Enterochelin acquisition in *Campylobacter coli*: characterization of components of a binding-protein-dependent transport system

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Siderophore-mediated iron uptake systems play a central role in the pathogenesis of infection for many bacterial pathogens. *Campylobacter* species are not thought to produce siderophores, yet they are able to utilize both ferrichrome and enterochelin as sources of iron. Part of an operon named *ceuBCDE*, encoding components of a periplasmic binding-protein-dependent transport (PBT) system for the uptake of a ferric siderophore from *Campylobacter coli*, was cloned directly into *Escherichia coli* using a plasmid rescue technique. Phenotypic and genetic analyses of this system showed it to comprise two hydrophobic integral membrane proteins, CeuB (35.5 kDa) and CeuC (34.8 kDa), which may form the cytoplasmic membrane permease, an ATP-binding protein, CeuD (28.8 kDa), and a periplasmic substrate-binding protein, CeuE (34.5 kDa). *In vivo* labelling studies using [³H]palmitate demonstrated that CeuE, the periplasmic binding protein, is expressed as a lipoprotein in *C. coli*, which is unusual for a Gram-negative PBT system. Mutants of *C. coli*, defective in components of the transport mechanism, were severely impaired in the ability to utilize enterochelin as an iron source suggesting that this siderophore is a substrate for the transport system. This is the first molecular characterization of a PBT system in *Campylobacter* species.

Keywords: *Campylobacter coli*, enterochelin, lipoprotein, siderophore, *ceu*

INTRODUCTION

Campylobacter coli and *Campylobacter jejuni* are now recognized to be one of the major causes of bacterial gastroenteritis in the developed world (Tauxe, 1992). In addition, these pathogenic bacteria are also carried as commensals in a wide variety of animals which may form reservoirs for the human disease (Griffiths & Park, 1990). Despite the epidemiological significance of this group of organisms the pathophysiology of infection in this group of bacteria is poorly understood (Walker *et al.*, 1986). This is partly due to lack of characterization of the genetic elements important to virulence and inadequate animal models to test potential virulence determinant mutants.

Iron is essential for the growth of most bacteria but has very low solubility under physiological conditions. Furthermore, in humans the availability of iron for microbial

assimilation is limited because of its sequestration by high-affinity iron-binding proteins such as transferrin and lactoferrin which occur in serum and at mucosal surfaces (Weinberg, 1984). The ability to acquire this essential element under iron-limited conditions present within the host, therefore, is considered to be critical to the virulence and survival of a number of bacterial pathogens (Payne & Lawlor, 1990). As a response to this nutritional restriction bacteria have evolved highly efficient systems for sequestering iron (Briat, 1992; Wooldridge & Williams, 1993). A strategy for iron sequestration employed by many micro-organisms comprises the secretion of highly specific chelators for ferric ions, termed siderophores (Neilands, 1981; Braun & Hantke, 1991), followed by their re-internalization via a cognate high-affinity transport system and the subsequent release of iron into the cytoplasm (Crosa, 1989; Braun & Hantke, 1991).

Successful colonization of the gastrointestinal tract requires effective mechanisms for acquiring iron in this complex nutritional milieu. *Campylobacter* spp. do not, however, appear to produce detectable levels of siderophores (Baig *et al.*, 1986). It is likely, therefore, that these

Abbreviations: EDDHA, ethylenediamine-di-o-hydroxyphenylacetic acid; PBT, periplasmic binding-protein-dependent transport.

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bacteria can compete successfully in the heterogeneous competitive environment of the gastrointestinal tract by scavenging siderophores produced by the indigenous microflora. For instance, strains of both *C. coli* and *C. jejuni* are able to acquire iron from enterochelin (Baig *et al.*, 1986; Field *et al.*, 1986), the phenolate-type siderophore produced by *Escherichia coli* and other enteric bacteria (Neilands, 1981). The transport of siderophores in other Gram-negative bacteria is well characterized yet very little is known about the transport systems for their uptake in *Campylobacter* spp. Gram-negative bacteria have two membranes, an outer one and an inner one, hence transport of siderophores must occur in two steps. Firstly, the complex is transported across the outer membrane into the periplasm via a specific receptor. Transport of siderophores across the cytoplasmic membrane then occurs via a periplasmic binding-protein-dependent (PBT) system (Braun & Hantke, 1991). All known PBT systems consist of a periplasmic component, one or two hydrophobic components embedded within the cytoplasmic membrane, and a peripheral cytoplasmic component most likely involved in energy coupling (Ames *et al.*, 1990; Higgins, 1992). Analogous systems exist in Gram-positive bacteria (Alloing *et al.*, 1990; Schneider & Hantke, 1993) but, in the absence of an outer membrane, there is no requirement for outer-membrane receptors. Instead, the binding-protein component is lipophilically modified and this is thought to facilitate its anchorage to the cytoplasmic membrane preventing its escape into the extracellular environment (Gilson *et al.*, 1988).

In a previous study, we identified a lipophilically modified protein from *C. jejuni* which showed significant homology to periplasmic siderophore-binding proteins (Park & Richardson, 1995). Here we identify its homologue in *C. coli* and, by characterizing genes upstream of the siderophore-binding protein, demonstrate that it is a component of a binding-lipoprotein-dependent transport system for enterochelin uptake. This is the first molecular characterization of a PBT system for any *Campylobacter* species.

METHODS

Bacterial strains and culture. The strains and plasmids used in this study are listed in Table 1. *Campylobacter* cultures were routinely grown at 37 °C on Mueller-Hinton (MH) agar under microaerophilic conditions using the recommended gas generating kit (Oxoid). *E. coli* JM101, used during plasmid construction, was grown aerobically in LB-broth or on LB-agar (Gibco BRL). For testing the ability of strains of *C. coli* to utilize haemin and enterochelin, bacteria were seeded in MH-agar containing 56 µM ethylenediamine-di-*o*-hydroxyphenylacetic acid (EDDHA; Sigma) which had been deferrated by the procedure of Rogers (1973). Filter paper discs impregnated with 10 µl of 100 µM or 1 mM concentrations of either haemin (Sigma) or enterochelin (kindly provided by J. B. Neilands, University of California) were placed upon the agar and the plates incubated for 24 h to assess the growth stimulation. When necessary, the antibiotics ampicillin and tetracycline were used at concentrations of 100 µg ml⁻¹ and 10 µg ml⁻¹, respectively.

Cloning of the *ceuBCDE* operon by plasmid rescue. A DNA fragment containing part of the *ceuE* gene of *C. coli* UA585 was

generated using PCR. A commercial *Taq* DNA polymerase system (Perkin Elmer Cetus) was used to amplify target DNA (*C. coli* UA585 chromosomal DNA) in the presence of deoxynucleotides (50 µM). A total of 26 cycles was used, each including a 30 s denaturation step at 94 °C, a 30 s annealing step at 45 °C, followed by a 2 min extension step at 72 °C. The primers with homology to *ceuE* had the sequence: 5' CAGCTTGCGATTCTAAATCCA 3' and 5' TCTCTTGGT-GAAGAGAATGT 3'. A 513 bp *Sau3A* fragment, internal to the fragment generated by PCR, was then cloned into the *Bam*HI site of the integrational plasmid pSP105 (Dickinson *et al.*, 1995) and the resulting plasmid was designated pSP120. This plasmid was introduced into *C. coli* UA585 by natural transformation (Wang & Taylor, 1990). The chromosomal DNA from one tetracycline-resistant transformant, designated CCH1, was analysed by Southern hybridization and the *ceuE* gene was shown to contain a single integrated copy of pSP120. Chromosomal DNA was isolated from this strain using the method of Pitcher *et al.* (1989) and the DNA digested with either *Sac*I or *Pst*I to excise the integrated plasmid and flanking DNA. The restricted DNA was diluted to 20 ng µl⁻¹, then ligated and used to transform *E. coli* to ampicillin resistance. The plasmids pS1 and pP1, generated using this procedure, are described in Table 1. The same technique was used to excise the plasmid pS2 (Table 1) from the chromosome of CATP1 using the restriction enzyme *Sph*I.

Recombinant DNA techniques. Standard techniques including plasmid mini preparations, restriction endonuclease digestions, ligations and transformation into *E. coli* were according to standard procedures (Maniatis *et al.*, 1982). Large-scale plasmid preparations were prepared using affinity columns according to the manufacturer's instructions (Qiagen). Double-stranded DNA sequencing was performed using standard methods (Sanger *et al.*, 1977) with [³⁵S]dATPαS and using a Sequenase version 2.0 kit (United States Biochemical). The insert in pS1 was sequenced in its entirety using primers designed to bind to opposing strands at approximately 200 bp intervals allowing sequence data to be obtained independently from both strands. Regions of the nucleotide sequences in the inserts of pP1 and pS2 were determined in the same manner. Oligonucleotides were synthesized using a 391 DNA synthesizer (Applied Biosystems). Computer analysis of the sequence data was carried out using the University of Wisconsin molecular biology software package (Devereux *et al.*, 1984).

Analysis of plasmid-encoded proteins. The polypeptides encoded by pS1 were identified using an *E. coli* *in vitro* coupled transcription/translation system (Promega). Proteins were radiolabelled by incubation with 20 µCi (740 kBq) [³⁵S]methionine (Amersham). Polypeptides were separated on 10% (w/v) SDS-polyacrylamide gels (Laemmli, 1970) using Rainbow ¹⁴C-methylated markers (Amersham) as molecular size standards. Radiolabelled bands were visualized by fluorography at -70 °C.

***In vivo* labelling of iron-repressible lipoproteins with [³H]palmitate.** Lipoproteins were labelled specifically using [³H]palmitate. Cells of *C. coli* UA585 and CCH1 were grown in MH-broth to exponential phase, and iron-repressible protein expression was then induced by the addition of 20 µM deferrated EDDHA which restricted the availability of iron. At this point 20 µCi (740 kBq) [9,10 (n)-³H]palmitate ml⁻¹ (Amersham) was added and the incubation continued at 37 °C for 6 h. Cells were harvested and washed twice in methanol to remove unincorporated radioactive precursors and non-covalently bound lipids. The dried cell pellets were then boiled in SDS-loading buffer and analysed using SDS-PAGE gels as described

Table 1. Bacterial strains and plasmids

Strain/plasmid	Genotype/relevant characteristics	Source/reference
<i>E. coli</i>		
JM101	F' <i>traD36 lacI^q Δ(lacZ)M15 proAB/supE thi Δ(lac-proAB)</i>	Yanisch-Perron <i>et al.</i> (1985)
<i>C. coli</i>		
UA585	Parental strain	Wang & Taylor (1990)
CCH1	<i>ceuE</i> ::pSP120	Current study
CATP1	<i>ceuD</i> ::pSP180	Current study
CFED1	Δ(<i>ceuB-ceuD</i>)	Current study
Plasmids		
pSP105	<i>Campylobacter</i> integrative plasmid, Tet ^R	Dickinson <i>et al.</i> (1995)
pSP120	513 bp <i>Sau3A</i> fragment, internal to <i>ceuE</i> , cloned into pSP105	Current study
pSP180	370 bp <i>BglII/EcoRV</i> fragment, internal to <i>ceuD</i> , cloned into pSP105	Current study
pS1	Plasmid excised from CCH1 with <i>SacI</i> contains 3.2 kb of DNA upstream of <i>ceuE</i>	Current study
pS2	Plasmid excised from CATP1 using <i>SpbI</i> , contains 24 kb of DNA upstream of <i>ceuD</i>	Current study
pP1	Plasmid excised from CCH1 using <i>PstI</i> contains 10 kb of DNA downstream of <i>ceuE</i>	Current study
pCFF1	pTZ19R with a 2.1 kb <i>SacI/EcoRV</i> insert containing <i>cenC</i>	Current study
pCFED1	pCFF1 in which 1.47 kb of DNA internal to the insert was replaced by a Tet ^R marker	Current study
pTZ19R	General cloning vector	Mead <i>et al.</i> (1986)

previously (Laemmli, 1970). Radiolabelled bands were visualized by fluorography at -70°C .

DNA/DNA hybridizations. DNA/DNA hybridizations were carried out using a non-radioactive ECL gene detection kit (Amersham). Chromosomal DNA from various strains of *C. coli* UA585 and CFED1 was restricted using *BglII* and electrophoresed on a 0.8% agarose gel. Gel-electrophoresed DNA was depurinated by gently shaking the gel in 250 mM HCl for 30–45 min and the DNA transferred to Hybond N+ nitrocellulose membranes (Amersham) using an alkali transfer procedure (Maniatis *et al.*, 1982). The DNA insert in pCFF1 or the 1.30 kb *BglII* fragment internal to this was labelled and used as a DNA probe to detect cross-hybridizing species, following the manufacturer's guidelines.

RESULTS

Cloning of the enterochelin-uptake system from *C. coli* by plasmid rescue

An earlier study from this laboratory reported the characterization of lipoprotein from *C. jejuni* which has significant homology to siderophore-binding proteins (Park & Richardson, 1995). A 957 bp DNA fragment, containing part of the gene encoding the equivalent protein in *C. coli*, was isolated using PCR. Subsequently, a 513 bp *Sau3A* fragment, internal to the gene (Fig. 1), was subcloned into the *BamHI* site of the *Campylobacter*

integrational vector pSP105 (Dickinson *et al.*, 1995) to generate the plasmid pSP120. To clone the regions flanking the gene encoding the putative siderophore-binding protein a plasmid rescue method was used. The plasmid pSP120 was introduced into *C. coli* UA585 by natural transformation (Wang & Taylor, 1990) and tetracycline-resistant transformants were recovered. As the plasmid is not capable of extrachromosomal replication the only way for this antibiotic-resistant phenotype to arise is if the plasmid becomes integrated into the chromosome by a single point crossover driven by homologous recombination at the region of homology. One transformant, designated CCH1, was shown by Southern hybridization to contain such an integrated element (data not shown). The integrated derivative of pSP120 present in the chromosome of CCH1 contains an intact copy of the plasmid flanked by genomic DNA and, accordingly, will contain a *colE* replicon and antibiotic-resistance markers which are expressed in *E. coli*. Plasmid DNA containing the sequences flanking the site of integration is generated following ligation of chromosomal DNA which has been digested with any restriction enzyme that has a unique site within the multilinker of pSP120. Digestion of chromosomal DNA from CCH1 with the enzymes *SacI* or *PstI* released integrated plasmids including 3.2 kb of flanking upstream DNA and 10 kb of

Fig. 1. Partial restriction map of the *C. coli* chromosomal region containing the *ceu* loci. The 513 bp *Sau3A* fragment and the 370 bp *BglII*/*EcoRV* fragment cloned into the integrative vectors pSP120 and pSP180 are indicated by the hatched areas. Inserts contained in the plasmids, which were excised by plasmid rescue, and derivatives, are indicated by single lines beneath the restriction map. The extent of the deletion present in *C. coli* CFED1 is denoted by Δ CFED1.

flanking downstream DNA (Fig. 1) which were designated pS1 and pP1, respectively. The plasmid pS2, which contains 24 kb of DNA upstream of the site of integration DNA, was generated in a similar fashion but was excised from CATP1 (Table 1) using *Sph*I.

Sequence analysis and protein expression studies of the enterochelin-uptake operon

To establish whether the gene encoding the lipoprotein with homology to siderophore-binding proteins was part of an operon encoding a PBT system, the nucleotide sequences of the insert in pS1 and parts of inserts in pP1 and pS2 were determined. The operon subsequently characterized in the following pages was designated *ceu* (*Campylobacter enterochelin uptake*). The plasmid pS1 carried a 3.2 kb insert containing the 5' end of the *ceuE* gene (Fig. 1), encoding the lipoprotein with homology to siderophore-binding proteins. In addition, two complete ORFs, designated *ceuD* and *ceuC*, and the 3' end of an ORF termed *ceuB* were apparent in the insert (Figs 1 and 2). The nucleotide sequence of the 5' end of *ceuB* was determined from pS2 since this plasmid contained *C. coli* chromosomal DNA which was originally upstream of the *Sac*I site at which pS1 was excised from the chromosome (Fig. 1). Sequence analysis of part of the insert in pP1, which was proximal to the site of the integration of pSP120, showed it to contain the 3' end of *ceuE*. An unidentified ORF, designated ORFA, and an incomplete ORF (ORFB), which was orientated in the opposite direction, were apparent in the DNA which was downstream of *ceuE* (Figs 1 and 2).

The ORF for *ceuB*, which most likely starts at position 24 and is preceded by a well-defined Shine–Dalgarno sequence (Fig. 2), potentially encodes a protein of 322 amino acids with a calculated molecular mass of 35.5 kDa. The first codon of the next gene, *ceuC*, at position 985 occurs within the reading frame of *ceuB*, indicating that these proteins are translationally coupled. The start codon is preceded by a well-conserved Shine–Dalgarno sequence. *ceuC* encodes a protein of 311 amino acids, the calculated molecular mass of which is 34.8 kDa. This is in good agreement with a protein which was expressed from pS1, using an *in vitro* coupled transcription/translation system, with an observed mass of 34 kDa (Fig. 3). The termination codon of *ceuC* and the start of the next gene, *ceuD*, at position 1920 overlap, suggesting that these two genes are also translationally coupled. In this case, the start

codon again appears to be preceded by a well-defined Shine–Dalgarno sequence. The putative *CeuD* protein of 251 amino acids has a calculated mass of 28.8 kDa, which is consistent with a protein, migrating with an apparent molecular mass of 28 kDa, which is expressed *in vitro* from pS1 (Fig. 3). Thirteen bases separate the termination codon of *ceuD* and the start codon of the next gene, *ceuE*, at position 2689 (Fig. 2), which is preceded by a well-conserved Shine–Dalgarno sequence. *ceuE* encodes a protein of 328 amino acids, the first 21 amino acids of which have the characteristics of a typical lipoprotein signal sequence, with the signal peptidase II consensus recognition sequence $^{18}\text{L-T-A-}^{21}\text{C}$ (von Heijne, 1989; Pugsley, 1993). The mature protein with 308 amino acids (33.7 kDa) and with lipid modification (approximately 789 Da) would have an expected molecular mass of 34.5 kDa. The homologous gene from *C. jejuni*, which contains an identical signal sequence, was expressed as a 33.8 kDa lipoprotein in *E. coli* (Park & Richardson, 1995). The plasmid pS1 carries the 5' end of the *ceuE* gene extending to the *Sau3A* site at position 3589 (Figs 1 and 2), with the potential to encode a polypeptide of 300 amino acids. The immature version of this protein would have an expected molecular mass of 32.9 kDa. No protein of this size was apparent when pS1 was used as a template in an *in vitro* coupled transcription/translation system. Instead, two proteins of 26.0 kDa and 27.0 kDa were expressed uniquely from pS1, and not the parental plasmid pSP105 (Fig. 3). Since these are unlikely to originate from the other characterized ORFs in pS1 they may represent degradation products of the truncated *CeuE* protein. An intergenic region of 166 bp separates the termination codon of *ceuE* from the start codon of the next ORF, designated ORFA. As this region contains a inverted repeat capable of forming a stem–loop structure, typical of a rho-independent terminator, it is likely that *ceuE* represents the last gene in the *ceu* operon and that ORFA is transcribed independently. ORFA has the potential to encode a protein of 210 amino acids with a calculated molecular mass of 22.8 kDa. A search of the GenBank database using the amino acid sequence of the putative polypeptide revealed no significant homologies to any previously characterized protein. In addition, the 3' end of another ORF, designated ORFB was apparent 670 bp downstream of the termination codon of ORFA. Since this ORF is orientated in the opposite direction compared to that of ORFA and the *ceu* operon, it is likely that this gene is transcribed independently of either of these.

Inactivation of the *ceuB*, *ceuC*, *ceuD* and *ceuE* genes

Sequence analysis of the region upstream of *ceuE* suggested that the genes may form an operon encoding a PBT system for a ferric siderophore. To confirm this hypothesis and to determine the identity of the substrate(s) which were transported by this uptake system, genes encoding chosen components of the system were inactivated by mutation. The integration of plasmid pSP120 into the *ceuE* gene of *C. coli* CCH1 initially provided a means of cloning the DNA flanking this gene directly into *E. coli* by plasmid rescue. However, because the insert containing the region of homology in pSP120 was internal to the *ceuE* gene then the integrative event will also have generated a disrupted copy of *ceuE* (Dickinson *et al.*, 1995). *Campylobacter* spp. have been shown to be able to use both haemin and the siderophore enterochelin as sources of iron (Baig *et al.*, 1986; Field *et al.*, 1986; Pickett *et al.*, 1992). The capacity of the *ceuE* mutant CCH1 to utilize either of these iron sources was, therefore, assessed. Cells were added to MH-agar plates containing EDDHA, which binds iron to make it unavailable to *C. coli* (Field *et al.*, 1986). Growth can only occur, therefore, when exogenous iron sources are supplied and if the bacteria are able to utilize these. The stimulation of growth due to 1 mM and 100 µM haemin was equivalent for both mutant and wild-type cells, suggesting that *ceuE* mutation did not affect haemin uptake. In contrast, however, when enterochelin was supplied as an iron source at a concentration of 1 mM the zone of growth of the mutant strain was both smaller and less dense when compared to that observed for the parental strain (Table 2). When lower concentrations of enterochelin were available (100 µM), a very poor growth response was observed for CCH1 although a good growth stimulation was apparent for wild-type cells. The same mechanism of mutation by single site integration of plasmid DNA was utilized to disrupt the *ceuD* gene. To facilitate this, a 0.37 kb *Bgl*II/*Eco*RV fragment, internal to *ceuD* (Fig. 1), was cloned into pSP105 (Dickinson *et al.*, 1995). The resulting integrative plasmid, pSP180, was introduced into *C. coli* UA585 by natural transformation and the nature of the mutagenic integrative event in one transformant, designated *C. coli* CATP1, was confirmed by Southern hybridization (data not shown). When the capacity of CATP1 to utilize enterochelin as an iron source was assessed, no growth response was apparent when the siderophore was supplied at a concentration of 100 µM. A very weak zone of stimulation was observed when enterochelin was present at 1 mM. In contrast, the ability of *C. coli* to utilize haemin as an iron source was unaffected by the presence of the *ceuD* mutation (Table 2).

Allelic exchange has been utilized previously in *Campylobacter* species to generate mutation by introducing copies of genes disrupted by antibiotic markers into the chromosome (Labigne-Roussel *et al.*, 1988). In order to determine the role of the inner-membrane permease complex in the uptake of enterochelin, we decided to disrupt the expression of these components by generating a *ceuB-ceuD* deletion mutant by allelic exchange. To facilitate this, a 2.1 kb *Sac*I/*Eco*RV fragment (Fig. 1) was

cloned into pTZ19R (Mead *et al.*, 1986) and the resulting plasmid was designated pCFF1. The DNA between the two *Bgl*II sites at positions 659 and 2132 (Fig. 2), encompassing *ceuC*, the 3' end of *ceuB* and the 5' end of *ceuD* (Fig. 1), was then removed and replaced by a *Bgl*II tetracycline-resistance cassette (Dickinson *et al.*, 1995) to generate pCFED1. The disrupted sequence was isolated from pCFED1 as a *Bam*HI/*Pst*I cassette and then introduced into *C. coli* via natural transformation, and tetracycline-resistant transformants were recovered. To confirm the introduction of the deleted sequence element into the chromosome, total DNA from one transformant designated CFED1 was analysed by Southern hybridization (Fig. 4). When DNA isolated from the wild-type was probed with the entire insert in pCFED1 four bands of 5.00 kb, 1.30 kb, 0.78 kb and 0.16 kb showed homology to the probe. This pattern is predictable, given the arrangement of *Bgl*II sites in the cloned DNA (Fig. 1). In DNA from the CFED1, however, the 5.00 kb and 0.78 kb bands were still apparent, but the 1.30 kb and 0.16 kb bands were absent, demonstrating that the DNA between the *Bgl*II sites at positions 659 and 2131 (Figs 1 and 2) is absent from CFED1. To confirm this the 1.30 kb *Bgl*II fragment, removed during the construction of pCFED1, was used to probe DNA derived from the mutant and parental strains. In this case the probe hybridized to the 1.30 kb fragment in the DNA from the parent but not to DNA from the mutant. The Southern hybridization data confirm the absence of the chromosomal DNA encoding *ceuC*, the 3' end of *ceuB* and the 5' end of *ceuD* in the deletion mutant CFED1 and also demonstrate that the gene replacement had been very precise since the *Bgl*II sites and the DNA flanking the deletion remain unaltered. Phenotypic studies of CFED1 showed it to be deficient in the utilization of enterochelin as an iron source and it exhibited the same growth response to the siderophore as CATP1 (Table 2).

ceuE encodes a putative enterochelin-binding lipoprotein

The amino acid sequences of CeuE from both *C. coli* and *C. jejuni* show significant homology to the periplasmic siderophore-binding proteins from other bacterial iron acquisition systems. Furthermore, a region of amino acids from 118 to 133 (QVDFEAINALKPDLII, showing the invariably conserved residues in bold) shows strong conservation to an amino acid sequence which has been identified as a signature sequence for periplasmic siderophore-binding proteins (Park & Richardson, 1995; Tam & Saier, 1993). It is likely, therefore, that CeuE is the periplasmic binding protein of the enterochelin transport system. CeuE has a signal sequence which conforms to the consensus sequence recognized by signal peptidase II (von Heijne, 1989; Pugsley, 1993). We have shown previously that the homologue of this protein from *C. jejuni* is expressed as a lipoprotein in *E. coli* (Park & Richardson, 1995). To assess whether this protein was expressed as lipoprotein in *C. coli*, cells of the parental strain UA585 and CCH1 (CeuE⁻) were grown in the presence of [³H]palmitate to effect the specific labelling of

SD *ceuB*
 ttattattactataaggaataattgctttttaaacacttgcttagtttaaatattcttt 60
 L L F K H L F S L N I L L
 taattttactgtgtgtttttggaataataagcttatttataggggtatttcgtatcaatt 120
 I L L V V F G I I S L F I G V I R I N L
 tagacgatattttttcactcagcactaccagctcgaaatcattttacttacaagaattc 180
 D D I F S L S T T Q L E I I L L T R I P
 caagacttattgcaattttacttacaggtatgagctcttagtatatcggaactcatcatgc 240
 R L I A I L L T G M S L S I C G L I M Q
 aacaactcagcgaataaattttgttctccaaccactcgaggactatggattgtgcta 300
 Q L T Q N K F V S P T T A G T M D C A K
 aatttggcattttgatttcttaataattttttacaggagcttcttttttactcaagctc 360
 F G I L I S L I F F T G A S F F T Q A L
 tcatcgcatctattttgtcttttttaggttcttttatatttccaatttctaagaaaa 420
 I A S I F A L L G S F I F I Q I L R K I
 ttaaaactaaagatgtgattttttgtgctttgataggtaataattgttggggattataa 480
 K L K D V I F V P L I G L M F G G I I N
 atgctataaccacttttttgccttatgctttaaattatatacaaaatccaaggctggc 540
 A I T T F F A Y A L N Y I Q N I Q G W L
 tacaggcgagtagggcaaatgttatgcagggaattatgaattgctttatatttctttac 600
 Q G S M A N V M Q G N Y E L L Y I S L P
 cgtttttatacttgcctttttttagcctataaaatcactatagcaggtatgggagaag 660
 L F I L A Y F L A Y K I T I A G M G E D
 atcttgccttaaatcttggagtttcttaataccaatcttatttttaggcttaataatag 720
 L A L N L G V S Y N P I L F L G L I I V
 tttctattatcactagcgtagtgattgtaagcattggagttataccttttttaggtctta 780
 S I I T S V V I V S I G V I P F L G L I
 tcatccctaatcttgttgccttatacgcggggataatcttaagaaaaatcttatctata 840
 I P N L V A I Y R G D N L K K N L I Y I
 tagcattatgcggggcttggtttttacttatttgcgatattatctcaagacttggattt 900
 A L C G A L F L L I C D I I S R L V I F
 ttcttttgaatgccttttagtataaccacaggtgttttgggctctttaatttttattt 960
 P F E M P L S I T T G V L G S L I F I F
 SD *ceuC*
 ttcttttactcaaaaggaaaacttatgcgttaaaaaaatcttaattcttagcttttttaacc 1020
 L L L K R K T Y A *
 M R K K I L I L A F L T
 cttaaatggcacttgtttttgattttgcagggtttaaagattttgatgaatacgaatta 1080
 L I M A L V F V F A G L K D F D E Y E L
 aaaaatcgctttttgcaaatcgagctattgttatagtggtctatttgtatagctatatca 1140
 K N R F L Q I A A I V I V A I C I A I S
 actgtgatttttcaaactcttttgcaatacaaaaattttaaccctcgccatcatagggcttg 1200
 T V I F Q T L C N N K I L T P A I I G L
 gattctttatataatgcttcttcaaagcgccctgtcttttcttaggttctcgaaatttg 1260
 D S L Y M L L Q S A L V F S L G S A N L
 agtgtatatagaatgatattaatttttaatacactctagctgtatggtgtattttct 1320
 S V Y R N D I N F L I T L A C M V V F S
 ttaggactttataaaattcttttagcagtgataaaagtatttatcttatcatgctttta 1380
 L G L Y K I L F S S D K S I Y L I M L L
 ggtttgatttttgaacacttttttagcactaagctcttttttgaatttcttatcgat 1440
 G L I F G T L F S T L S S F F E I L I D
 cctgatgaatttatgattattcaaggaagaatggttgcaggttttgataatgtagctttt 1500

 P D E F M I I Q G R M F A S F D N V A F
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 D V L A L A Y I V T L L S F I W I F R Y
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 M K F L D P L S L G K D L S I N L G I N
 tatcaaaaaatttcaaacatcttatgataataattgctatttttaacttctattagcaca 1680
 Y Q K I S K H L M I I I A I L T S I S T
 gcacttgggtgcttatcaccttccaggacttttagtagtaaatatcacttacgaactt 1740
 A L V G P I T F L G L L V V N I T Y E L
 tttaaaaactgcaagcatagcatttttacttacttgccttgcataactcatttagcatttagct 1800
 F K T A K H S I L L L A C I L I S I L A
 ttacttgggggagtagttttttgtctctagaatttttgattacaatacaaccataagcgtt 1860
 L L G G V F F V S R I F D Y N T T I S V
 attatcaatttttaggtggaataatttttatctatcttgtattaaaagggaataaacta 1920
 SD *ceuD*
 I I N F L G G I Y F I Y L V L K G N K L
 tgataagattacaaaatatcaccaaatgctacgatcaaaaggctatcatttcagatctta 1980
 *
 I R L Q N I T K C Y D Q K A I I S D L S
 gtatcgattttcataaaggaaaaatttacttccattataggggcaaatggagcagaaaaat 2040
 I D F H K G K I T S I I G A N G A E K S
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 T L L A L A S R L I K P D S G R I Y I D
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 G M N L A L Y K E Q D L A Q K I S I L K
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 I I Q E D V L R Q I Y D M D I P V S Q I
 SD *ceuE*
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 M K K S
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 I P K N P S K V V I L D L G I L D T F H

Fig. 2. For legend see facing page.

gcctaagacttaacgataaagtgtcaggagttccagctaaaaacttgccaaatactta 3000
A L R L N D K V A G V P A K N L P K Y L
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N A L K P D L I I I S G R Q S K F Y E K
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L K I A P T M F V G L D N A N F L S S F
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SD
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L F A S K K D R Q K Q L A T L G A G V V
atcactatacttttagcttattcttagctttaaatttttttatctagcttttttaagctag 4500

I T I L L A Y L S L *
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* K K D I
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V Q Y N Y P A L N K D H E L L E K P P N
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G N V E I E D G Y Y R E G A K H G T L M
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K A N Y Y L E G P N F K Q M D E F G L N
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T K A M D V A Q L L M R S A E R P N H F
OrfB
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G T S N E A N V F D A R M Q S K R H L E

Fig. 2. Nucleotide sequence of the *C. coli* *ceuB*, *ceuC*, *ceuD* and *ceuE* genes and the deduced amino acid sequences. The putative Shine-Dalgarno sequences are denoted by SD. An inverted repeat representing a potential rho-independent terminator is underlined.

Fig. 3. Analysis of proteins expressed from the plasmid pS1. A fluorograph of [^{35}S]methionine-labelled proteins expressed in an *in vitro* coupled transcription/translation system using the parental plasmid pSP105 (lane 1) and pS1 (lane 2) as templates is shown. The positions of standard proteins are given in kDa.

lipoproteins. The expression of components of siderophore-uptake systems is often repressed during growth in iron-replete conditions. The expression of lipoproteins was therefore assessed in cells with respect to the availability of iron. When lipoprotein expression in cells of the parental strain was examined in this manner, a 34 kDa lipoprotein, expressed only when the availability of iron was restricted by the addition of 20 μM EDDHA, was apparent (Fig. 5). In contrast, this iron-repressible protein was not apparent in lysates of CCH1 cells which had been grown under iron-deficient conditions. Instead a faint band which represented a protein of 31 kDa was seen. These observations suggest that the iron-repressible

Fig. 4. Confirmation of the deletion in *C. coli* CFED1 by Southern analysis. Genomic DNA from *C. coli* UA585 (lanes 1 and 3) and *C. coli* CFED1 (lanes 2 and 4) was digested with *Bgl*II and probed with the 2.1 kb insert in pCFF1 (lanes 1 and 2) or with the 1.3 kb *Bgl*II fragment removed during the construction of pCFED1 (lanes 3 and 4). The size of molecular size standards is shown in kb.

34 kDa lipoprotein represents CeuE since it is absent from the *ceuE* mutant CCH1. The appearance of a 31 kDa protein in CCH1 cells is consistent with this as the integrative event, which led to the mutation in CCH1, would have generated a truncated *ceuE* gene which could potentially encode a mature protein of 30.6 kDa.

***ceuB* and *ceuC* encode the putative enterochelin-specific permease**

The deduced amino sequences of CeuB and CeuC reveal that these proteins are very hydrophobic and contain a

Table 2. Haemin and enterochelin utilization by *C. coli*

Growth response to iron compounds on MH-plates containing 56 μM EDDHA was classified as: + + +, very good growth; + +, good growth; +, weak growth; $\pm$, very weak growth; —, no growth.

Strain (genotype)	Growth with enterochelin		Growth with haemin	
	1 mM	100 μM	1 mM	100 μM
UA585	+ + +	+ +	+ +	+ +
CCH1 (<i>ceuE</i>)	+ +	$\pm$	+ +	+ +
CATP1 (<i>ceuD</i>)	+	—	+ +	+ +
CFED1 (Δ <i>ceuB-ceuD</i>)	+	—	+ +	+ +

Fig. 5. Lipophilic modification of CeuE. Cells of *C. coli* UA585 grown in iron-replete broth (lane 3) or broth containing 20 μ M EDDHA (lane 2), and cells of *C. coli* CCH1 grown in broth containing 20 μ M EDDHA (lane 1), were incubated in the presence of [3 H]palmitate and the labelled proteins were analysed by SDS-PAGE and fluorography. The positions of standard proteins are given in kDa.

number of potential membrane-spanning segments. These characteristics are consistent with the hypothesis that CeuB and CeuC are integral membrane protein components of the PBT system. There is often significant homology between the inner-membrane permeases of PBT systems (Staudenmaier *et al.*, 1989). A pair-wise comparison of the amino acid sequences of CeuB and CeuC revealed that 26.3% of their amino acids were identical. Furthermore, both sequences showed significant homology to the group of proteins that are the subunits of the cytoplasmic membrane permeases for the transport of ferric siderophores. CeuB showed 40.9% homology (identical amino acids) to FatD (*Vibrio anguillarum* anguibactin transport; Koster *et al.*, 1991) and 26.7% homology to FepD (*E. coli* enterochelin transport; Shea & McIntosh, 1991). Similarly, CeuC displayed 31.6% homology to FatC (*V. anguillarum* anguibactin transport; Koster *et al.*, 1991) and 22% to HemU (*Yersinia enterocolitica* haemin uptake; Stojiljkovic & Hantke, 1994). Specifically, the conserved regions for the integral membrane proteins identified by Staudenmaier *et al.* (1989) are partially conserved in CeuB and CeuC. The tetrapeptide in the first conserved region RLPR is represented by RIPR in CeuB but is not apparent in CeuC. The highly conserved FIGL in region five (Staudenmaier *et al.*, 1989) is present as FLGL in both CeuB and CeuC. The homology box in the third region of conservation starting with Q and containing NP preceded by a charged amino acid was represented by QQLTQNK and QTLCNNK in CeuB and CeuC, respectively.

ceuD encodes the putative ATP-binding component of the enterochelin uptake system

The *ceuD* gene encodes a protein with significant homology to the ATP-binding proteins from other PBT systems for ferric siderophores. It shows 36.5% homology (identical amino acids) with FhuC (*E. coli* hydroxymate uptake; Burkhardt & Braun, 1987), 32.9%

homology with FecE (*E. coli* ferric-citrate uptake; Staudenmaier *et al.*, 1989) and 32.6% homology with FepC (*E. coli* enterochelin uptake; Shea & McIntosh, 1991). In support of the concept that CeuD is the ATP-binding protein for the enterochelin transport system, it also contains the sequence GANGAGKST, which compares well with GxxGxGKS/T which is the consensus sequence motif associated with the glycine-rich loop of ATP-binding proteins (Walker *et al.*, 1982). In addition, it contains a second conserved region, containing the invariant residue DEP, which is typical of ATP-binding proteins (Walker *et al.*, 1982).

DISCUSSION

In a variety of bacterial pathogens iron availability and the sequestration of this essential element are intimately associated with virulence (Payne & Lawlor, 1990; Wooldridge & Williams, 1993). A description of the factors which enable *Campylobacter* to become established within the host and cause disease would, therefore, be incomplete without an understanding of the mechanisms through which this group of pathogens are able to acquire iron. Prior to this study information concerning the biochemistry and genetics of siderophore uptake in *Campylobacter* species was lacking. As a first step towards a molecular description of iron uptake in this pathogen we have used a genetic approach to characterize a PBT system for the uptake of the catechol type siderophore enterochelin.

Previously, a lipoprotein, isolated from *C. jejuni* NCTC 11351 and with significant homology to siderophore-binding proteins, was characterized in our laboratory (Park & Richardson, 1995). The function of this protein could not, however, be confirmed by the generation of isogenic mutants because this strain has proven refractory to the introduction of recombinant DNA (K. A. Grant & S. F. Park, unpublished data). In this study we have identified the homologue in *C. coli* UA585, which is naturally competent and amenable to genetic manipulation (Wang & Taylor, 1990). Plasmid rescue was used to clone the DNA sequences flanking the gene encoding this protein from this strain. Analysis of this region identified four ORFs encoding proteins with significant homology to the components of PBT systems for the transport of ferric siderophores. Mutational analysis showed that these components were essential for the efficient uptake of the siderophore enterochelin. Genes which encode other bacterial PBT systems are often transcribed as operons (Staudenmaier *et al.*, 1989). Given that the *ceuBCDE* genes are all orientated in the same direction and that only relatively small intergenic distances separate the reading frames or the genes overlap, it is likely that the *ceuBCDE* genes are transcribed as part of an operon. It is also probable that *ceuE* is the last gene in the *ceu* operon for two reasons. Firstly, the deduced protein product of ORFA bears no homology to any known protein associated with iron acquisition and secondly, the intergenic region between *ceuE* and ORFA is much larger than that for any of the other *ceu* genes and it contains a potential rho-independent transcriptional terminator. It is highly likely,

therefore, that the phenotypic effects observed in the various *ceu* mutants are a result of the disfunction of components of the putative PBT system and are not caused by polar effects.

All known bacterial PBT systems consist of at least three components: a periplasmic binding protein, one or two integral membrane components and one or two ATP-binding proteins (Ames *et al.*, 1990). According to its deduced amino acid sequence and phenotypic characterization, it is likely that CeuE is the periplasmic enterochelin-binding protein. Mutants in *ceuE*, defective in the putative enterochelin-binding component, were still able to utilize enterochelin, although the stimulation of growth due to the siderophore was significantly reduced compared to the wild-type. Ferric siderophores are needed in very small amounts so it is possible that they can be accumulated to a sufficient concentration to allow a degree of growth even if part of the transport system has been disabled. Our data also indicate that CeuE is a lipoprotein since an iron-repressible [³H]palmitate-labelled 34 kDa protein, corresponding to the predicted size of the processed CeuE protein, was absent in cells containing a *ceuE* mutation. The presence of a lipophilically modified substrate-binding protein is unusual in a Gram-negative organism. In Gram-positive organisms, however, this is a common adaptation (Alloing *et al.*, 1990; Schneider & Hantke, 1993), and in the absence of an outer membrane the lipid moiety is thought to interact with the cytoplasmic membrane to form an anchor to prevent the escape of the protein (Gilson *et al.*, 1988). The reason for the presence of a substrate-binding lipoprotein in *C. coli* is at present unclear. Recently, however, a protein, identified as a cell-binding factor of *C. jejuni*, has been shown to be homologous to the binding proteins of amino acid transport systems in Gram-negative bacteria (Pei & Blaser, 1993). This protein is predicted to have both signal peptidase I and signal peptidase II cleavage sites and it has been suggested that this characteristic allows the protein to occupy different locations so that it can perform multiple functions. The two-step cleavage of the leader peptide of the protein may distinguish it from amino acid binding proteins and make it accessible to the bacterial surface where it can perform cell binding functions as well (Pei & Blaser, 1993). We have shown that CeuE, which contains a signal peptidase II site and is lipophilically modified, is likely to function as a siderophore-binding protein in a PBT system for enterochelin uptake. Whether it undergoes the two-step processing suggested by Pei & Blaser (1993), enabling it to perform another function on the cell surface, is not known but it is interesting to note that *ceuE* contains a weak signal peptidase I recognition site (³¹S-X-³³A; Fig. 2).

The amino acid sequence similarities between CeuB, CeuC and CeuD and components of cytoplasmic membrane permease complexes of siderophore-binding-protein-dependent transport systems from other bacteria is striking given the phylogenetic distances between campylobacters and the other species. Generally these assemblies comprise one or two hydrophobic trans-

membrane components and one or two hydrophilic components with ATP-binding domains (Ames *et al.*, 1990). In view of the structural characteristics predicted from their amino acid sequences it is likely that CeuB and CeuC form the integral membrane components and that CeuD is the ATP-binding protein. When the genes encoding this complex were disrupted, enterochelin utilization was severely impaired. The mutants showed no growth response when enterochelin was supplied at 100 µM, but at higher concentrations of the siderophore both the *ceuD* insertion mutant and the *ceuBCD* deletion mutant exhibited a very weak zone of growth stimulation. A similar phenomenon has been observed for haemin PBT-system-deficient mutants of *Y. enterocolitica* (Stojiljkovic & Hantke, 1994). In this situation it has been proposed that the formation of a haemin gradient between the periplasm and cytoplasm may facilitate the leakage of this hydrophobic compound through the cytoplasmic membrane into the cell. It is possible, therefore, that the same mechanism could account for the weak growth stimulation, due to enterochelin, that was observed in the permease-defective mutants of *C. coli*.

In conclusion, we have identified and characterized a PBT system for the uptake of enterochelin in *C. coli*. Although this bacterium does not appear to produce enterochelin or any other known siderophore (S. F. Park, unpublished data; Baig *et al.*, 1986; Field *et al.*, 1986) it seems likely that this pathogen can utilize this transport system to scavenge the enterochelin produced in the gastrointestinal tract by *E. coli* and other enteric bacteria. The transport-defective mutants generated in this study will allow the role of the enterochelin-uptake system in promoting the virulence of *C. coli* to be assessed.

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