, Arg, Glu, Lys-Arg-Orn, Glu-Asp, Leu-Ile-Val-Thr-Ala-Ser and glycine betaine-proline (Hosie *et al*., 2001; Hosie and Poole, 2001). So far as we are aware, there have been no reports of ABC-type cysteine uptake systems in bacteria. Indeed, cysteine uptake in bacteria is very poorly characterized, though the fact that *E. coli* can utilize cysteine as a sole source of sulphur points to the existence of systems for the uptake of this amino acid. In contrast, such systems have been well characterized in eukaryotes, including yeast (During-Olsen *et al*., 1999).

Two efflux systems, YfiK and YdeD, which export *O*-acetylserine and cysteine, respectively, have been described in *E. coli*, together with a third, CydDC, an ABC-type system that exports cysteine. CydDC mutation is associated with defects in the assembly of periplasmic cytochromes and increased sensitivity to high temperature and hydrogen peroxide (Delaney and Georgopoulos, 1992; Pittman *et al*., 2002). The efflux systems, in contrast to the uptake systems, are binding protein independent.

If, as we propose, Cj0982 is involved in cysteine uptake, it is likely to have an important role in sulphur metabolism in *C. jejuni*. Many bacteria can import sulphur in its most abundant form, sulphate, which they then reduce in a series of energetically expensive steps to sulphide prior to its incorporation into cysteine. Cysteine is then used in protein synthesis, converted to glutathione, or its sulphur is donated to methionine and other essential sulphur-containing factors such as biotin, thiamine and lipoic acid (Verschueren and Wilkinson, 2005). The pathway for cysteine biosynthesis from sulphate appears to be incomplete in *C. jejuni*; there is no obvious orthologue of sulphate-binding protein nor are there putative genes encoding PAPS reductase or sulphite reductase, although a putative ATP sulphurylase and a putative APS kinase are present (CampyDB; http://campy.bham.ac.uk/).

Sulphur may also be taken up in more reduced forms such as sulphonates and sulphate esters (Kertesz, 2001). There also exist systems for the uptake of methionine and cystine, the disulphide bonded pairing of two cysteines, with *E. coli*, *S. typhimurium* and *B. subtilis* possessing multiple systems for the uptake of the latter (Burguiere *et al*., 2004). It is not clear in what other forms sulphur is taken up in *C. jejuni*, though it has been observed that omission of methionine from cultures of *C. jejuni* grown *in vitro* decreases cell yield (Velayudhan *et al*., 2004).

Putative cysteine-binding proteins are found in related pathogens

The discovery that *C. jejuni* possesses a cysteine-binding protein led us to search for putative cysteine-binding proteins in other organisms. A database search using the

Cj0982 sequence as a query produced a main group of hits with TblastN Expect values of $<10^{-50}$. A set of representative hits is presented in Fig. 5. These proteins are derived from both Gram-positive and Gram-negative bacteria but they are not genera-specific. For example, *Neisseria gonorrhoeae* and *N. lactamica* appear but *N. meningitidis* does not, similarly *Streptococcus pneumoniae* and *S. gordonii* are represented but *S. pyogenes* is not. All of the organisms represented are anaerobes, facultative anaerobes or capnophiles. The presence of genes with such high sequence similarity to Cj0982 suggests that these organisms have cysteine uptake systems.

The sequences fall into three clear phylogenetic clusters. Cluster 1 includes a number of microaerophilic Gram-negative human pathogens (*Campylobacter* and *Helicobacter* species), the CO_2-loving *N. gonorrhoeae*, a ruminant gut bacteria (*Mannheinia succiniciproducens*) and a facultatively anaerobic Gram-positive swine pathogen (*Actinobacillus pleuropneumoniae*). Cluster 2 includes a set of lactic acid bacteria as well as an aerotolerant anaerobe (*S. pneumoniae*) and a bacterium associated with periodontitis and invasive human infections (*Fusobacterium nucleatum*). Cluster 3 includes a spore-former associated with food poisoning (*B. cereus*), a pathogen in frogs, fish and mammals (*Aeromonas hydrophila*) and a plant pathogen (*Pseudomonas syringae*).

The set of hits achieved with Cj0982 is quite distinct from that produced when *Ec*GlnBP is used as the query. Each does pick out the other's set of hits but with significantly lower *e*-values in the range 10^{-20}–10^{-30}. Thus the residues involved in binding the amino and carboxylate group, Arg^{96} and Asp^{177} in Cj0982 are fully conserved in both sets. Exclusive to the Cj0982 set are the invariant residues Arg^{74}, which forms an interaction with the thiol group, and Phe^{27}, which appears to exclude larger ligands. Figure 5 also highlights the invariant GTTA motif at residues 137–140 in the Cj0982 set. The main chain >NH of Thr^{139} forms a polar contact with the ligand's carboxylate while the side chain of Thr^{138} lines the ligand's side chain pocket. In Cj0982, a nearby salt bridge between Asp^{141} and Lys^{155} appears to be conserved. These residues are flanked by two other conserved polar residues in Cj0982, Asn^{135} and Asn^{159}, which form hydrogen bonds to the water molecule in the ligand-binding pocket. The only polar residue in contact with the cysteine and its associated water molecule that is not conserved across the sequences presented in Fig. 5 is Lys^{30}. In *Leuconostoc lactis* and *Lactobacillus johnsonii*, this residue is a leucine.

Identification of the membrane components of the cysteine transporter

ATP binding cassette transporters comprise a pair of integral membrane proteins that form a channel through which the substrate passes and a pair of ATPase subunits peripherally associated with the cytoplasmic face of the membrane, which couple ATP hydrolysis to solute translocation (Holland *et al*., 2003). ESRs, such as Cj0982, constitute a fifth component present in solute uptake systems (and occasionally in efflux systems) that defines the specificity of the transporter, capturing extracytoplasmic substrates and delivering them to the membrane components for transport (Hosie *et al*., 2001; Wilkinson and Verschueren, 2003). The three to five genes (depending on whether the ATPase and channel components are homo- or hetero-dimeric) encoding the ABC transporter are usually found in a single operon. The *cj0982c* gene is not genetically linked to genes encoding other ABC transporter components. This is not so surprising as breakdown of operons into smaller transcriptional units is common in the *C. jejuni* genome (Parkhill *et al*., 2000). Interestingly however, the orthologue of Cj0982 in *H. pylori*, HP1172, is part of an operon, also containing HP1169, HP1170 and HP1171, encoding a complete ABC transporter (Fig. 6). The orthologues of the latter three genes form an operon, Cj0467, Cj0468 and Cj0469, in *C. jejuni* that lacks the gene for an ESR. This implies that Cj0982 is the receptor for a transporter made up of Cj0467, Cj0468 and Cj0469. Analysis of orthologous transporters in related bacteria reveals that the translocation of the ESR gene away from the other ABC components also occurs in the *H. hepaticus* and *H. mustelae* genomes.

Analysis of the genome context of these orthologous systems provides some speculative support for their function in L-cysteine transport. The genes for the putative transporter in *M. succiniciproducens* are contained in a potential operon with a gene for glutathione synthase (*ghsA*). This is a cytoplasmic enzyme that uses L-cysteine for the synthesis of gamma-glutamylcysteine in the production of glutathione, suggesting a possible role in coupling of cysteine transport to its utilization in glutathione biosynthesis. Furthermore, the gene directly downstream and in the same direction as the orthologous system from *Bifidobacterium longum* is *metC*, which also uses L-cysteine as a substrate in a deamidation reaction forming pyruvate. There appears also to be an orthologous transporter system encoded in the genome of *S. thermophilus*, however, the upstream gene in this bacterium encodes a putative D-alanine-D-alanine ligase, which is consistent with this being an amino acid transporter but not necessarily with L-cysteine being the ligand.

The *H. pylori* proteins HP1169, HP1170, HP1171 and HP1172 have been annotated as components of a glutamine transporter. A recent study has demonstrated that two of these genes are induced when *H. pylori* is exposed to acid (Merrell *et al*., 2003). It now appears

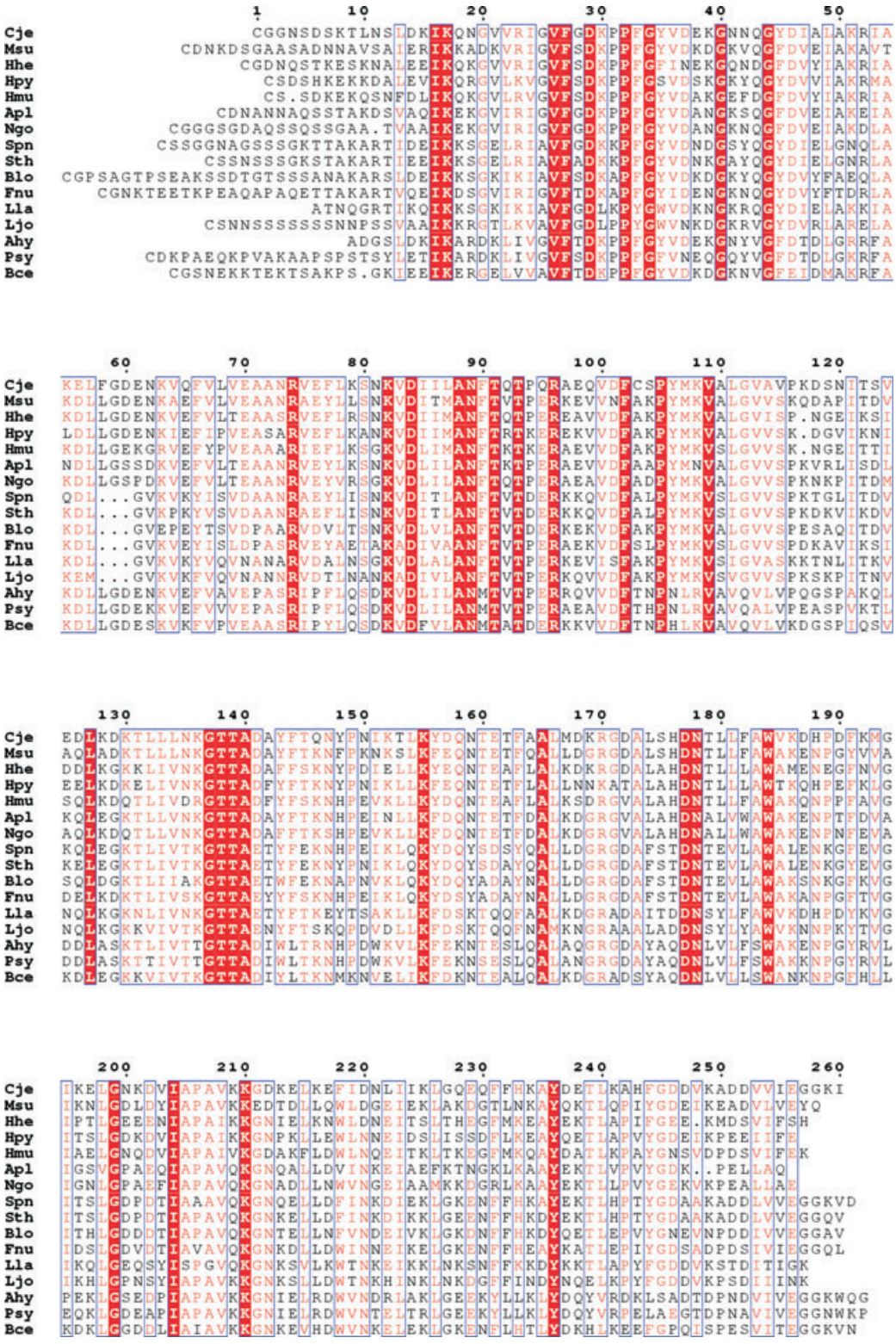

Fig. 5. Sequence alignment of putative cysteine-binding proteins. A representative set of sequences that are highly homologous to Cj0982 (top sequence) were collated from publicly available databases. The figure was drawn using ESPript (Gouet *et al*., 1999). Species codes: Hmu, *Helicobacter mustelae*; Sth, *Streptococcus thermophilus*; Fnu, *Fusobacterium nucleatum*; Ahy, *Aeromonas hydrophila*; Cje, *Campylobacter jejuni*; Msu, *Mannheimia succiniciproducens*; Ngo, *Neisseria gonorrhoeae*; Spn, *Streptococcus pneumoniae*; Hhe, *Helicobacter hepaticus*; Hpy, *Helicobacter pylori*; Apl, *Actinobacillus pleuropneumoniae*; Blo, *Bifidobacterium longum*; Lla, *Leuconostoc lactis*; Ljo, *Lactobacillus johnsonii*; Psy, *Pseudomonas syringae*; Bce, *Bacillus cereus*.

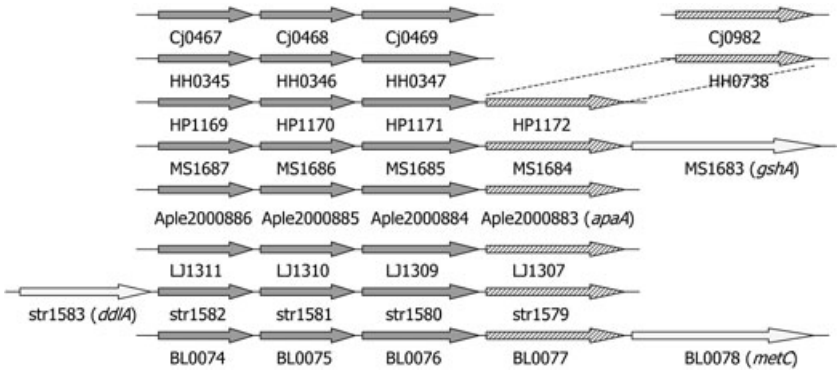

Fig. 6. Schematic representation of the organization of the genes encoding the Cj0467/0468/0469/0982 ABC transporter and its orthologues from other bacteria. The ESR proteins orthologous to Cj0982 are indicated by hatched arrows, while the membrane and ATP-binding cassette components are represented by solid arrows. The organisms are (from the top): *C. jejuni*, *H. hepaticus*, *H. pylori*, *M. succiniciproducens*, *A. pleuropneumoniae*, *Lactobacillus johnsonii*, *S. thermophilus* and *B. longum*. Interesting flanking genes are indicated for three organisms, which are described in detail in the text.

unlikely that the function of these genes during acid stress is to provide additional glutamine that would subsequently be deaminated to release ammonia in the cytoplasm (Merrell *et al*., 2003). Instead, the similarity of HP1172 and Cj0982 suggests that these components are part of a cysteine transporter (Fig. 6). An enzyme that uses L-cysteine as a substrate, *metB*, is also induced under these conditions, supporting the notion of an, as yet, undefined role for cysteine at low pH.

Antigenicity

Cj0982 is a major immunogenic protein in *C. jejuni* and a promising vaccine candidate. ApaA, a Cj0982 orthologue, is highly immunogenic in *A. pleuropneumoniae*, a bacterium that causes a highly contagious and often fatal bronchopneumonia in pigs (Martin and Mulks, 1999). Oral immunization of chickens with an avirulent *Salmonella* vaccine strain expressing plasmid-encoded Cj0982 elicits specific humoral immune responses associated with protection against challenge by wild-type *Campylobacter* (Wyszynska *et al*., 2004). The structure presented here does not identify directly the relevant antigenic surfaces of the protein, although it does provide a framework for interpreting results of future epitope-mapping experiments. An obvious candidate epitope is the antiparallel β-ribbon, which protrudes prominently from the larger lobe of the molecule (Fig. 1A). A similar protruding feature is also expected to be present in CjaC (Cj0734), a putative histidine-binding protein, which is also immunogenic and cross-reacts with antiserum raised against Cj0982.

If the protruding β-hairpin is a key determinant of immune recognition, it may contribute to the immunodominance of Cj0982. In the related histidine-binding protein (HisJ) from *S. typhymurium*, the corresponding protruding antiparallel β-ribbon is implicated in interactions with the membrane components of the histidine transporter that lead to ligand translocation to the cytoplasm. It may therefore not be possible to evade the immune system without loss of the cysteine transport function. The alternative sorting of Cj0982, giving rise to an outer membrane pool of protein, in addition to the periplasmic pool, presumably also contributes to immunogenicity. The role of Cj0982 on the outside of the cell is unknown. Interestingly, two other *C. jejuni* proteins that are expected to have ESR-like folds, Peb1 and Peb3, are crucial for adhesion and virulence (Pei *et al*., 1998).

Concluding remarks

Cj0982 is an ESR for cysteine as revealed by X-ray crystallography and fluorescence spectroscopy. This suggests that *C. jejuni* possesses an ABC transporter for cysteine. The importance of cysteine for metabolism in this species has been demonstrated (Velayudhan *et al*., 2004). Omission of single amino acids from the medium of cultures of *C. jejuni* grown *in vitro* decreases cell yield with the largest effects observed for serine followed by methionine and then cysteine. Comparative analysis of the genomes of other bacteria with Cj0982 orthologues points to Cj0467, Cj0468 and Cj0469 as being the membrane components of the cysteine transporter.

Experimental procedures

Cloning, expression and purification

The *cj0892c* (CampyDB; http://campy.bham.ac.uk/) coding sequence was amplified by polymerase chain reaction (PCR) from *C. jejuni* NCTC11168 genomic DNA using the oligonucleotide primers 5′-CCGGCGATGGCCATGGCTAGCGGAG GAAATTCTGACTCTAAAACTTTAAA-3′ and 5′-GGTGGTG GTGCTCGAGAATTTTTCCACCTTCAATCACTACAT-3′. To facilitate handling of the protein, the TGT codon for Cys^{20} was replaced by an AGC serine codon. For overproduction of recombinant Cj0982 in *E. coli*, the coding sequence for the mature form of the protein was inserted into the plasmid pET22b (Novagen) between sequences coding for (i) the PelB leader peptide, which will direct the expressed protein to the periplasm, and (ii) a C-terminal hexa-histidine tag to aid protein purification.

After linearization of the pET22b vector DNA with *Nco*I and *Xho*I, recombination with the PCR products was catalysed

using the BD-InFusion enzyme (BD Biosciences-Clontech). The recombination mix was mixed with competent *E. coli* NovaBlue cells and resultant colonies harbouring the recombinant pETCj0982 plasmid were identified among the ampicillin-resistant transformants.

For expression, the purified plasmid was introduced into *E. coli* BL21-Gold cells, which were grown with shaking in 1.6 l of Luria–Bertani medium supplemented with 50 μg ml^{-1} carbenicillin at 37°C until the OD_{600} reached 0.6. Production of recombinant protein was induced by addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.6 mM and growth was continued for a further 2.5 h. Periplasmic proteins were prepared by an osmotic shock procedure and then loaded onto a Ni-NTA column. The protein was eluted with 50 mM imidazole in 50 mM Tris-HCl pH 8.0 and 300 mM NaCl buffer. The eluate containing the purified protein was dialysed against 20 mM Tris-HCl pH 8.5 and stored at 4°C.

Crystallization and data collection

Crystallization experiments were initially carried out at the Oxford Protein Production Facility (Brown *et al*., 2003; Walter *et al*., 2003). Crystallization was then repeated using a Mosquito nanoliter dispensing robot. Crystals appeared after 2–3 weeks in sitting drops made by mixing 100 nl of Cj0982 at a concentration of 20 mg ml^{-1} in 20 mM Tris-HCl pH 8.5 and 100 nl of reservoir solution containing 0.2 M NaCl, 0.1 M MES pH 6.5 and 25% (w/v) PEG 3350.

Crystals were transferred in a series of four steps into a solution of mother liquor plus 20% glycerol. Subsequently the crystals were mounted in a rayon loop and immediately plunged into a stream of liquid nitrogen. A data set extending to 2.0 Å spacing was collected on beam line 14.1 at the ESRF, Grenoble. Data were indexed and integrated with MOSFLM and scaled with SCALA (Leslie, 1992) (Table 1). The crystals belong to space group *C*2 with unit cell dimensions $a = 95.3$ Å, $b = 88.5$ Å, $c = 72.2$ Å and $\beta = 109.0°$. With two molecules in the asymmetric unit, the Matthews coefficient is 2.4 Å^3 Da^{-1}. As a result of crystal lifetime in the beam, and only a single strongly diffracting crystal being available, we were able to collect only 79% of the unique data set. However, these data are of good quality with a mean $I/\sigma(I)$ of 14.7 and an overall R_{merge} of 7%.

Structure solution and refinement

Among the entries in the Protein Data Bank (PDB), glutamine-binding protein from *E. coli* has the closest sequence similarity (27% identity) to Cj0982 (Fig. 1C). It was therefore chosen as the search model for molecular replacement calculations performed in the program MOLREP (Vagin and Teplyakov, 1997). Co-ordinates for the open unliganded (1ggg) and closed liganded (1wdn) protein were explored with the latter yielding convincing solutions.

As expected, the MOLREP calculations showed that there are two molecules in the asymmetric unit. The correct solutions to the rotation function have R_f/σ values of 5.56 and 4.48, respectively, corresponding to the best and sixth highest solutions. The translation function for the first monomer produces a correlation coefficient of 0.137 and an R_{factor} of 0.584. For the second molecule the discrimination against wrong solutions improves the R_{factor} to 0.567 with a correlation coefficient of 0.198. This compares with values of 0.581 and 0.153, respectively, for the next best solution.

This initial molecular replacement model was rebuilt using RESOLVE (Terwilliger, 2003) and ARP (Perrakis *et al*., 1997). The electron density maps were of good quality and allowed the unambiguous introduction of the Cj0982 sequence. The structure was refined using iterative cycles of REFMAC5 (Murshudov *et al*., 1997) and Coot (Emsley and Cowtan, 2004) until the R_{factor} values converged (Table 1). The co-ordinates and structure factors have been deposited in the PDB (Code 1xt8).

Fluorescence spectroscopy

All protein fluorescence experiments used a FluoroMax 3 fluorescence spectrometer with connecting water bath at 25°C. Because of the presence of only a single tryptophan and multiple tyrosine residues, the protein was excited at 280 nm with slit widths of 5 nm. Cj0982 was used at a concentration of 0.2 μM in 10 mM Tris pH 8.0 for all fluorescence experiments. The binding of other potential amino acid ligands presented to Cj0982 at a final concentration of 100 μM was also tested. Cumulative fluorescence changes from titration of the protein with ligand were plotted in SigmaPlot and fitted to a single rectangular hyperbola. The K_d values reported were averaged from three separate ligand titration experiments.

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