The *Campylobacter jejuni* PEB1a adhesin is an aspartate/glutamate-binding protein of an ABC transporter essential for microaerobic growth on dicarboxylic amino acids

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Summary

The PEB1a protein of the gastrointestinal pathogen *Campylobacter jejuni* mediates interactions with epithelial cells and is an important factor in host colonization. Cell fractionation and immunoblotting showed that PEB1a is most abundant in the periplasm of *C. jejuni*, and is detectable in the culture supernatant but not in the inner or outer membrane. The protein is homologous with periplasmic-binding proteins associated with ABC transporters and we show by fluorescence spectroscopy that purified recombinant PEB1a binds L-aspartate and L-glutamate with sub μM $K_d$ values. Binding of L-$^{14}$C-aspartate or L-$^{14}$C-glutamate was strongly out-competed by excess unlabelled aspartate or glutamate but only poorly by asparagine and glutamine. A mutant in the Cj0921c gene, encoding PEB1a, was completely unable to transport 5 μM L-$^{14}$C-glutamate and showed a large reduction (~20-fold) in the rate of L-$^{14}$C-aspartate transport compared with the wild type. Although microaerobic growth of this mutant was little affected in complex media, growth on aspartate or glutamate in defined media was completely prevented, whereas growth with serine was similar to wild type. $^1$H-NMR analysis of the culture supernatants of the Cj0921c mutant showed some utilization of aspartate but not glutamate, consistent with the transport data. It is concluded that in addition to the established role of PEB1a as an adhesin, the PEB1 transport system plays a key role in the utilization of aspartate and glutamate, which may be important in vivo carbon sources for this pathogen.

Introduction

*Campylobacter jejuni* is one of the most important causes of human enteric disease worldwide and continues to be a major public health and economic burden (Friedman et al., 2000). Although a pathogen of humans, *C. jejuni* is part of the normal commensal flora of many bird species, and ingestion of contaminated poultry is a common route for infection. Acute symptoms of *C. jejuni* infection in humans include diarrhoea, fever and abdominal pain but the complications can include reactive arthritis and neurological sequelae such as the Miller-Fisher and the Guillaine–Barré syndromes (Skirrow and Blaser, 2000).

The molecular mechanisms of pathogenesis of *C. jejuni* are still not completely understood, but the interactions of the bacteria with host intestinal epithelial cells and phagocytes are clearly crucial in the disease process, especially as a prerequisite to invasion (Hu and Kopecko, 2005; Raphael et al., 2005). The first step in such an interaction is adherence, and there have been several studies describing the adhesion properties of various strains of *C. jejuni* to different host cell-lines in vitro (Hu and Kopecko, 2005). Binding to INT407 (a human intestinal epithelial cell line) and Caco-2 (a human colonic cell line) have been investigated most intensively, as these are similar to the cells encountered by *C. jejuni in vivo*. Molecules proposed to serve as adhesins include lipooligosaccharide (McSweegan and Walker, 1986), flagellin (Grant et al., 1993), the fibronectin-binding protein CadF (Konkel et al., 1997) and a variety of outer membrane proteins (Moser et al., 1997).

Fauchere et al. (1989) characterized two fractions thought to be derived from the outer membrane (CBF1 and CBF2) that bound preferentially to HeLa cells. In a study designed to identify proteins suitable for use in serology and as vaccine candidates, Pei et al. (1991) identified four proteins of 28, 29, 30 and 31 KDa in acidic glycine extracts of whole cells of strain 81–176. These
were termed PEB1 to PEB4 respectively, and it later became clear that PEB1 was identical to CBF1 and PEB4 identical to CBF2 (Kervella et al., 1993). The structural gene for PEB1 (peb1a) was cloned by Pei and Blaser (1993) by using polyclonal antibody raised against purified PEB1a protein, in a screen of a λgt11 library of genomic DNA from strain 81–176. The gene was mutated and the null mutant strain showed 50- to 100-fold less adherence to and 15-fold less invasion of epithelial cells in culture (Pei et al., 1998). Moreover, mouse challenge studies indicated that the rate and duration of intestinal colonization was significantly lower and shorter, respectively, compared with the wild-type strain. These data have established the PEB1a protein as an important colonization and virulence factor in C. jejuni. Western blot analysis showed that PEB1a is present in all C. jejuni and Campylobacter coli strains examined, but appears to be absent from Campylobacter fetus, Campylobacter lari and Helicobacter pylori (Pei et al., 1991).

From database searching using the deduced amino acid sequence of PEB1a, Pei and Blaser (1993) showed that the protein is homologous to the periplasmic-binding protein component of amino acid ABC transporters, for example GlnH (27.8% identity) and HisJ (28.9% identity). A linked open-reading frame encodes a protein with homology with the membrane proteins GlnQ and HisP, and the genome sequence of strain NCTC 11168 clearly reveals that peb1a in this strain (Cj0921c) is part of a four-gene operon encoding a typical ABC transporter. A potential dual role for PEB1a as both a surface exposed adhesin and a periplasmic soluble-binding protein can be rationalized because of the unusual existence of two predicted processing sites in the signal sequence for signal peptidase I and II, which may be responsible for the localization of the protein both in the periplasm and on the cell surface (Pei and Blaser, 1993), although the mechanism of export across the outer membrane is unknown.

No previous studies have been carried out on the transport function of PEB1a and the importance of the PEB1 system in amino acid transport in C. jejuni is therefore unknown. In this article we establish the identity of the amino acids that bind to purified PEB1a and provide evidence that the PEB1 transport system is of crucial importance in the growth of C. jejuni on both aspartate and glutamate. The results suggest that the transport function of PEB1a could be a significant factor in animal host colonization in addition to its role as an adhesin.

Results

Overexpression and purification of PEB1a

The Cj0921c gene encodes the PEB1a protein in the NCTC 11168 strain of C. jejuni and this was polymerase chain reaction (PCR)-amplified and cloned into the expression vector pET21a, such that the recombinant protein would be expressed from the T7 promoter with the original C. jejuni signal sequence and without any tags. Induction of Escherichia coli BL21(DE3)(pMK3) with IPTG resulted in the overproduction of a 25 kDa protein which was purified to homogeneity from periplasmic extracts using cation-exchange chromatography (Fig. 1A). The location of the protein in the E. coli periplasm indicates correct export across the cytoplasmic membrane, and the single-step purification took advantage of the fact that whereas most E. coli proteins did not bind to the cation-exchange column under the conditions used, the basic PEB1a (pI 8.4) bound tightly and was eluted at a moderate salt concentration as a single species. N-terminal amino acid sequencing gave a sequence of AEGKLESIKSK which is identical to residues 27–37 of the deduced sequence and consistent with correct cleavage after the ANA signal peptidase I recognition site. However, attempts to obtain an accurate mass of the protein by electrospray mass spectrometry were unsuccessful. Western blots using the original polyclonal antibody described by Pei et al. (1991), which was raised against PEB1a purified from acidic glycine extracts of C. jejuni 81–176, showed specific cross-reaction with the overexpressed and purified recombinant protein (data not shown).

Identification of L-aspartate and L-glutamate as ligands for PEB1a by steady-state fluorescence spectroscopy

Fluorescence spectroscopy has proven to be an extremely useful tool in the characterization of periplasmic-binding proteins (Thomas et al., 2006), most sensitively exploiting changes in intrinsic tryptophan fluorescence upon ligand binding. Although the deduced amino acid sequence of PEB1a shows that the mature protein contains just one tryptophan at the C-terminus, it contains nine tyrosine residues which can also be used as a reporter of conformational changes upon interactions with potential ligands. Excitation of the protein at 280 nm resulted in an emission maximum at 345 nm (Fig. 1B). All 20 L-amino acids were individually added at a final concentration of 100 µM to 0.1 µM aliquots of PEB1a and changes in the fluorescence intensity at the emission maximum monitored. Only two amino acids, L-aspartate and L-glutamate gave a significant change in fluorescence, a quench of about 12–15% in each case (Fig. 1B). Titration of the fluorescence change using 0.05 µM protein with these amino acids resulted in a typical hyperbolic saturation curve, from which $K_d$ values of 0.5 ± 0.05 µM for L-aspartate (Fig. 1C) and 0.79 ± 0.06 µM for L-glutamate (Fig. 1D) were determined. These low $K_d$ values are consistent with L-asp and L-glu being the physiological ligands for PEB1a. As noted above, the sequence of
PEB1a is most similar to glutamine and histidine-binding proteins from other ABC systems. No evidence was obtained for fluorescence changes upon addition of L-his at concentrations up to 500 µM, but a small quench of 3–6% was observed with 500 µM L-gln or L-asn, suggesting weak binding. However, we were not able to accurately titrate the fluorescence quench with either of these amino acids to obtain a $K_d$ value, due to the small magnitude of the changes and problems with protein photobleaching.

L-$^{14}$C-aspartate and L-$^{14}$C-glutamate binding to PEB1a and competition with asparagine and glutamine

Figure 2 shows the binding of radiolabelled L-asp (Fig. 2A) or L-glu (Fig. 2B) to PEB1a, detected after ammonium sulphate precipitation, filtration and liquid scintillation counting. Specific binding of either amino acid was readily detected. Binding of each radiolabelled amino acid was almost completely out-competed by the addition of a 10-fold molar excess of unlabelled L-aspartate or L-glutamate to PEB1a. A 10- or 100-fold excess of L-asn or L-gln showed little evidence of competition with L-$^{14}$C-aspartate (Fig. 2A), but 100-fold excess L-asn or L-gln did inhibit binding of L-$^{14}$C-glutamate (Fig. 2B) to a significant extent ($P < 0.05$ in a Students $t$-test), suggesting weak binding consistent with the data from the fluorescence quenching. L-his did not compete significantly.
Mutagenesis, Western blotting and 2D-gel analysis show that PEB1a is a highly abundant periplasmic protein in C. jejuni

The Cj0921c gene is contained within the Cj0919c–Cj0922c operon, which encodes the remaining components of the PEB1 ABC transporter. A mutant in Cj0921c was constructed by the insertion of a chloramphenicol resistance cassette into a unique SpeI site within the cloned gene. After electroporation into wild-type cells, several chloramphenicol resistant colonies were selected and a PCR with gene-specific primers showed that the mutant construction had been successful (data not shown). The absence of the PEB1a protein in this mutant

is shown by the SDS-PAGE gel and Western blot shown in Fig. 3A and B respectively. In periplasmic extracts of the C. jejuni wild-type parent, a prominent band of about 25 kDa, which strongly cross-reacted with the PEB1a antibody on the Western blot is clearly visible. As expected, this protein is completely missing in the mutant.

Two additional mutants were isolated from independent transformations and these showed the absence of PEB1a and identical phenotypes to those described below, eliminating any possibility that second site mutations were responsible for the phenotypes observed. Immediately downstream of the Cj0919c–Cj0922c operon and transcribed in the same direction, is the prsA gene (Cj0918c) encoding phosphoribosyl-pyrophosphate (PRPP) synthase. Any polar effect of the chloramphenicol cassette on the expression of this gene can be ruled out because PRPP synthases are essential enzymes needed for synthesis of purines, pyrimidines, NAD, histidine and tryptophan (Hove-Jensen, 1988). The Cj0921c mutant was viable and did not show any specific requirement for growth supplementation with these precursors in minimal media.

The SDS-PAGE and immunoblot data in Fig. 3B and B suggest that PEB1a is an abundant protein in the periplasm of C. jejuni, and this was further confirmed by a 2D-gel separation of wild-type cell-free extract (Fig. 3C) and periplasm (Fig. 3D). In periplasmic samples, MALDI-TOF mass spectroscopy of tryptic digests of spot picks confirmed that two very abundant isoforms (approximate pl values of 7.8 and 8.4) of the correct molecular size were both PEB1a (Fig. 3D). One of these was also positively identified by mass spectroscopy of spot picks from a total-cell free extract gel (Fig. 3C).

PEB1a is not found in the inner or outer membrane but is detectable in culture supernatants

In addition to a periplasmic location, some PEB1a must be exposed on the cell surface in order for the protein to act as an adhesin. Previous evidence for this has been obtained from immunogold labelling studies (Kervella et al., 1993) and the fact the protein was originally isolated from acidic glycine extracts (a treatment thought to preferentially release surface proteins; Pei et al., 1991; Kervella et al., 1993). However, the presence of a signal peptidase II recognition site (predicted by LipoP v1.0 at the sequence 13-FALGA/CVAFS with a log-odds score of 2.6) may result in the protein being anchored in the inner or outer membrane via acylation of Cys18. Figure 4 shows a fractionation experiment where cytoplasmic, periplasmic, inner and outer membrane fractions, as well as an acid-glycine extract of intact cells and a concentrated culture supernatant were subjected to SDS-PAGE and immunoblotting with PEB1a antibody. PEB1a was detected in
the periplasm, glycine extract and culture supernatant, but not in the inner or outer membrane fraction. Some PEB1a was present in the cytoplasmic fraction but this is almost certainly due to inevitable periplasmic contamination (see Experimental procedures). The lack of significant amounts of PEB1a in either membrane fraction suggests that the protein may have only a transient lipid membrane anchor, but the presence of the protein in the culture supernatant and glycine extract are consistent with the conclusion of Pei and Blaser (1993) that at least some PEB1a can be translocated across the outer membrane to the cell surface, although the mechanism for this is unknown.
In order to determine the importance of the PEB1 system in amino acid uptake, transport rates of L-14C-asp and L-14C-glu into *C. jejuni* wild type and mutant strains were measured in cells grown to early stationary phase under standard microaerobic conditions in BHI-FCS medium. Assay conditions were established with wild-type cells using 5 µM substrate, which gave linear uptake rates proportional to the amount of cell protein added. As clearly shown in Fig. 5A and B, mutation of *Cj0921c* resulted in a dramatic reduction in the transport rates of both L-asp and L-glu. For L-glu no uptake could be observed over the course of the assay and for L-asp, the rate was ∼20-fold lower than the wild-type parent strain (0.3 ± 0.12 and 6.7 ± 1.4 nmol min⁻¹ mg cell protein⁻¹ respectively). These data suggest that the PEB1 system is responsible for essentially all of the high-affinity glutamate and most of the aspartate transport in *C. jejuni* grown under microaerobic conditions. This enabled an estimation to be made of the affinity ($K_T$) of the PEB1 system for L-asp and L-glu in intact wild-type cells, by measuring uptake rates over a range of amino acid concentrations. For both amino acids, the data showed monophasic hyperbolic saturation kinetics, with fitted $K_T$ values of 4.7 ± 1.7 µM for L-asp and 1.4 ± 0.6 µM for L-glu. The corresponding $V_{max}$ values were similar for both amino acids; 12.6 ± 6.3 and 7.8 ± 1.8 nmol min⁻¹ mg⁻¹ cell protein for L-asp and L-glu respectively.

**Specificity of the PEB1 system**

Figure 5C and D show results of a competition experiment, where the cellular uptake of L-14C-asp (Fig. 5C) or L-14C-glu (Fig. 5D) was determined in the absence or presence of an excess of unlabelled competitor amino acid. As expected, a 10-fold excess of unlabelled L-glu resulted in a greatly lowered uptake rate of L-14C-asp and vice versa ($P < 0.05$ in Students t-test). However, despite some evidence for albeit weak binding of L-asn and L-gln to purified PEB1a (Fig. 2B), these amino acids did not compete significantly for uptake of either L-14C-asp (Fig. 5C; $P > 0.7$) or L-14C-glu in intact cells (Fig. 5D; $P > 0.2$). These data would suggest that the PEB1 system is specific for only L-asp and L-glu transport.

**PEB1a is essential for microaerobic growth of *C. jejuni* on both L-aspartate and L-glutamate**

The dramatic difference in uptake rates seen with the *Cj0921c* mutant implies that the PEB1 system is the major transporter for dicarboxylic amino acids in *C. jejuni*. The impact of removal of this system on cell growth was investigated by experiments in complex and defined media. In complex BHI-FCS media, the growth rate (doubling time of ∼2 h) and final cell density reached after 10 h was the same for both wild-type and mutant strains, although the mutant strain showed a reproducible slight lag in initiating growth. However, in MEM-$\alpha$ (Fig. 6) with either L-asp or L-glu as the major added carbon sources, no growth occurred above the level of the control with no
added carbon source, whereas with an unrelated amino acid (L-serine) the final cell-densities reached for both mutant and wild type were similar and much higher than the control. It should be noted that some background growth always occurs in commercial MEM-α medium (see Fig. 6, first panel), as it contains a low concentration of several amino acids. Nevertheless, the data clearly show that the PEB1 system is essential for growth under microaerobic conditions on either L-asp or L-glu but not L-ser.

**1H-NMR analysis of amino acid utilization**

The cell-free supernatants from the growth experiment in Fig. 6 after 16 h were analysed by 1H-NMR spectroscopy to determine the residual concentrations of the amino acid substrates and the appearance of any metabolic end-products. The spectrum of MEM-α with no added carbon sources shows resonances in the 3.4–4.0 p.p.m. region mainly due to glucose, which is not used by C. jejuni (Velayudhan and Kelly, 2002) and additional small resonances at 2.4 p.p.m and below due to amino acids (mainly glutamine) present in the medium (Fig. 7A). In Fig. 7B, analysis of the spectra from control cultures where 40 mM serine was initially present clearly shows essentially complete utilization of this amino acid during growth by both the wild type and Cj0921c mutant (approximate residual concentrations of 0.7 and 0.4 mM after 16 h as determined by integration of the Hα + Hβ peaks at 3.9 p.p.m). Acetate was produced as a significant end-product in both strains (accumulating to 11.4 mM in the wild type and 7.8 mM in the mutant), presumably via the phosphotransacetylase-acetate kinase pathway from acetyl-CoA produced via pyruvate, which is the direct deamination product of serine (Velayudhan et al., 2004).
Campylobacter jejuni PEB1 transport system

Growth on 40 mM aspartate (Fig. 7C) resulted in incomplete utilization in the wild type (compare top and middle spectra in Fig. 7C) with 17 mM aspartate still remaining after 16 h. However, although the Cj0921c mutant was unable to grow on aspartate (Fig. 6) integration of the aspartate H_β+H_β peaks at 2.6–2.9 p.p.m. showed that the cells metabolized this amino acid to some extent, with 27 mM remaining after 16 h (compare the top and bottom spectra in Fig. 7C). Some succinate (2.4 p.p.m. resonance) was produced as an end-product by wild-type cells, but this was barely detectable in the mutant. Succinate results from conversion of aspartate to fumarate via aspartase, followed by reduction via fumarate reductase.

In contrast, analysis of the ¹H-NMR spectra of the supernatants of glutamate grown cells (Fig. 7D) showed that the Cj0921c mutant did not utilize this amino acid at all, with 41 mM remaining in the supernatant after 16 h. As with aspartate, incomplete utilization of glutamate was observed in the wild type (23 mM remaining after 16 h). A small amount of acetate was produced as an end-
product in the case of the wild type but not the mutant cells.

Discussion

In this article we have built upon the work of Kervella et al. (1993) and Pei and Blaser (1993) who originally identified PEB1a as an adhesin but also recognized it as a homologue of the periplasmic-binding component of amino acid ABC transporters. However, the exact transport function of PEB1a has not been investigated in previous studies. Using overexpressed and purified PEB1a we have shown using both fluorescence spectroscopy and radiolabelled ligand-binding experiments that the protein binds the two dicarboxylic amino acids L-aspartate and L-glutamate with high and approximately equal affinity ($K_d$ values for both ligands below 1 µM). Two lines of evidence suggested that asparagine and glutamine can bind to PEB1a with very low affinity; (i) a small degree of fluorescence quenching observed at high concentrations of these amino acids (ii) competition with L-glutamate binding observed at a 10-fold molar excess. Nevertheless, this weak interaction appears not to be physiologically significant as neither asparagine nor glutamine competed with the transport of aspartate or glutamate in uptake assays.

The identification of the PEB1a protein on 1D and 2D gels (Fig. 3) clearly showed that this is one of the most abundant periplasmic proteins in C. jejuni grown to early stationary phase in complex media. Two isoforms were confirmed by MALDI-TOF, with approximate pI values of 7.8 and 8.4. The predicted pI of mature PEB1a processed at the signal peptidase I cleavage site is 8.44, and that for the protein processed at the signal peptidase II cleavage site is 8.41, too small a difference to account for the less basic of the observed isoforms, so it is not clear how this species originates. The vast majority of PEB1a in C. jejuni is clearly periplasmic, consistent with its solute-binding function, but some must be surface exposed in order for the protein to act as an adhesin. The presence of a signal peptidase II recognition site might suggest that the protein could be anchored in the outer membrane, but we could find no evidence for this from our fractionation and localization study (Fig. 4). Rather, we confirmed the presence of PEB1a in acidic glycine extracts and also detected some protein in concentrated culture supernatant samples, supporting the suggestion of Pei and Blaser (1993) that the protein can be exported across the outer membrane. Although the mechanism for this is unclear, the two-component signal peptide of PEB1a is extremely similar to that of endoglucanase (Egl) from Pseudomonas solanacearum (Huang and Schell, 1992; Pei and Blaser, 1993), the two-step export of which has been studied in detail. Egl is first directed across the inner membrane to the periplasm by the lipoprotein signal peptide, and is then further cleaved at the signal peptidase I site during export across the outer membrane (Huang and Schell, 1992). A similar process may apply to PEB1a, although in this case most of the fully mature protein appears to remain in the periplasm. Interestingly, PEB1a is not the only differentially localized solute-binding protein in C. jejuni with an unusual signal peptide. Recently, another C. jejuni immunodominant surface antigen and vaccine candidate, CjaA (Cj0982) has been shown to be a cysteine-binding protein component of an ABC-system (Müller et al., 2005). The signal peptide of CjaA contains a very strongly predicted recognition sequence for signal peptide II (LipoP v1.0 log odds score of 22.5) but a much less convincing atypical signal peptidase I site (Signal P v3.0 probability of 0.2), so the sorting mechanism may not be the same as for PEB1a. Indeed preliminary data suggest that CjaA is mainly located in the inner membrane (Godlewski et al., 2005).

Willis and Furlong (1975a,b) originally identified a dual glutamate-aspartate-binding protein from osmotic shock fluid of E. coli, with $K_d$ values of 0.7 and 1.2 µM, respectively, for glutamate and aspartate. This is the product of the $gltI$ (ybeJ) gene. PEB1a shares 28% amino acid identity with GltI, and other GltI homologues are widely distributed in sequenced bacterial genomes, although none has been characterized in detail. The organization of the $gltIJKL$ operon in E. coli is similar to Cj0921c–Cj0924c, with two genes encoding the integral membrane proteins and a single gene encoding the ABC-protein. As expected, the corresponding E. coli and C. jejuni gene products share significant sequence similarity. However, the relative importance of this type of transport system in the two bacteria appears to be very different.

In E. coli, there are three genetically distinct systems for L-glutamate transport, but the combined activity of all three systems in wild-type cells does not allow growth of E. coli on L-glutamate as a sole carbon source. However, growth does occur if the expression of any one of them is increased (Halpern and Lupo, 1965; Schellenberg and Furlong, 1977). In addition to GltJKL, there are two binding protein-independent secondary transporters, encoded by the $gltS$ and $gltP$ genes. GltS is an L-glutamate-specific, sodium-dependent system, which has been estimated to account for about 25% of the total uptake of glutamate by intact cells grown in batch cultures (Schellenberg and Furlong, 1977; McFall and Newman, 1996). GltP in contrast is able to transport both L-glutamate and L-aspartate in a sodium-independent manner but with a slightly lower affinity (estimated $K_d$ values of 1.5 µM for GltS vs. 5 µM for GltP; McFall and Newman 1996). This appears to be the major glutamate transporter in E. coli, accounting for about 60% of the total uptake rate (Schellenberg and Furlong, 1977; McFall and Newman, 1996). Thus, the binding protein-dependent GltIJKL system

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transport function of PEB1a and the PEB1 system in addition to its well characterized role as an adhesin, the catabolized. From the results reported here, it is clear that serine, proline, aspartate, asparagine, glutamate and glutamine are capable of being fully catabolized. Results obtained with glutamate, NMR analysis revealed no evidence for metabolism of even high concentrations of glutamate over a 16 h period in the mutant. Nevertheless, although BLAST searches of the C. jejuni genome sequence with the E. coli GltS sequence show no similar proteins, when using GltP as the query good hits are obtained to several proteins which may yet prove to have a role in glutamate transport under different conditions; Cj1192 (annotated as similar to the aerobic C4-dicarboxylate transporter, DctA), Cj0025c (putative proton symporter) and Cj1097 (putative transport protein).

Mutation of Cj0921c reduced significantly, but did not completely abolish L-aspartate transport. This impairment prevented microaerobic growth of the mutant, at least within the 16 h time frame of this experiment, on 40 mM L-aspartate as the major carbon source. In contrast to the results obtained with glutamate, NMR analysis revealed some metabolism of aspartate was still occurring in these cultures, consistent with the low rate of uptake observed in the transport experiments. These results show that the PEB1 system is of prime importance for aspartate uptake, but is not the only transport route. Additional potential systems for aspartate transport include DctA (Cj1192) and the DcuA (Cj0088) and DcuB (Cj0671), proteins, which are homologues of the well characterized E. coli anaerobic C4-dicarboxylate transporters involved in fumarate respiration (Six et al., 1994; Unden and Kleefeld, 2004). In C. jejuni, dcuA is located immediately downstream of the aspA gene encoding aspartase and is upregulated under oxygen-limited conditions (Woodall et al., 2005), consistent with a role in transporting aspartate as a precursor for fumarate respiration.

Due to the absence of a phosphofructokinase gene, C. jejuni is apparently unable to catabolize sugars, and the inability to utilize glucose has been shown previously (Velayudhan and Kelly, 2002). Amino acids are thought to be important carbon and energy sources for in vitro as well as in vivo growth from both experimental data (Leach et al., 1997; Velayudhan et al., 2004) and predictions of catabolic pathways from genome analysis (Kelly, 2005), which indicates that only serine, proline, aspartate, asparagine, glutamate and glutamine are capable of being fully catabolized. From the results reported here, it is clear that in addition to its well characterized role as an adhesin, the transport function of PEB1a and the PEB1 system in toto is key to the ability of C. jejuni to catabolize aspartate and glutamate, two common amino acids likely to be present in gut contents. This suggests a re-interpretation of the in vivo significance of PEB1a, as the colonization defect previously reported for a peb1a mutant in the mouse model of infection (Pei et al., 1998) could actually be due to one or both of these functions. In order to assess the in vivo contribution of adhesion versus transport, it would be informative to study the colonization potential of a strain which produced an engineered PEB1a unable to bind its ligands but otherwise wild type for localization and adhesion.

Experimental procedures

Bacterial strains, media and culture conditions

Campylobacter jejuni strain NCTC 11168 was routinely cultured at 37°C under microaerobic conditions (10% [v/v] O2, 5% [v/v] CO2 and 85% [v/v] N2) in a MACS growth cabinet (Don Whitley Scientific, Shipley, UK) on Columbia agar containing 5% (v/v) lysed horse blood and 10 μg ml−1 of each amphotericin B and vancomycin. LC cultures of C. jejuni were routinely grown microaerobically at 200 r.p.m., either in brain heart infusion supplemented with 5% (v/v) foetal calf serum (BHI-FCS) or in the defined medium MEM-α (In vitroGen. Catalogue number 41061–029, containing glutamine and deoxyribonucleotides but no phenol red), both containing the above antibiotics and 50 μM FeSO4. For cell fractionation experiments, Mueller-Hinton broth (Scientific Laboratory Supplies, Nottingham, UK) containing 20 mM sodium pyruvate was used as an alternative serum-free complex medium which allowed good growth. To select for C. jejuni Cj0921c mutants, chloramphenicol was added to media at a final concentration of 30 μg ml−1. E. coli DH5α was cultured in Luria–Bertani (LB) supplemented with appropriate antibiotics at 37°C. For growth assays, C. jejuni overnight starter cultures were prepared in BHI before inoculation into fresh BHI or MEM-α to which 40 mM of the corresponding amino acids were added. Growth was monitored at 600 nm in an Amer sham Pharmacia Biotech Ultrospec 2000 spectrophotometer. No differences in results were obtained if cells grown in MEM-α plus serine were used as the inoculum instead of BHI grown cells.

Cell fractionation and isolation of periplasmic proteins from C. jejuni

A procedure to isolate cytoplasm, periplasm and a total membrane pellet was modified from the method of Myers and Kelly (2005). Cultures grown in Mueller-Hinton media containing 20 mM sodium pyruvate (4 × 250 ml) were grown for 16 h at 37°C under microaerobic conditions, harvested by centrifugation at 4800× g for 30 min and the cell pellet resuspended in 20 ml of ST buffer (20% w/v sucrose, 30 mM Tris-HCl pH 8.0) at room temperature. EDTA to a final concentration of 1 mM was then added and the cell suspension was shaken gently at room temperature for 10 min. The cells were centrifuged at 8000× g for 10 min at room temperature. The supernatant was removed and the pellet resuspended in 10 ml of

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ice-cold 10 mM Tris-HCl pH 7.5 and stirred gently at 4°C for 10 min. Finally, the osmotically shocked cell suspension was centrifuged at 15 000 × g at 4°C for 15 min. The supernatant corresponded to the periplasmic fraction, as judged by high soluble cytochrome c content (periplasmic marker) and low isocitrate dehydrogenase activity (cytoplasmic marker), using the assays described by Myers and Kelly (2005). The pellet was resuspended in 5 ml of ice-cold 10 mM Tris-HCl pH 7.5, sonicated (Soniprep 150, Sanyo, Japan) by three bursts of 30 s at 6 µm amplitude. The suspension was then centrifuged (4°C for 10 min at 13 000 × g) and the broken cell pellet discarded. The clarified supernatant was then ultracentrifuged at 100 000 × g at 4°C for 1 h. The supernatant was retained as the cytoplasmic protein fraction. The dark pellet obtained was the total membrane fraction, which was further treated as described below. Quantification of total cytochrome c (Myers and Kelly, 2005) showed that the cytoplasmic fraction was contaminated with about 5–10% of the periplasmic fraction.

Isolation of inner and outer membranes

Differential solubilization of the inner and outer membrane using sarkosyl has previously been shown to be suitable for \textit{C. jejuni} (Logan and Trust, 1982) and was used here according to the method of Carlone et al. (1986). The total membrane pellet obtained above was washed several times with ice-cold 10 mM Tris-HCl, pH 7.5 and thoroughly suspended in 0.2 ml of 10 mM HEPES buffer (pH 7.4). The inner membranes were solubilized by addition of an equal volume of 2% sodium N-lauroylsarcosinate (Fluka-BioChemika) in 10 mM HEPES (pH 7.4) and incubated at room temperature for 30 min with intermittent mixing. The outer membranes were then pelleted by centrifugation at 15 600 × g for 30 min at 4°C; the supernatant was recovered as the solubilized inner membrane fraction and the pellet was washed with 0.5 ml of 10 mM HEPES buffer, before final resuspension in 200 µl HEPES buffer. This fraction corresponded to the outer membrane; SDS-PAGE showed a characteristic protein profile very similar to that reported by Logan and Trust (1982) and distinct from the inner membrane.

Acidic glycine extraction of surface proteins

The method followed was a modification of that described by McCoy et al. (1975). Stationary-phase cells grown microaerobically at 37°C in Mueller-Hinton medium supplemented with 20 mM sodium pyruvate, were harvested by centrifugation at 6000 × g at room temperature. The supernatant was filtered through a 0.2 µm nitrocellulose filter to remove residual cells and dialysed exhaustively against 10 mM Tris-HCl pH 8.0 at 4°C. After dialysis, the supernatant was concentrated about 50-fold by using Aquacide I (Calbiochem) and stored at –20°C.

DNA isolation and manipulation

Plasmid DNA was isolated by using the Qiagen miniprep kit (Qiagen, Crawley, UK). \textit{C. jejuni} chromosomal DNA was extracted by using the Wizard® Genomic DNA purification kit (Promega, Madison, USA). Standard techniques were employed for the cloning, transformation, preparation, and restriction analysis of plasmid DNA from \textit{E. coli} (Sambrook et al., 1989).

Overexpression and purification of PEB1a

For the Overexpression of the \textit{Cj0921c} gene product, primers (forward, 5'–ATCGGAATTCCATAGTTTTTTGGGAAAATCTTT GTTA-3' and reverse 5'–ATCGGAATTCCGAATCTCATTATA AACCCCCATTTTTTTC-3') EcoRI sites underlined, Ndel and BamHI restriction sites in italics) were designed to amplify the complete \textit{Cj0921c} gene from \textit{C. jejuni} 11168 chromosomal DNA by PCR using a proofreading DNA polymerase enzyme (Tgo, Promega, UK). The PCR product was cloned into the EcoRI site of pGEM3ZF (Promega, UK), the gene excised with Ndel-BamHI digest, then cloned into similarly restricted pET21a(+) to give pMK3. Automated DNA sequencing (Lark Technologies, Saffron Walden, UK) showed that the sequence of the \textit{Cj0921c} gene in pMK3 was correct. pMK3 was transformed into \textit{E. coli} BL21 (DE3) cells which were grown aerobically at 25°C in LB medium containing ampicillin (100 µg ml⁻¹) to an optical density at 600 nm of 0.6 and then 1 mM isopropyl-β-D-thiogalactopiranoside (IPTG) was added. Induced cells were then grown for a further 16 h before harvesting by centrifugation (30 min, 4°C, 4200 × g). Cell pellets were treated for isolation of periplasmic proteins by osmotic shock; briefly, the cell pellet was resuspended in 10 ml of STE buffer (0.5 M sucrose, 10 mM Tris-HCl pH 8.0, 2 mM EDTA), 100 µg ml⁻¹ of lysozyme was added and the suspension was kept on ice for 30 min 20 ml deionized water was then added and the mixture centrifuged (30 min, 4°C, 10 000 × g). The supernatant was recovered as the periplasmic fraction and fractionated on a CM-Sepharose® Fast flow column (Pharmacia Biotech AB, Uppsal, Sweden) by ion-exchange chromatography. The protein was eluted from the resin by a step gradient from 0 to 500 mM NaCl in 10 mM Tris-HCl, pH 7.0 before being desalted by dialysis against 10 mM Tris-HCl, pH 7.0 and concentrated using Aquacide I (Calbiochem). The identity of the purified protein was confirmed by N-terminal sequencing by the automated Edman method, performed by Dr A. Moir, Molecular Biology and Biotechnology Department, University of Sheffield, and by Western blot using a polyclonal anti-PEB1a antibody raised in rabbits (a gift from Dr Z. Pei) and described in Pei et al. (1991).
Inactivation of the Cj0921c gene

For inactivation of the Cj0921c gene, a 1020 bp fragment containing the entire coding region plus 240 bp of upstream sequence was PCR-amplified (forward primer, 5′-ATCGGAAATTGTTAATGATTGTA-3′; and reverse primer, 5′-ATCGGAATTCCGATCTTGTTATATAACCCATTATTTCGC-3′, EcoRI sites underlined) from C. jejuni 11689 chromosomal DNA, using a proof-reading DNA polymerase (Pwo; Promega, UK), cut with EcoRI and cloned into pGEM3ZF (Promega) to form pMK4. A chloramphenicol acetyltransferase (cat) cassette originating from C. coli (Wang and Taylor, 1990) was then cloned into a unique SpeI site in Cj0921c to form pMK5. Antibiotic-resistant constructs were introduced into C. jejuni 11689 by electroporation.

Transformants were selected on Columbia blood agar plates supplemented with chloramphenicol at a final concentration of 30 µg ml\(^{-1}\). Single colonies were then restreaked, and chromosomal DNA was extracted for screening by PCR, using the above primers, to verify that allelic exchange by double homologous recombination had occurred. In all cases, PCR analysis of chloramphenicol resistant colonies showed an increase in amplicon size of ~800 bp compared with that obtained with the wild type, due to insertion of the cat cassette.

**L-\(^{14}\)C-glutamate and L-\(^{14}\)C-aspartate-binding assays**

The purified PEB1a protein (2 µM), diluted in 10 mM Tris-HCl pH 8.0, was mixed with either L-\(^{14}\)C-glutamate or L-\(^{14}\)C-aspartate (6.9–9.36 GBq mmol\(^{-1}\)) alone at a final concentration of 5 µM or with unlabelled L-asp, L-glu, L-asn, L-gln, or L-his at final concentrations of 50 or 500 µM before addition of either radiolabelled L-asp or L-glu to a final concentration of 5 µM. BSA at 2 µM was used as a negative control in place of PEB1a. The reactions (total volume 0.1–0.2 ml) were left on ice for 15 min, after which time 1 ml of saturated ammonium sulphate was added and left on ice for a further 20 min. The samples were filtered through 0.45 µM nitrocellulose membranes and washed with 4 ml of saturated ammonium sulphate. Filters were then immersed in Filter-Count scintillation cocktail (Perkin Elmer; Boston, MA) and were counted in a Beckman Coulter\textsuperscript{TM} LS6500 (USA) scintillation counter.

**L-\(^{14}\)C-glutamate and L-\(^{14}\)C-aspartate transport assays**

Campylobacter jejuni cells grown for 16 h in 20 ml of BHI-FCS batch cultures were harvested by centrifugation, washed twice, and resuspended in 1 ml of M9 minimal medium, pH 7.4 and kept on ice for no longer than 4 h. An aliquot (100–50 µl) of the cell suspension was added to 2 ml of M9 minimal medium containing 0.5% (w/v) sodium lactate and was allowed to equilibrate by gently stirring at 37°C for 3 min. The assay was then initiated by the addition of the specified concentration of the \(^{14}\)C-labelled amino acid (6.9–9.36 GBq mmol\(^{-1}\)). In competition experiments, unlabelled amino acid was added to the assay mixture 1 min before the labelled amino acid. Samples (0.1 ml) were withdrawn at the indicated time points, collected by vacuum filtration through membrane filters (0.45-µm pore size), and washed twice with stop buffer (M9 minimal medium supplemented with 10 mM of the unlabelled amino acid). Sample filters were then immersed in Filter-Count scintillation cocktail (Perkin Elmer; Boston, MA) and were counted in a Beckman Coulter\textsuperscript{TM} LS6500 (USA) scintillation counter.

**Steady-state fluorescence spectroscopy**

Slow timescale fluorescence spectroscopy was performed using a Hitachi F-2500 spectrofluorimeter with an excitation wavelength of 280 nm (slit width 5 nm) and an emission wavelength of 345 nm (slit width 10 nm). The assay mixture consisted of either 0.1 µM (for screening) or 0.05 µM (for titration) of PEB1a protein in 1.5 ml 10 mM Tris-HCl pH 8.0. The sample cuvette was maintained at 25°C in the spectrofluorimeter housing and the assay mixture was continuously stirred. Fluorescence changes upon the addition of ligands were monitored until the fluorescence change stabilized and were corrected for dilution. For calculation of \(K_d\) values, data were averaged from at least three separate titrations and the hyperbolic curve fitting algorithms in SigmaPlot used to analyse the data.

**Proteome analysis by 2D-gel electrophoresis**

Methods for the analysis of C. jejuni proteins on 2D gels closely followed those described in Holmes et al. (2005). For the first dimension, 100–200 µg total cell-free extract or periplasmic protein was mixed with an IPG rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 18 mM dithiothreitol (DTT), bromophenol blue and 2% pH 3-10 NL non-linear IPG buffer (final volume 400 µl) before loading onto 18 cm 3–10NL Immobiline DryStrips (Amersham Biosciences). Following overnight rehydration, IEF was performed for 80 kVh at 20°C over 24 h using the pHaser system (Genomic Solutions). The focussed strips were conditioned in Tris-acetate equilibration buffer (Genomic Solutions) containing 5 mg ml\(^{-1}\) SDS, 360 mg ml\(^{-1}\) urea and 300 mg ml\(^{-1}\) glycerol. To reduce and alkylate cysteine residues, the strips were sequentially treated with equilibration buffer containing 8 mg ml\(^{-1}\) DTT, and then equilibration buffer containing 25 mg ml\(^{-1}\) iodoacetamide (each treatment 9 ml of buffer, 30 min with shaking). Second dimension 10% duracryl gels (28 × 23 cm, 1 mm thick) were prepared for use in the Investigator 2nd Dimenson Running System (Genomic Solutions), with electrophoresis at 500 V or 20 W per gel. Proteins were stained by Sypro-Ruby (Bio-Rad) and the gels imaged using the ProX-PRESS Proteomics Imaging system with ProFinder Imaging software (Perkin Elmer Life Sciences). Protein spots were excised from the gel using the ProPick excision robot (Genomic Solutions) and in-gel tryptic digestion performed as described by Holmes et al. (2005). Tryptic digests were analysed using a Reflex III MALDI-TOF instrument (Bruker). Proteins were identified by the Protein Mass Fingerprint technique using the Mascot search tool (Matrix Science; http://matrixscience.com).

**\(^{1}\)H-NMR spectroscopy**

Culture samples (1.5 ml) were centrifuged to remove cells (13 800× g, 5 min) and the supernatants used for NMR analysis. \(^{1}\)H-NMR was carried out using a Bruker DRX500 spectrometer operating at 500 MHz. Spectra were acquired into
4096 complex points over a spectral width of 12.5 kHz and the solvent \((\text{H}_2\text{O})\) signal reduced by presaturation for 2 s. Samples (0.45 ml of culture supernatant plus 0.05 ml of \(\text{D}_2\text{O}\)) were run in 5 mm diameter tubes at 25°C. Chemical shifts and concentrations were established by reference to 1 mM trimethylsilylpropionate (TSP; 0 p.p.m) added to the samples.

For quantitation, integration was performed by measurement of peak area using FELIX (Accelerys, San Diego, CA). For amino acid quantitation, the following signals were used: serine \((\text{H}_2\text{N} + \text{H}_4\text{O})\) at 3.9 p.p.m. (using only the left hand side of the peak to avoid overlap with signals from medium at 3.8 p.p.m); aspartate \((\text{H}^\beta + \text{H}_3\text{O})\) at 2.6–2.9 p.p.m.; glutamate \((\text{H}^\beta + \text{H}_2\text{O})\) at 2.0–2.4 p.p.m.

**Prediction of signal peptides**

For prediction of lipoproteins and signal peptide II cleavage sites, LipiP version 1.0 was used (Juncker et al., 2003) and for prediction of signal peptide I cleavage sites SignalP ver 3.0 (Bendtsen et al., 2004) was used, both at http://www.cbs.dtu.dk/services/.

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