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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<sup>-7</sup> 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 ABCtype 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 warmblooded 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 <sup>17</sup>LAAC<sup>20</sup> 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 22residue 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 $\alpha/\beta$ 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 C $\alpha$  atoms of residues 10–257 of chains A and B of Cj0982, the rms $\Delta$  in C $\alpha$  positions is 0.31 Å.

The Cj0982 polypeptide forms nine  $\alpha$ -helices and 15  $\beta$ strands, assembled as two domains, each with a central five-stranded  $\beta$ -pleated sheet against which the  $\alpha$ -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  $\beta 5 - \beta 1 - \beta 6 - \beta 15 - \beta 7$  with strand  $\beta 15$  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  $\beta 11 - \beta 10 - \beta 12 - \beta 9 - \beta 13$ with strand  $\beta 9$  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 threestranded antiparallel  $\beta$ -sheet  $\beta 2-\beta 3-\beta 4$ , 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 <sup>a</sup>
Beamline	ESRF 14-1
Wavelength (Å)	0.93400
Space group	C2
Cell dimensions	<i>a</i> = 93.9 Å, <i>b</i> = 88.8 Å,
	<i>c</i> = 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 <sub>merae</sub> <sup>b</sup>	0.07 (0.53)
I/σ(Ĭ)	14.7 (1.9)
Refinement	
R <sub>factor</sub>	18.0 (24.5)
R <sub>free</sub> <sup>c</sup>	23.0 (32.2)
Rms∆ bonds (target value 0.022)	0.011 Å
Rms∆ angles (target value 1.966)	1.317°
Average isotropic B value (Å <sup>2</sup> )	
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.**  $R_{\text{merge}} = \Sigma(|I - \langle I \rangle|)/\Sigma \langle I \rangle$ , 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.**  $R_{\rm free}$  is the  $R_{\rm factor}$  calculated with 4.9% of the reflections chosen at random and omitted from refinement.

segments ( $\beta 8$  and  $\beta 14$ ) 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-tohigh ligand affinity ( $K_{d}$  values in the range 0.1–10  $\mu$ M) 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 ligandbinding 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  $\alpha$ -NH<sub>3</sub>+ group forms an ion pair with the carboxylate of Asp<sup>177</sup> and further polar interactions with the side chain hydroxyl of Thr<sup>91</sup> and the main chain carbonyl of Asn<sup>91</sup>. The  $\alpha$ -CO<sub>2</sub><sup>-</sup> group on the cysteine ligand forms a pair of bidentate ionic interactions with the side chains of Arg<sup>74</sup> and Arg<sup>96</sup> with each of the carboxylate oxygens forming an additional interaction with the main chain amide >N-H groups of Thr<sup>91</sup> and Thr<sup>139</sup> respectively.

The side chain sulphur is in close proximity to the charged side chains of Lys<sup>30</sup> and Arg<sup>74</sup> (mean S<sup>...</sup>N $\zeta$  and S<sup>...</sup>NH1 distances of 3.3 Å and 3.4 Å respectively). There is a nearby water molecule (mean S<sup>...</sup>O distance 3.1 Å) that is itself hydrogen-bonded to the side chains of His<sup>176</sup>, Asn<sup>159</sup> and Asn<sup>135</sup> (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.

Ligand group		Distance (Å)		
	Protein group	Mol A	Mol B	
$\alpha$ -NH <sub>3</sub> <sup>+</sup>	Thr <sup>91</sup> Ογ	2.8	2.8	
$\alpha$ -NH <sub>3</sub> <sup>+</sup>	Asp <sup>177</sup> OD2	2.8	2.7	
$\alpha$ -NH <sub>3</sub> <sup>+</sup>	Asn <sup>89</sup> O	2.8	2.9	
α-CO <sub>2</sub> - OXT	Arg <sup>96</sup> NH1	2.7	2.8	
$\alpha$ -CO <sub>2</sub> <sup>-</sup> OXT	Thr <sup>91</sup> >NH	2.8	3.0	
$\alpha$ -CO <sub>2</sub> <sup>-</sup> OXT	Arg <sup>74</sup> NH1	3.3	3.1	
$\alpha$ -CO <sub>2</sub> <sup>-</sup> O	Arg <sup>74</sup> NH2	3.0	3.0	
$\alpha - CO_2^- O$	Arg <sup>96</sup> NH2	3.1	2.9	
$\alpha$ -CO <sub>2</sub> <sup>-</sup> O	Thr <sup>139</sup> >NH	2.9	3.0	
-SH	Lys <sup>30</sup> Nζ	3.1	3.4	
-SH	Arg <sup>74</sup> NH1	3.5	3.3	
-SH	H <sub>2</sub> O	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, cysteinyltRNA synthetase though in this system the thiolate is coordinated 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-

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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<sup>30</sup>, Phe<sup>27</sup>, Arg<sup>74</sup>, Arg<sup>96</sup>, Thr<sup>91</sup> and Asp<sup>177</sup>. 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 proteinligand 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  $\mu$ M DTT was added to keep the sample reduced. L-cysteine bound to Cj0982 with a  $K_d$  of 0.14  $\pm$  0.04  $\mu$ M (Fig. 3B).

The addition of serine resulted in a larger fluorescence quench of around 6% and an excess of bound serine (200  $\mu$ M) could be displaced by the addition of a small amount of L-cysteine (10  $\mu$ M in Fig. 3C) indicating that L-cysteine binds with higher affinity. Subsequent titration of serine binding revealed a  $K_d$  of  $32.2 \pm 2.4 \mu$ M, 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  $\mu$ M Cj0982 excited at 280 nm in the absence (upper spectrum) or presence (lower spectrum) of 100  $\mu$ M L-cysteine.

B. Titration of 0.2  $\mu$ M 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  $\mu M$  L-serine to 0.2  $\mu M$  Cj0982 were followed by two additions of 10  $\mu M$  L-cysteine. The signal peaks are artefacts attributed to external light during sample addition.

D. Titration of 0.2  $\mu$ M 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 (EcGInBP) have closely similar structures. Following alignment and superposition, 148 pairs of equivalent C $\alpha$  atoms in Cj0982 and EcGInBP 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 EcGInBP are clearly different as shown in Fig. 4B and C. When the structures are superposed on the protein  $C\alpha$  atoms (Fig. 4B), it can be seen that the glutamine and cysteine ligand side chains explore their surroundings in quite different ways. The ligands'  $C\beta$ 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  $\chi$ 1 angles of the side chain (Fig. 4C). Thus, even though the Cj0982 and GInBP 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<sup>176</sup> of Cj0982 and His<sup>156</sup> 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'  $C\alpha$  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*GInBP 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, GluAsp, 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 ABCtype 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<sub>2</sub>-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 aero-tolerant anaerobe (*S. pneumoniae*) and a bacterium associated with periodontitis and invasive human infections (*Fusobacterium nucleatum*). Cluster 3 includes a sporeformer 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 Ci0982 is guite distinct from that produced when EcGInBP 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<sup>96</sup> and Asp<sup>177</sup> in Cj0982 are fully conserved in both sets. Exclusive to the Cj0982 set are the invariant residues Arg<sup>74</sup>, which forms an interaction with the thiol group, and Phe<sup>27</sup>, 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<sup>139</sup> forms a polar contact with the ligand's carboxylate while the side chain of Thr<sup>138</sup> lines the ligand's side chain pocket. In Cj0982, a nearby salt bridge between Asp<sup>141</sup> and Lys<sup>155</sup> appears to be conserved. These residues are flanked by two other conserved polar residues in Ci0982. Asn<sup>135</sup> and Asn<sup>159</sup>, 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<sup>30</sup>. 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 homoor 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

		1	10	20	30	40	50
Cje		CGGNS	DSKTLNSLDK	IKQNGVVRIGV	FGDKPPFGY	V D E K G N N Q G	YDIALAKRIA
Hhe	CDNKI	CGDNOS	DNNAVSAIER	IKKADKVRIGV IKOKGVVRIGV	FSDKPPFGY	V D K D G K V Q G I N E K G O N D G	FDVEIAKAVT
Hpy		CSDS	HKEKKDALEV	IKQRGVLKVGV	FSDKPPFGS	VDSKGKYQG	YDVVIAKRMA
Hmu	C1	CS.S	DKEKQSNFDL	IKQKGVLRVGV	FSDKPPFGY	V D A K G E F D G	FDVYIAKRIA
Ngo	CGGGS	GDAQSSQ	SSGAA.TVAA	IKEKGVIRIGV	FGDKPPFGY	VDANGKNOG	FDVEIAKDLA
Spn	CSSGGN	AGSSSGK	TTAKARTIDE	IKKSGELRIAV	FGDKKPFGY	VDNDGSYQG	YDIELGNQLA
Blo	CGPSAGTPSEAKSSI	SNSSSGR DTGTSSS	STAKARTIEE	IKKSGELRIAV IKKSGKIKIAV	FADKKPFGY	V D N K G A Y Q G V D K D G K Y O G	YDIELGNRLA YDVYFAEOLA
Fnu	CGNKTEETKPE	AQAPAQE	TTAKARTVQE	IKDSGVIRIGV	FTDKAPFGY	IDENGKNQG	YDVYFTDRLA
Lla	C 51	MNCCCCC	ATNOGRTIKO	IKKSGKIKIAV	FGDLKPYGW	VDKNGKRQG	YDIELAKKIA
Ahy	0.01	1100000	ADGSLDK	IKARDKLIVGV	FTDKPPFGY	VDEKGNYVG	FDTDLGRRFA
Psy	CDKPAEQ	KPVAKAA	PSPSTSYLET	IKARDKLIVGV	FSDKPPFGF	VNEQGQYVG	FDTDLGKRFA
BCe	CGSNE	VVIEVIS	AKPS.GKLEE	INERGELVVAV	FIDEPPEGE		E E I DMANKE A
	60	70	80	90	100	110	120
Cje	KELFGDENKVQFVL	VEAANRV	EFIKSNKVDI	ILANFTOTPOR	AEQVDECSP	YMK <mark>V</mark> ALGVA	VPKDSNITSV
Hhe	KDLLGDENKAEFVL KDLLGDENKVEFVL	VEAANRA TEAASRU	EYLLSNKVDI	TMANE TVTPER	KEVVNFAKP EAVVDFAKP	YMKVALGVV YMKVALGVI	SKQDAPITDV SP.NGEIKSI
Hpy	LDLLGDENKIEFIP	VEASARV	EFLKANKVDI	IMANFTRTKER	EKVVDFAKP	YMKVALGVV	SK.DGVIKNI
Hmu	KDLLGEKGRVEFYP	VEAAARI	EFLKSGKVDL	IMANFTKTKER	AEVVDFAKP	YMKVSLGVV	SK.NGEITTI
Ngo	KDLLGSPDKVEFVL	TEAANRY	EYVRSGKVDL	ILANFTOTPER	AEAVDFADP	YMKVALGVV	SPKNKPITDM
Spn	QDLGVKVKYIS	VDAANRA	EYLISNKVDI	TLANFTVTDER	KKQVDFALP	YMK <mark>V</mark> SLGVV	SPKTGLITDV
Blo	KDLGVEPEYTS	VDAANRA	DVLTSNKVDL	VLANETVTDER	KEKVDFALP	YMKVALGVV	SPESAOITDV
Fnu	KDLGVKVEYIS	LDPASRV	EYAETAKADI	VAANFTVTPER	AEKVDFSLP	YMK <mark>V</mark> SLGVV	SPDKAVIKSI
Lla	KDLGVKVKYVQ KEM GVKVKFVQ	VNANARV	DALNSGKVDL	ALANFTVTPER	KEVISFAKP	YMK <mark>V</mark> SIGVA	SKKTNLITKV
Ahy	KDLLGDENKVEFVA	VEPASRI	PFLQSDKVDL	ILANMTVTPER	RQVVDFTNP	NLRVAVQVL	VPQGSPAKQL
Psy	KDLLGDEKKVEFVV	VEPASRI	PFLQSDKVDL	I LANMTVTPER	AEAVDFTHP	NLRVAVQAL	VPEASPVKTL
BCe	KDTTPOPPSKAVL AL	VEAAS	FILVENAVOL	VEANETATUER	KKVVUEINE	HPKAVAAA	VKDGSPUQSV
89205	130 1	4 0	150	160	170	180	190
Cje	130 1 EDUKDKTLLLNKGT	4 0 TADAYFI	150 QNYPNIKTLK	160 YDONTETFAAL	170 MDKRGDALS	180 H <mark>DN</mark> TLLFAW	190 VKDHPDFKMG
Cje Msu Hhe	130 1 EDIKDKTLLLNKGT AQLADKTLLLNKGT DDIKGKKLIVNKGT	4 0 TADAYFT TADAYFT TADAYFT	150 QNYPNIKTLK KNFPKNKSLK KNYPDIELLK	160 YDONTETFAAL FEONTETFOAL YEONTEAFLAL	170 MDKRGDALS LDGRGDALS KDKRGDALA	180 HDNTLLFAW HDNTLLFAW HDNTLLFAW	190 VKDHPDFKMG AKENPGYVVA AMENEGFNVG
Cje Msu Hhe Hpy	130 1 EDIKDKTLLLNKGT AQIADKTLLLNKGT DDIKGKKLIVNKGT EIKDKELIVNKGT	4 0 TADAYFT TADAYFT TADAFFS TADFYFS	150 QNYPNIKTIK KNFPKNKSIK KNYPDIELIK KNYPNIKLIK	160 YD ONTETFAAL FEONTETFOAL YE ONTEAFLAL FEONTETFLAL	170 MDKRGDALS LDGRGDALS KDKRGDALA LNNKATALA	180 HDNTLLFAW HDNTLLFAW HDNTLLLAW HDNTLLLAW	190 VKDHPDFKMG AKENPGYVVA AMENEGFNVG TKOHPEFKLG
Cje Msu Hhe Hpy Hmu Apl	130 1 EDIKDKTLLLNKGT AQIADKTLLLNKGT DDIKGKKLIVNKGT EEIKDKELIVNKGT SQIKDQTLIVDRGT KQEGKTLLVNKGT	4 0 TADAYFT TADAYFT TADAFFS TADFYFT TADFYFS TADFYFS	150 QNYPNIKTIK KNFPKNKSIK KNYPDIELIK KNYPNIKLIK KNHPEVKLIK KNHPEINLIK	160 YD QNTETFAAL FE QNTETFQAL YE QNTEAFLAL FE QNTETFLAL FD QNTETFDAL FD QNTETFDA	17 Q MDKRGDALS LDGRGDALS KDKRGDALA LNNKATALA KSDRGVALA KDGRGVALA	180 HDNTLLFAW HDNTLLFAW HDNTLLLAW HDNTLLLAW HDNTLLFAW HDNALVWAW	190 VKDHPDFKMG AKENPGYVVA AMENEGFNVG TKQHPEFKLG AKQNPFAVG AKENPTFDVG
Cje Msu Hhe Hpy Hmu Apl Ngo	130 1 EDIKDKTLLINKGT AQIADKTLLINKGT DDIKGKKLIVNKGT EEIKDKELIVNKGT SQIKDQTLIVDRGT KQEGKTLIVNKGT AQIKDQTLIVNKGT	4 Q TAD AYFT TAD AYFT TAD AFFS TAD FYFS TAD FYFS TAD AYFT TAD AFFT	150 QNYPNIKTIK KNFPKNKSIK KNYPDIELIK KNYPNIKLIK KNHPEVKLIK KNHPEVKLIK	160 YD ONTETFAAL FE ONTETFOAL YE ONTETFLAL FE ONTETFLAL FD ONTETFDAL FD ONTETFDAL	170 MDKRGDALS LDGRGDALS KDKRGDALA LNNKATALA KSDRGVALA KDGRGVALA KDGRGVALA	180 HDNTLIFAW HDNTLIFAW HDNTLILAW HDNTLIFAW HDNALVWAW HDNALVWAW	190 VKDHPDFKMG AKENPGYVVA AMENEGFNVG TKQHPEFKLG AKQNPPFAVG AKENPTFDVA AKENPNFEVA
Cje Msu Hhe Hpy Hmu Apl Ngo Spn Sth	130 1 EDIKDKTLLINKGT AQIADKTLLINKGT DDIKGKKLIVNKGT EDIKDQTLIVDRGT KQLEGKTLLVNKGT AQIKDQTLLVNKGT KQLEGKTLIVTKGT	4 0 TADAYFI TADAYFI TADFYFI TADFYFI TADFYFI TADAYFI TADAYFI TAETYFE	150 QNYPNIKTIK KNFPKNKSIK KNYPDIELIK KNYPNIKLIK KNHPEINLIK KNHPEIKLQK KNHPEIKLQK	160 YD QNTETFAAL FE QNTETFQAL YE QNTEAFLAL FE QNTETFLAL FD QNTETFDAL FD QNTETFDAL YD QYSDSYQAL YD QYSDAYQAL	17 Q MDKRCDALS LDGRGDALS KDKRCDALA KSDRCVALA KSDRCVALA KDGRGVALA LDGRGDAFS	180 HDNTLLFAW HDNTLLFAW HDNTLLFAW HDNTLLFAW HDNALVWAW HDNALWAW TDNTEVLAW	190 VKDHPDFKMG AKENPGYVVA AMENEGFNVG TKQHPEFKLG AKENPTFDVA AKENPNFEVA ALENKGYEVG ALENKGYEVG
Cje Msu Hhe Hpy Hmu Apl Ngo Spn Sth Blo	130 1 EDIKDKTLLINKGT AQLADKTLLINKGT DDIKGKLIVNKGT EELKDKELVNKGT KQLEGKTLIVNKGT KQLEGKTLIVNKGT KQLEGKTLIVTKGT SQLDGKTLIIAKGT	4 0 TADAYFI TADAYFI TADFYS TADFYFS TADAYFI TADAYFI TADTYFE TAETYFE TAETYFE	150 QNYPNIKTIK KNFPKNKSIK KNYPDIELIK KNYPNIKLIK KNHPEVKLIK KNHPEVKLIK KNHPEVKLIK KNHPEILOK KNYPNIKLOK	160 YD QNTETFAAL FE QNTETFQAL YE QNTETFLAL FE QNTETFLAL FD QNTETFDAL FD QNTETFDAL YD QYSDAYQAL YD QYSDAYQAL YD QYSDAYQAL	17 Q MDKRCDALS LDGRGDALS KDKRCDALA KSDRGVALA KSDRGVALA KDGRGVALA LDGRGDAFS LDGRGDAFS	180 HDNTILIFAW HDNTILIFAW HDNTILIFAW HDNTILIFAW HDNTILIFAW HDNAILWAW TDNTEVLAW TDNTEVLAW	190 VKDHPDFKMG AKENPGYVVA AKENPGYVVA TKQHPEFKLG AKQNPPFAVG AKENPNFEVA AKENPNFEVA ALENKGFEVG AKSNKGFKVG
Cje Msu Hhe Hpy Hmu Apl Ngo Spn Sth Blo Fnu Lla	130 LINK CKTLLLNKGT AQLADKTLLLNKGT DDLKGKKLIVNKGT DDLKGKLIVNKGT KQLEGKTLLVNKGT KQLEGKTLLVNKGT SQLDGKTLIVKGT SQLDGKTLIVKGT	4 Q TADAYFT TADAYFY TADAYFY TADFYFY TADAYFT TADAYFT TADAYFT TAETYFE TAETYFE TAETYFE	150 QNYPNIKTIK KNFPKNKSIK KNYPDIELIK KNYPNIKLIK KNHPEINLIK KNHPEINLOK KNHPEIKLOK KNAPNVKLOK KNAPNVKLOK	160 YD ONTETFAAI FE ONTETFOAI YE ONTETFLAI FE ONTETFAFLAI FD ONTETFDAI FD ONTETFDAI YD OYSDSYQAI YD OYSDAYQAI YD OYADAYNAI YD SYADAYNAI	170 MDKRGDALS LDGRGDALS KDKRGDALA LNNKATALA KSDRGVALA KDGRGVALA LDGRGDAFS LDGRGDAFS LDGRGDAFS LDGRGDAFS	180 HDNTLLFAW HDNTLLFAW HDNTLLAW HDNTLLAW HDNTLLFAW HDNALWAW HDNALWAW TDNTEVLAW TDNTEVLAW TDNTEVLAW TDNTEVLAW	190 VKDHPDFKMG AKENPGYVVA AKENPGYVVA TKOHPEFKLG AKQNPPFAVG AKENPNFEVA AKENPNFEVA ALENKGFEVG AKSNKGFKVG AKSNKGFKVG AKSNKGFKVG
Cje Msu Hpy Hmu Apl Ngo Spn Sth Blo Fnu Lla Ljo	130 1 E D I K D KTLLLNK GT A Q LA D KTLLLNK GT D D L K G K K L I V N K GT D D L K G K K L I V N K GT K Q L E G KTLL V N K GT K Q L E G KTLL V N K GT S Q L D G KTLI V T K GT S Q L D G KTLI V T K GT D D L K G K N L I V N K GT N Q L K G K N L I V N K GT	4 Q TADAYFI TADAYFS TADFYFS TADFYFS TADFYFS TADAYFI TADAYFI TAETYFE TAETYFE TAETYFE TAETYFS TAETYFS TAETYFS	150 QNYPNIKTIK KNFPKNKSIK KNYPDIELIK KNYPDIELIK KNYPNIKIK KNHPEVKLIK KNHPEVKLIK KNHPEIKLQK KNHPEIKLQK KNHPEIKLQK KNHPEIKLQK KNHPEIKLQK KNHPEIKLQK	1 6 0 YD ONTETFAAL FE ONTETFOAL YE ONTETFLAL FE ONTETFDAL FD ONTETFDAL FD ONTETFDAL YD OYSDSYQAL YD OYSDSYQAL YD OYSDAYQAL YD OYADAYNAL FD SKTQQFAAL FD SKTQQFNAM	170 MDKRGDALS LDGRGDALS KDKRGDALA LNNKATALA KSDRGVALA KDGRGVALA KDGRGVALA LDGRGDAFS LDGRGDAFS LDGRGDAFS LDGRGDAFS KNGRAAALA	ISO HDNTLLFAW HDNTLLFAW HDNTLLFAW HDNTLLFAW HDNALWAW HDNALWAW HDNALWAW TDNTEVLAW TDNTEVLAW TDNTEVLAW DDNTEVLAW	190 VKDHPDFKMG AKENPGYVVA AKENPGYVVA TKOHPEFKLG AKQNPPFAVG AKENPFFVA AKENPNFEVA ALLENKGFEVG AKSNKGFKVG AKSNKGFKVG VKNNPKYTVG
Cje Msu Hhe Hpy Hmu Apo Spn Sth Blo Lla Ljo Aby Pey	130 EDIKDKTLLLNKGT AQIADKTLLLNKGT DDIKGKKLIVNKGT SQIKDQTLIVNKGT KQIEGKTLIVNKGT KQIEGKTLIVNKGT KQIEGKTLIVKGT NQIKGKNIVNKGT NQIKGKNIVNKGT DDIASKTIVTGT	4 Q TADAYFT TADAYFT TADFYFS TADFYFS TADFYFS TADAYFT TADTYFE TAETYFE TAETYFE TAETYFS TAETYFS TAETYFS TAETYFS	150 QNYPNIKTIK KNFPKNKSIK KNYPDIELIK KNYPDIELIK KNYPNIKIIK KNHPEVKLIK KNHPEVKLIK KNHPEIKLQK KNHPEIKLQK KNHPEIKLQK KNHPEIKLQK KNHPEIKLQK SKOPDVDLIK SKOPDVDLIK	160 YD0NTETFAAL FE0NTETFQAL YE0NTETFLAL FE0NTETFLAL YD0NTEAFLAL FD0NTETFDAL YD0YSDSYQAL YD0YSDSYQAL YD0YSDAYQAL YD0YADAYNAL FD5KTQ0FNAL FD5KTQ0FAAL FE5KNTESL0AL	170 MDKRGDALS LDGRGDALS KDKRGDALA LNNKATALA KDGRGVALA KDGRGVALA LDGRGDAFS LDGRGDAFS LDGRGDAFS LDGRGDAFS KDGRADAIT KNGRAAALA	180 HDNTLLFAW HDNTLLFAW HDNTLLFAW HDNTLLFAW HDNTLLFAW HDNALVWAW HDNALVWAW HDNTEVLAW TDNTEVLAW TDNTEVLAW TDNTEVLAW TDNTEVLAW DDNSYLFAW DDNSYLFAW QDNLVFSW	VKDHPDFKMG AKENPGYVVA AKENPGYVVA TKQHPEFKLG AKQNPPFFVA AKENPFFVA ALENKGFEVG AKSNKGFKVG AKSNKGFKVG AKSNKGFKVG AKSNKGFKVG AKSNPGFTVG VKNNPKYTVG AKSNPGYRVL
Cje Msu Hhe Hpy Hmu Ngo Spn Sth Blo Fnu Lla Ljo Ysy Bce	130 LUNK GENERAL STILLINK GT AQLAD KTLLLNK GT DDLKGKKLIVNKGT EELKDKELIVNKGT KQLEGKTLLVNKGT KQLEGKTLLVNKGT KQLEGKTLIVKGT SQLNGKTLIVKGT NQLKGKNLIVNKGT NQLKGKNLIVNKGT NQLKGKNLIVTKGT NQLKGKNLIVTKGT KDLASKTIVTGT KDLASKTIVTGT	4 Q TADAYFI TADAYFS TADFYFS TADFYFS TADAYFI TADAYFI TAETYFE TAETYFE TAETYFE TAETYFE TAETYFE TAETYFI TAENYFI TAENYFI TADIWLI TADIWLI	150 QNYPNIKTIK KNYPNIKTIK KNYPNIKIIK KNYPNIKIIK KNHPEVKLIK KNHPEVKLIK KNYPNIKLQK KNYPNIKLLK	I 60 YD 0 NT E TF AAL FE 0 NT E TF QAL YE 0 NT E AF LAL YE 0 NT E AF LAL FD 0 NT E AF LAL YD 0 NT E TF DAL YD 0 NT E TF DAL YD 0 YS DAYQAL YD 0 YS DAYQAL YD 0 YADAYNAL FD SKT Q0 FAAL FD SKT PAAL FD SKT PAAL FD SKT PAAL FD SKT PAAL FD SKT PAAL FD SKT FAAL FD SKT FAAL FAAL FD SKT FAAL FA	170 MDKRGDALS LDGRGDALS KDKRGDALA LNNKATALA KSDRGVALA KDGRGVALA KDGRGVALA LDGRGDAFS LDGRGDAFS LDGRGDAFS LDGRGDAFS KNGRAAALA ANGRGDAYA KDGRADAYA	180 HDNTLIFAW HDNTLIFAW HDNTLIFAW HDNTLIFAW HDNTLIFAW HDNALIWAW HDNALIWAW TDNTEVLAW TDNTEVLAW TDNTEVLAW TDNTEVLAW DDNSYIFAW DDNSYIFAW QDNLVIFSW QDNLVIFSW	190 VKDHPDFKMG AKENPGYVVA AKENPGYVVA AKENPFFVVA AKENPFFVA AKENPFFVA AKENPFFVA AKENPFFVA AKENPGFTVG VKNNPKYTVG VKNNPKYTVG AKKNPGFHL
Cje Msu Hhe Hmu Apl Ngo Spn Sth Blo Lla Ljo Ahy Bce	130 LUX LAD KDKTLLLNKGT AQLADKTLLLNKGT DDLKGKKLIVNKGT ELKDKLIVNKGT KQLEGKTLLVNKGT KQLEGKTLIVTKGT KQLEGKTLIVTKGT KQLGKKLIVNKGT NQLKGKNLIVNKGT NQLKGKNLIVNKGT NQLKGKNLIVTKGT KDLEGKTLIVTKGT KDLEGKTLIVTKGT	4 9 TADAYFT TADAYFT TADFYFS TADFYFS TADFYFS TADAYFT TACTYFE TACTYFE TACTYFE TACTYFS TACTYFS TACTYFS TACTYFS TACYFS TACYFS TACYFS TACYFS TACYFS	150 ONYPNIKTIK KNYPNIKLIK KNYPNIKLIK KNYPNIKLIK KNHPEINLIK KNHPEIKLOK KNHPEIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK	I 60 YD 0 NT E TF AAL FE 0 NT E TF QAL YE 0 NT E AF LAL YE 0 NT E AF LAL YD 0 NT E TF LAL YD 0 NT E TF DAL YD 0 YS DAY QAL YD 0 YS DAY QAL YD 0 YS DAY QAL YD 0 YS DAY QAL FD SKT Q 0 F AAL FD SKT Q 0 F AAL FD SKT SL QAL FE KNT E SL QAL FD KNT E AL QAL	17 Q MDKRCDALS LDGRGDALS KDKRCDALA KNKATALA KSDRGVALA KDGRGVALA LDGRCDAFS LDGRCDAFS LDGRCDAFS KDGRADAIT KNGRAAALA AQGRCDAYA ANGRCDAYA KDGRADSYA	180 HDNTLLFAW HDNTLLFAW HDNTLLLAW HDNTLLFAW HDNALVWAW HDNALVWAW TDNTEVLAW TDNTEVLAW TDNTEVLAW DDNSYLFAW DDNSYLFAW QDNLVLFSW QDNLVLFSW QDNLVLSW	190 VKDHPDFKMG AKENPGYVVA AKENPGYVVA AKENPFFVA AKENPFFVA AKENPFFVA ALENKGFEVG AKSNKGFKVG AKSNKGFKVG KNNPKYTVG VKNNPKYTVG AKENPGFHLL
Cje Msu Hhe Hmu Apl Spn Sth Blo Fnu Lla Ljo Psy Bce	130 L X DKTLLLNKGT A QLADKTLLLNKGT D DLKGKKLIVNKGT E LKDKLLVNKGT K QLEGKTLLVNKGT K QLEGKTLLVNKGT K QLEGKTLIVKGT K QLEGKTLIVKGT N QLKGKNLIVNKGT N QLKGKNLIVNKGT N QLKGKNLIVNKGT N QLKGKNLIVNKGT K DLEGKKVIVTKGT K DLEGKKVIVTKGT	4 9 TAD AYFT TAD AYFT TAD AYFT TAD FYFS TAD FYFS TAD AYFT TAD FYFS TAD TYFS TAD TYFS TAE TYFS TAE TYFS TAE TYFS TAE TYFS TAE TYFT TAD IWLT TAD IWLT TAD IWLT	150 QNYPNIKTIK KNYPNIKLIK KNYPNIKLIK KNYPNIKLIK KNYPNIKLIK KNYPNIKLIK KNYPNIKLIK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK KNYPNIKLOK	I 60 YD 0 NT E TF AAL FE 0 NT E TF QAL YE 0 NT E TF QAL YE 0 NT E TF LAL FD 0 NT E TF LAL FD 0 NT E TF DAL YD 0 YS DSYQAL YD 0 YS DSYQAL YD 0 YAD AYNAL FD SKT 00 FAAL FD SKT 00 FAAL FD SKT 00 FAAL FE KNTESLQAL FE KNTESLQAL	17 Q MDKRGDALS LDGRGDALS KDKRGDALS KDKRGDALS KDGRGVALA KDGRGVALA LDGRGDAFS LDGRGDAFS LDGRGDAFS LDGRGDAFS KDGRADAIT KNGRAAALA ANGRGDAYA ANGRGDAYA	180 HDNTLLFAW HDNTLLFAW HDNTLLFAW HDNTLLFAW HDNTLLFAW HDNALVWAW TDNTEVLAW TDNTEVLAW TDNTEVLAW TDNTEVLAW DDNSYLFAW DDNSYLFAW QDNLVLFSW QDNLVLFSW	190 VKDHPDFKMG AKENPGYVVA AMENEGFNVG AKENPFFVG AKONPFFVG AKENPTFDVA ALENKGFEVG ALENKGFEVG AKSNKGFKVG VKDHPDYKVG VKDHPDYKVG VKDHPDYKVG AKKNPGYRVL AKKNPGFHLL
Cje Msu Hhey Hmu Apl Spn Sth Blo Fnu Lla Ljo Psy Bce	130 1. EDIKDKTLLLNKGT AQIADKTLLLNKGT DDIKGKKLIVNKGT SQIKDQTLIVDRGT KQLEGKTLIVNKGT KQLEGKTLIVKGT KQLEGKTLIVKGT NQIKGKNVIVKGT NQIKGKNVIVTKGT NQIKGKNVIVTGT DDIASKTIVTIGT DDIASKTIVTIGT	49 TADAYFI TADAYFI TADAYFI TADFYFS TADAYFI TAETYFE TAETYFE TAETYFE TAETYFE TAETYFI TAETYFI TADIWLI TADIWLI	150 ONYPNIKTIK KNFPKNKSIK KNFPKNKSIK KNFPLNIKLIK KNHPEVKLIK KNHPEVKLIK KNHPEVKLIK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPDWKVLK KNHPDWKVLK KNHPDWKVLK KNHPDWKVLK	160 YD0NTETFAAL FE0NTETFQAL YE0NTEAFLAL FE0NTEAFLAL YD0NTEAFLAL YD0NTEAFLAL YD0NTEAFLAL YD0NTETFDAL YD0YSDSYQAL YD0YSDSYQAL YD0YSDSYQAL YD0YADAYNAL YDSYADAYNAL YDSYADAYNAL FDSKT00FAAL FEKNTESLQAL FEKNTESLQAL	17 Q MDKRGDALS LDGRGDALS KDKRGDALA KDGRGVALA KDGRGVALA LDGRGDAFS LDGRGDAFS LDGRGDAFS KDGRADAIT KNGRAAAIT KNGRAAAIT	180 HDNTLLFAW HDNTLLFAW HDNTLLLAW HDNTLLLAW HDNTLLLAW HDNALVWAW TDNTEVLAW TDNTEVLAW TDNTEVLAW TDNTEVLAW DDNSYLFAW QDNLVLFSW QDNLVLFSW QDNLVLFSW	190 VKDHPDFKMG AKENPGYVVA AMENEGFNVG TKQHPEFKLG AKENPFFAVG AKENPFFVG ALENKGFEVG ALENKGFEVG ALENKGFKVG ALENKGFKVG ALENKGFKVG AKSNKGFKVG AKSNPGYRVL AKKNPGYRVL AKKNPGFHLL
Cje Msu Hhpy Hmu Apl Spn Sth Spn Sth Fnu Lla Ljo Ahy Psy Bce	130 L X D KTLLLNKGT A Q LA D KTLLLNKGT D D LK G K KL V NKGT S Q LK D Q TL V D R GT K Q LE G KTL V V K GT K Q LE G KTL V V K GT K Q LE G KTL I V X K GT N Q LK G K N I V NKGT N Q LK G K N I V NKGT N Q LK G K N V V X GT D D LA S KTL V V T GT K D LE G K K V V X GT 200 200 21 K E L C N K D V A P A M	49 TADAYFI TADAYFI TADAYFI TADFYFS TADAYFI TADFYFS TADAYFI TAETYFE TAETYFE TAETYFE TAETYFE TAETYFI TAETYFI TAETYFI TAETYFI TADIWLI TADIWLI	150 QNYPNIKTIK KNFPKNKSIK KNYPDIELIK KNHPEVKLIK KNHPEVKLIK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKKVLK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKVLK KNHPIKK KNHFIKK KNHPIKK KNHFIK KNHF	160 YD ONTE TFAAL FEONTE TFOAL YE ONTE AFLAL FEONTE AFLAL YD ONTE AFLAL YD ONTE AFLAL YD ONTE AFLAL YD OYSD SYQAL YD OYSD SYQAL YD OYSD AYNAL YD SYAD AYNAL YD SYAD AYNAL YD SYAD AYNAL YD SYAD AYNAL FD SKT QOFAAL FE KNTE SLQAL FE KNTE SLQAL	17 0 MDKRGDALS LDGRGDALS KDKRGDALA LDGRGVALA KDGRGVALA LDGRGDAFS LDGRGDAFS LDGRGDAFS LDGRGDAFS KDGRADAIT KNGRAAAIT XNGRAAAIT XNGRAAAIT XNGRAAYA	180 HDNTLIFAW HDNTLIFAW HDNTLILAW HDNTLILAW HDNTLILAW HDNTLVFAW TDNTEVLAW TDNTEVLAW TDNTEVLAW TDNTEVLAW TDNTEVLAW DDNSYLFAW DDNSYLFAW QDNLVIFSW QDNLVIFSW QDNLVIFSW QDNLVIFSW	190 VKDHPDFKMG AKENPGYVVA AMENEGFNVG TKQHPFFVG AKENPTFVA ALENKGFEVG AKENPTFVA ALENKGFEVG AKSNKGFKVG AKSNKGFKVG AKSNPGYRVL AKNPGYRVL AKNPGFHIL
Cje Msu Hhey Hmu Apl Spn Sth Spn Sth Fnu Lla Ljo Ahy Psy Bce Cje Msu	130 LINC LINC LINC LINC CINC LINC CINC LINC	49 TADAYFI TADAYFI TADAYFI TADFYFS TADAYFI TADFYFS TADAYFI TAETYFE TAETYFE TAETYFE TAETYFE TAETYFI TAETYFI TADIWLI TADIWLI	150 QNYPNIKTIK KNFPKNKSIK KNFPKNKSIK KNFPKNKSIK KNPPUKLIK KNHPEVKLIK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPIKLIK KNHPIKVIK KNHPIKVIK KNHPIKVIK KNHPIKVIK KNHPIKVIK KNHPIKVIK KNHPIKVIK KNHPIKVIK KNHPIKVIK KNHPIKVIK KNHPIKVIK KNHPIKVIK KNHPIK KNHPIKVIK KNHPIK	160 YD ONTE TFAAL FEONTE TFOAL YE ONTE AFLAL FEONTE AFLAL YD ONTE AFLAL YD ONTE AFLAL YD ONTE AFLAL YD ONTE AFLAL YD OYSD SYQAL YD OYSD SYQAL YD OYSD AYNAL YD SYAD AYNAL YD SYAD AYNAL YD SYAD AYNAL YD SYAD AYNAL FE KNTE SLOAL FE KNTE SLOAL FE KNTE SLOAL FE KNTE SLOAL	17 Q MDKRGDALS LDGRGDALS LDGRGDALA LDGRGDALA LNNKATALA KDGRGVALA LDGRGDAFS LDGRGDAFS LDGRGDAFS LDGRGDAFS LDGRGDAFS KDGRADAIT KNGRAAAIT KNGRAAJAIT KNGRAAJAIT KNGRAAJAIT KNGRAAJAIT	180 HDNTLIFAW HDNTLIFAW HDNTLILAW HDNTLILAW HDNTLILAW HDNTLILAW HDNALVWAW HDNALVWAW TDNTEVLAW TDNTEVLAW TDNTEVLAW TDNTEVLAW DDNSYLFAW QDNLVIFSW QDNLVIFSW QDNLVIFSW QDNLVIFSW QDNLVIFSW QDNLVIFSW QDNLVIFSW QDNLVIFSW	190 VKDHPDFKMG AKENPGYVVA AMENEGFNVG TKQHPFFVG AKENPTFVA ALENKGFEVG ALENKGFEVG ALENKGFEVG ALENKGFKVG ALENKGFKVG AKSNKGFKVG AKSNPGYRVL AKSNPGYRVL AKSNPGYRVL AKSNPGYRVL IEGGKI VEYQ
Cje Msu Hhey Hmu Apl Spn Sth Spn Sth Fnu Lla Ljo Psy Bce Cje Msu Hhey	130 LXDKTLLLNKGT AQIADKTLLLNKGT AQIADKTLLLNKGT DDLKGKKLIVNKGT SQIKDQTLIVDRGT KQLEGKTLIVNKGT KQLEGKTLIVTKGT KQLEGKTLIVTKGT NQLKGKNLIVNKGT NQLKGKNVIVTKGT DDLASKTIVTIGT DDLASKTIVTIGT DDLASKTIVTIGT KDLEGKKVVIXKGT	49 TADAYFI TADAYFI TADAYFI TADFYFS TADTYFS TADFYFS	150 QNYPNIKTIK KNFPKNKSIK KNFPKNKSIK KNYPDIELIK KNHPEVKLIK KNHPEVKLIK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPDWKVIK KNHP	160 YD ONTETFAAL FE ONTETFQAL YE ONTEAFLAL FD ONTEAFLAL YD ONTEAFLAL YD ONTEAFLAL YD ONTEAFLAL YD ONTEAFLAL YD OYSDSYQAL YD OYSDSYQAL YD OYSDAYNAL YD SYADAYNAL YD SYADAYNAL FD SKT QOFAAL FE KNTESLQAL FE KNTESLQAL FE KNTESLQAL FE KNTEALQAL FE KNTEALQAL FE KNTEALQAL FE KNTEALQAL	17 Q MDKRGDALS LDGRGDALS LDGRGDALA LDGRGVALA KDGRGVALA LDGRGDAFS LDGRGDAFS LDGRGDAFS LDGRGDAFS LDGRGDAFS KDGRADAIT KNGRAAAIT XNGRAAAT XNGRAAAT XNGRAAAIT XNGRAAAIT XNGRAAAIT XNGRAAAIT XNGRAAAIT XNGRAAAIT XNGRAAAIT XNGRAAAIT XNGRAAAIT XNGRAAAIT XNGRAAAIT XNGRAAAT XNG	180 HDNTLLFAW HDNTLLFAW HDNTLLLAW HDNTLLLAW HDNTLLLAW HDNTLLLAW HDNALVWAW TDNTEVLAW TDNTEVLAW TDNTEVLAW TDNTEVLAW DDNSYLFAW DDNSYLFAW QDNLVLFSW QDNLVLFSW QDNLVLFSW QDNLVLFSW QDNLVLFSW QDNLVLFSW QDNLVLFSW QDNLVLFSW QDNLVLFSW QDNLVLFSW QDNLVLFSW QDNLVLFSW QDNLVLFSW QDNLVLFSW	190 VKDHPDFKMG AKENPGYVVA AMENEGFNVG TKQHPFFVG AKENPTFDVA ALENKGFEVG ALENKGFEVG ALENKGFEVG ALENKGFKVG ALENKGFKVG AKSNKGFKVG AKSNPGYRVL AKSNPGYRVL AKSNPGYRVL AKSNPGYRVL IEGGKI VEYQ FSH FE
Cje Msu Hhey Hmu Apl Spn Sth Spn Sth Fnu Lla Ljo Psy Bce Cje Msu Hhe Hpy Hmu	130 LING LING LING LING CONT LING CONT LING CONT LING CONT LING CONT LING L	49 TADAYFI TADAYFI TADAYFI TADFYFS TADAYFI TADFYFS TADAYFI TAETYFE TAETYFE TAETYFE TAETYFE TAETYFE TAETYFI TADIWLT TADIWLT TADIWLT TADIWLT	150 QNYPNIKTIK KNFPKNKSIK KNFPKNKSIK KNFPKNKSIK KNFPUKLIK KNHPEVKLIK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPDWKVIK KNHPDWKVIK KNHPDWKVIK KNHPDWKVIK KNHPDWKVIK KNHPDWKVIK KNHPDWKVIK KNHPDWKVIK KNHPDWKVIK KNHPDWKVIK KNHPDWKVIK KNHPDWKVIK KNHPDWKVIK KNHPDWKVIK KNHPDWKVIK KNHPDWKVIK	160 YD0NTETFAAL FE0NTETFQAL YE0NTEAFLAL FE0NTEAFLAL YD0NTEAFLAL YD0NTEAFLAL YD0NTEAFLAL YD0NTEAFLAL YD0YSDSYQAL YD0YSDSYQAL YD0YSDAYNAL YD0YADAYNAL YD0YADAYNAL YD0YADAYNAL FDSKT00FAAL FEKNTESLQAL FEKNTESLQAL FEKNTESLQAL FEKNTEALQAL YD0YACAL	17 Q MDKRGDALS LDGRGDALS LDGRGDALS LDGRGDALS LNNKATALA KDGRGVALA LDGRGDAFS LDGRGDAFS LDGRGDAFS LDGRGDAFS LDGRGDAFS KDGRADAIT KNGRAALI AQGRGDAYA ANGRADAYA ANGRADAYA ANGRADAYA ANGRADAYA	180 HDNTLIFAW HDNTLIFAW HDNTLILFAW HDNTLILAW HDNTLILAW HDNTLILAW HDNTLVFWW HDNALVWAW TDNTEVLAW TDNTEVLAW TDNTEVLAW TDNTEVLAW DDNSYLFAW QDNLVIFSW QDNLVIFSW QDNLVIFSW QDNLVIFSW QDNLVIFSW QDNLVIFSW QDNLVIFSW DDSYLFAW DDSYLFAW DDNSYLFSW QDNLVIFSW QDNLVIFSW QDNLVIFSW QDNLVIFSW QDNLVIFSW QDNLVIFSW QDNLVIFSW QDNLVIFSW QDNLVIFSW QDNLVIFSW	190 VKDHPDFKMGA AKENPGYVVA AMENEGFNVG TKQHPFFVG AKENPTFEVG AKENPTFEVG ALENKGFEVG ALENKGFEVG ALENKGFKVG AKSNKGFKVG AKSNPGYRVL AKSNPGYRVL AKSNPGYRVL IEGGKI VEYQ FSH FE FE
Cje Msu Hhey Hmu Apl Spn Sth Fnu Lla Ljoy Psy Bce Cje Msu Hhe Hpy Hmu Apl	130 LING LING LING LING CONT LING CONT LING CONT LING CONT LING CONT LING LING CONT LING LING CONT LING CONT LING CONT LING CONT LING CONT LING CONT LING CONT LING CONT LING CONT LING CONT LING CONT LING CONT LING CONT C	49 TADAYFI TADAYFI TADAYFI TADFYFS TADFYFS TADFYFS TADFYFS TADFYFS TADFYFS TAETYFE	150 QNYPNIKTIK KNFPKNKSIK KNFPKNKSIK KNYPDIELIK KNHPEVKLIK KNHPEVKLIK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPDWKVLK KNHPDWKVLK KNHPDWKVLK KNHPDWKVLK KNHPDWKVIK KNHPOK KNHPDWKVIK KNHPOK KNHPDWKVIK KNHPO	160 YD ONTE TFAAL FE ONTE TFOAL FE ONTE TFOAL YE ONTE AFLAL FD ONTE AFLAL YD ONTE AFLAL YD ONTE AFLAL YD ONTE AFLAL YD OYSD SYQAL YD OYSD SYQAL YD OYSD SYQAL YD OYSD AYNAL YD SYAD AYNAL YD SYAD AYNAL FE KNTE SLOAL FE KNTE SLOAL F	17 0 MDKRGDALS LDGRGDALS LDGRGDALA LDGRGDALA LNNKATALA KDGRGVALA LDGRGDAFS LDGRGDAFS LDGRGDAFS LDGRGDAFS LDGRGDAFS KDGRADAIT KNGRAAT KNGRAAAIT KNGRAAA	180 HDNTLLFAW HDNTLLFAW HDNTLLLAW HDNTLLLAW HDNTLLLAW HDNTLLLAW HDNALVWAW HDNALVWAW TDNTEVLAW TDNTEVLAW TDNTEVLAW TDNTEVLAW DDNSYLFAW DDNSYLFAW QDNLVLFSW	190 VKDHPDFKMG AKENPGYVVA AMENEGFNVG TKQHPFFVG AKENPTFDVA ALENKGFEVG AKENPTFDVA ALENKGFEVG AKSNKGFKVG AKSNFGFTVG VKDHPDYKVG VKDHPDYKVG AKSNPGYRVL AKSNPGYRVL AKSNPGYRVL IEGGKI VEYQ FSH FE FE
Cje Msu Hhey Hmu Apl Spn Sth Bru Lla Ljoy Psy Bce Cje Msu Hhe Hpu Apl Spn Spn Sca Spn	130 LXDKTLLLNKGT AQIADKTLLLNKGT AQIADKTLLLNKGT DDLKGKKLIVNKGT SQIKDQTLIVDRGT KQLEGKTLIVNKGT KQLEGKTLIVNKGT NQLKGKNLIVNKGT NQLKGKNVIVIKGT NQLKGKNVIVTGT DDLASKTIVTIGT DDLASKTLIVTIGT KDLEGKVVIVTKGT KDLEGKVVIVTKGT KDLEGKVVIVTKGT IKNLGDLDYIAPAV IFTLGEEENIAPAI ISLGNDVIAPAI IGNLGPAEFIAPAV IGNLGPAEFIAPAV IFTLGEPIT	4 9 TADAYFI TADAYFI TADAYFI TADFYFS TADFYFS TADFYFS TADFYFS TADFYFS TADFYFS TADFYFS TADFYFS TADFYFS TADFYFS TADIYFS TADIYFS TADYFS TADYFS TADYFS TADYFS TADYFS TADYFS TADYFS TADYFS TADYFS TADYFS TADYFS TADYFS TADFYS TADFYS TADYFS TADFYS TA	150 QNYPNIKTIK KNFPKNKSIK KNFPKNKSIK KNFPKNKSIK KNFPLKIKIK KNHPEVKLIK KNHPEVKLIK KNHPEVKLIK KNHPEIKLOK KNHPEIKLOK KNHPDWKVIK KNHPDWKVIK KNHPDWKVIK KNHPDWKVIK KNHPDWKVIK KNHPDWKVIK KNHPDWKVIK LOVLDGEIEK KNWLDNEITS LEWLNEITS LDWLNGEITK LDVINKEIAE LNWVNGEIAA LDFINKDIEA	160 YD Q NTE TFAAL FE QNTE TFQAL YE QNTE AFLAL FE QNTE AFLAL YE QNTE AFLAL YD QNTE AFLAL YD QNTE AFLAL YD QNTE AFLAL YD QYSD SYQAL YD QYSD SYQAL YD QYSD AYNAL YD QYAD AYNAL YD SYAD AYNAL FE KNTE SLQAL FE KNTE SLQAL	17 Q MDKRGDALS LDGRGDALS KDKRGDALA KDGRGVALA KDGRGVALA LDGRGDAFS LDGRGDAFS LDGRGDAFS LDGRGDAFS KDGRADAIT KNGRADAIT AQGRGDAYA ANGRADAYA ANGRADAYA ANGRADAYA ANGRADAYA ANGRADAYA ANGRADAYA	180 HDNTLLFAW HDNTLLFAW HDNTLLLAW HDNTLLLAW HDNTLLLAW HDNTLLLAW HDNALVWAW HDNALVWAW TDNTEVLAW TDNTEVLAW TDNTEVLAW TDNTEVLAW DDNSYLFAW QDNLVLFSW QDNLVLFW DEIKEADVL EEKKPELAL DKPELAL ZKVKPEALL DAAKADDLV	190 VKDHPDFKMGA AKENPGYVVA AMENEGFNVG TKQHPFFVGA AKENPTFDVA ALENKGFEVGA ALENKGFEVGA ALENKGFKVG AKSNKGFKVG AKSNPGYRVL AKSNPGYRVL AKSNPGYRVL AKSNPGYRVL IEGGKI VEYQ FSH FE FE FE FE K Q AE VEGGKVD
Cje Msu Hhey Hmu Apl Spn Sth Bru Lla Ljoy Psy Bce Cje Msu Hhe Hpu Apl Spn Sth Spn Sth Sca Spn Sth Sth Sth Spn Sth Sth Sth Sth Sth Sth Sth Sth Sth Sth	130 LXDKTLLLNKGT AQIADKTLLLNKGT AQIADKTLLLNKGT DDLKGKKLIVNKGT SQIKDQTLIVDRGT KQLEGKTLIVNKGT KQLEGKTLIVKGT KQLEGKTLIVKGT NQLKGKNVIVKGT NQLKGKNVIVKGT NQLKGKNVIVTGT DDLASKTLIVTGT DDLASKTLIVTGT KDLEGKKVIVTKGT KDLEGKKVIVTGT DDLASKTLIVTGT LSGCKVIVTGT ISIGDEDTIAPAV ISIGDEDTIAPAV ISIGDEDTIAPAV ISIGDEDTIAPAV	4 9 TADAYFI TADAYFI TADAYFI TADAYFI TADFYFS TADAYFI TADFYFS TADAYFI TADTYFE TAETYFE	150 QNYPNIKTIK KNFPKNKSIK KNFPKNKSIK KNYPDIELLK KNHPEVKLIK KNHPEVKLIK KNHPEVKLIK KNHPEIKLOK KNHPEIKLOK KNHPEIKLOK KNHPDWKVLK KNHPDWKVLK KNHPDWKVLK KNHPDWKVLK KNHPDWKVLK KNHPDWKVLK KNHPDWKVLK LOVLDGEIEK KNWLDNEITS LEWLNQEITK LDVINKEIAE LNWVNGEIAA LDFINKDIKK	160 YD ONTETFAAL FE ONTETFQAL YE ONTEAFLAL FE ONTETFLAL YE ONTEAFLAL YD ONTEAFLAL YD ONTEAFLAL YD ONTEAFLAL YD ONTEAFLAL YD ONTEAFLAL YD OYSD SYQAL YD OYSD SYL YD OYSD SYL	17 Q MDKRGDALS LDGRGDALS KDKRGDALA KDGRGVALA KDGRGVALA LDGRGDAFS LDGRGDAFS LDGRGDAFS LDGRGDAFS KDGRADAIT AQGRGDAYA ANGRADAIT KNGRAALI AQGRGDAYA ANGRADAYA ANGRADAYA ANGRADAYA ANGRADAYA KDGRAV EXTLAPIYG EXTLAPIYG EXTLAPIYG EKTLHPIYG EKTLHPIYG	180 HDNTLLFAW HDNTLLFAW HDNTLLFAW HDNTLLLAW HDNTLLLAW HDNTLLFAW HDNALVWAW TDNTEVLAW TDNTEVLAW TDNTEVLAW TDNTEVLAW DDNSYLFAW QDNLVLFSW QDNLVLFW DDNSVDQU EXKADDV DENKFELL SKVKPELL	190 VKDHPDFKMGA AKENPGYVVA AMENEGFNVG TKQHPFFVG AKENPFFVG AKENPFFVG AKENFFVG ALENKGFEVG ALENKGFKVG VKDHPDYKVG VKDHPDYKVG VKDHPCYRVL AKSNPGYRVL AKSNPGYRVL AKSNPGYRVL AKSNPGYRVL IEGGKI VEYQ FSH FE FE FE FE FE VEGGVV
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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*.

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	Cj0467	Cj0468	Cj0469		Cj0982
	HH0345	HH0346	HH0347		HH0738
-	HP1169	HP1170	HP1171	HP1172	
	MS1687	MS1686	MS1685	MS1684	MS1683 (gshA)
	Aple2000886	Aple2000885	Aple2000884	Aple2000883 (apaA)	
	L1311	LJ1310	L)1309	LJ1307	
str1583 (ddlA)	str1582	str1581	str1580	str1579	
_	BL0074	BL0075	BL0076	BL0077	BL0078 (metC)

**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 Lcysteine 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  $\beta$ 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  $\beta$ -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  $\beta$ -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<sup>20</sup> 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 lineariziation of the pET22b vector DNA with *Ncol* and *Xhol*, 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 I of Luria–Bertani medium supplemented with 50 µg ml<sup>-1</sup> carbenicillin at 37°C until the OD<sub>600</sub> 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<sup>-1</sup> 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  $\beta = 109.0^{\circ}$ . With two molecules in the asymmetric unit, the Matthews coefficient is 2.4 Å<sup>3</sup> Da<sup>-1</sup>. 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  $l/\sigma(l)$  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  $R/\sigma$  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  $R_{\text{factor}}$  of

0.584. For the second molecule the discrimination against wrong solutions improves the  $R_{\text{factor}}$  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  $R_{\rm factor}$  values converged (Table 1). The coordinates 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  $\mu$ M 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  $\mu$ M was also tested. Cumulative fluorescence changes from titration of the protein with ligand were plotted in Sigma-Plot and fitted to a single rectangular hyperbola. The *K*<sub>d</sub> values reported were averaged from three separate ligand titration experiments.

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