Transposon mutagenesis of *Campylobacter jejuni* identifies a bipartite energy taxis system required for motility

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Summary

Campylobacter jejuni* constitutes the leading cause of bacterial gastroenteritis in the United States and a major cause of diarrhoea worldwide. Little is known about virulence mechanisms in this organism because of the scarcity of suitable genetic tools. We have developed an efficient system of *in vitro* transposon mutagenesis using a *mariner*-based transposon and purified *mariner* transposase. Through *in vitro* transposition of *C. jejuni* chromosomal DNA followed by natural transformation of the transposed DNA, large random transposon mutant libraries consisting of ≈ 16 000 individual mutants were generated. The first genetic screen of *C. jejuni* using a transposon-generated mutant library identified 28 mutants defective for flagellar motility, one of the few known virulence determinants of this pathogen. We developed a second genetic system, which allows for the construction of defined chromosomal deletions in *C. jejuni*, and demonstrated the requirement of σ^{28} and σ^{54} for motility. In addition, we show that σ^{28} is involved in the transcription of *flaA* and that σ^{54} is required for transcription of three other flagellar genes, *flaB* and *flgDE*. We also identified two previously uncharacterized genes required for motility encoding proteins that we call CetA and CetB, which mediate energy taxis responses. Through our analysis of the Cet proteins, we propose a unique mechanism for sensing energy levels and mediating energy taxis in *C. jejuni*.

Introduction

Campylobacter jejuni, a microaerophilic Gram-negative bacterium, is one of the leading causes of bacterial gastroenteritis throughout the world. In the United States, *Campylobacter* species (with *Campylobacter jejuni* accounting for a vast majority of the isolates) have been implicated in almost half of all laboratory-confirmed cases of bacterial gastroenteritis, surpassing the number of cases resulting from *Salmonella* and *Shigella* species combined (Altekruse *et al.*, 1999). It has been estimated that one out of every 100 individuals in the United States and Great Britain develops a *Campylobacter* infection each year (Kendall and Tanner, 1982; Tauxe, 1992).

C. jejuni is a common commensal organism of the gastrointestinal tract of many animals and birds. Humans are often exposed to the organism through handling or consuming contaminated poultry meats. Despite the prevalence of *C. jejuni* in poultry colonization and human disease, our knowledge about colonization and pathogenic mechanisms of this bacterium is sparse. A unipolar flagellum conferring motility is the best-characterized virulence factor. A flagellum or flagellar motility is required for efficient colonization of chickens and humans (Black *et al.*, 1988; Wassenaar *et al.*, 1993) and cellular invasion in tissue culture models of infection (Grant *et al.*, 1993; Yao *et al.*, 1994).

The major limitation in studying *C. jejuni* has been lack of suitable genetic systems developed for the organism, including a system of random transposon mutagenesis. The *mariner* family of transposable elements has recently been shown to undergo both *in vitro* and *in vivo* transposition into several bacterial chromosomes, including *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Mycobacterium smegmatis*, creating new methods of studying these pathogens (Akerley *et al.*, 1998; Rubin *et al.*, 1999). An *in vivo* system for *mariner* transposition was described recently for *C. jejuni*, but it generated only a few random mutants per transposition reaction (Golden *et al.*, 2000), indicating the need for a more efficient mutagenesis system to create large transposon mutant libraries.

We have developed a system for *in vitro* transposon mutagenesis of *C. jejuni* using a derivative of the *mariner* transposon, *Himar1*. We generated isogenic random transposon mutant libraries consisting of as many as

16 000 individual mutants. As an initial test of the utility of this system, we identified 28 transposon mutants encompassing 21 different genes required for wild-type flagellar motility, including *fliA* (encoding σ^{28}) and *rpoN* (encoding σ^{54}). We also identified and characterized two genes encoding proteins with homology to domains of the aerotaxis and energy response proteins of *Escherichia coli* and *Pseudomonas putida*, suggesting an unusual bipartite system for regulating motility in *C. jejuni*. This work describes the first genetic screen of *C. jejuni* using a random transposon insertion library to identify genes required for a virulence determinant.

Results

In vitro *transposon mutagenesis of* C. jejuni

To construct minitransposons containing antibiotic resistance cassettes expressed in *C. jejuni*, we modified *magellan3*, which had previously been used for transposition in *M. smegmatis* (Rubin *et al.*, 1999). The kanamycin resistance cassette from *magellan3* in pFD1 was replaced with *aphA-3* (encoding kanamycin resistance) and *cat* (encoding chloramphenicol resistance) originating from *Campylobacter* species (Trieu-Cuot *et al.*, 1985; Wang and Taylor, 1990). Plasmid pFalcon contains the *solo* transposon (containing *aphA-3*), and pEnterprise contains the *picard* transposon (containing *cat*).

These plasmids were used initially to mediate *in vitro* transposition of *solo* or *picard* into the chromosome of *C. jejuni* 81-176 (see *Experimental procedures*). We typically obtained 600−1450 individual transposon mutants per transposition reaction using 2 µg of 81-176 chromosomal DNA. Mutants obtained from 15 independent reactions were combined to create a large library containing ≈ 16 000 individual 81-176 *solo* mutants. Southern hybridization analysis of 19 individual 81-176 *solo*

mutants revealed that the transposon had inserted once randomly into the chromosome of each mutant (Fig. 1). The libraries generated with *picard* also showed random insertions of the transposon in the chromosome of 81-176 (data not shown).

Identification of solo *mutants defective in motility*

The 81-176 *solo* mutants were screened for mutants defective in flagellar motility, an important virulence and colonization determinant of *C. jejuni*. Approximately 1400 mutants were screened for motility defects in MH motility media after growth in microaerophilic conditions at 37°C, and 28 mutants that were non-motile or displayed reduced or altered motility phenotypes were isolated (Table 1). Chromosomal DNA from each mutant was used to transform wild-type 81-176, giving rise to kanamycin-resistant transformants with motility defects similar to the originally identified *solo* mutants (data not shown), verifying that acquisition of the DNA fragment harbouring *solo* was linked to a defective motility phenotype.

Sequence analysis of insertion sites revealed genes that had previously been implicated in motility of *Campylobacter* species, such as *flaA*, *flhA* and *fliM* (Guerry *et al.*, 1990; Wassenaar *et al.*, 1991; Miller *et al.*, 1993; Bleumink-Pluym *et al.*, 1999). Certain genes were identified multiple times with different *solo* insertion points, such as *flhA*, *cheA*, *rpoN* and *Cj1189c*. Whereas many of the insertion sites of *solo* were found in genes that appear to be involved in flagellar motility based on homologies to known flagellar genes in other bacteria, we have not ruled out the possibility in all cases that the defect in motility arises from polar effects on the transcription of genes downstream of the site of the transposon insertion. These results verify the utility of our mutagenesis system and screening method for identifying genes required for motility.

Involvement of *fliA* and rpoN *in the transcription of flagellar genes*

Insertions of *solo* into *fliA* (encoding σ^{28}) or *rpoN* (encoding σ^{54}) were identified in our motility screen (Table 1). These two σ-factors along with σ^{70} are the only σ-factors known to be encoded in the genome of *C. jejuni* (Parkhill *et al.*, 2000). σ^{28}, which transcribes flagellar genes in many bacteria (Helmann, 1991), was hypothesized to transcribe *flaA* (encoding the major flagellin) in *C. jejuni* and the closely related *Campylobacter coli* (Guerry *et al.*, 1990; Nuijten *et al.*, 1990). σ^{54}, which transcribes genes required for a broad set of functions in many bacteria (Shingler, 1996), was proposed to transcribe *flaB* (encoding a minor flagellin) in *C. jejuni* and *C. coli* (Guerry *et al.*, 1991; Wassenaar *et al.*, 1994) and *flgE* (encoding the flagellar
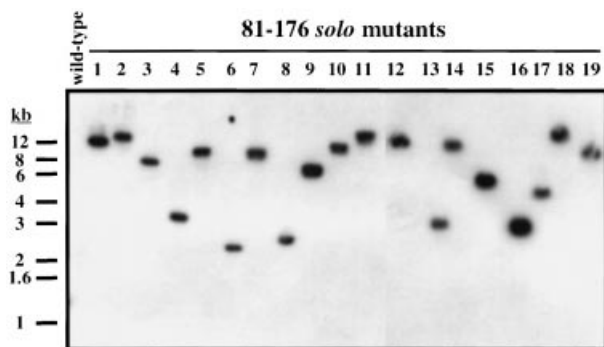

Fig. 1. Southern hybridization analysis of *C. jejuni* 81-176 *solo* mutants. Chromosomal DNA was isolated from 19 randomly selected 81-176 *solo* mutants and digested to completion with *Bgl*II, which does not cleave within the *solo* transposon. A ^{32}P-labelled probe consisting of *aphA-3* was used for hybridization.

Table 1. Location of *solo* in *C. jejuni* motility mutants.

Gene[ab]	Identified/proposed function[a]	Motility phenotype[c]
Flagellar structure/flagellar biosynthesis		
flaA	Major flagellin subunit	Altered
flhA (3)	Probable flagellar biosynthetic protein	Non-motile
flhB	Probable flagellar biosynthetic protein	Non-motile
fliI	Flagellum-specific ATP synthase	Altered
fliR	Probable flagellar biosynthesis protein	Non-motile
flgD	Possible hook-associated assembly protein	Reduced
flgH	Probable flagellar L-ring precursor protein	Non-motile
Flagellar motor		
motA	Probable flagellar motor proton channel	Non-motile
motB	Possible flagellar motor protein	Non-motile
fliM	Probable flagellar motor switch	Non-motile
Chemotaxis		
cheA (2)	Probable chemotaxis histidine kinase	Reduced
cheV	Probable chemotaxis protein	Altered
cheY	Chemotaxis regulatory protein	Non-motile
Flagellar transcription		
fliA	Probable RNA polymerase σ^{28}	Non-motile
rpoN (2)	Probable RNA polymerase σ^{54}	Non-motile
Undetermined		
Cj0062c (2)[d]	Probable integral membrane protein	Non-motile
Cj0248 (2)	Unknown/no identity	Altered
Cj0883c[e]	Unknown/no identity	Non-motile
Cj1026c	Probable lipoprotein	Non-motile
Cj1189c (2)	Possible signal transduction protein	Altered
Cj1190c	Probable signal transduction protein	Altered

a. Gene designation and proposed function are based on the annotated genome sequence from *C. jejuni* NCTC 11168 (Parkhill *et al.*, 2000).
b. Number indicates number of mutants identified with different *solo* insertions in the respective gene.
c. Non-motile, no migration; reduced, less migration with typical motility ring pattern; altered, less migration with atypical motility ring pattern.
d. *Cj0062c* is located immediately upstream of *fliA*, *fliM* and *fliY* (Parkhill *et al.*, 2000); the motility defect by the *solo* insertion in these mutants may result from polar effects on the transcription of these downstream genes.
e. The *solo* insertion in this mutant is located in the putative promoter for *Cj0883c*, which is immediately upstream of *flhA* (Parkhill *et al.*, 2000); the motility defect by the *solo* insertion in this mutant may result from polar effects on the transcription of *Cj0883c* and/or *flhA*.

hook protein) in *C. coli* (Kinsella *et al.*, 1997). These hypotheses were based on sequence analysis of the promoters for these genes, which showed typical σ^{28} binding sites or σ^{54} binding sites. Additionally, the transcriptional start sites for these genes map directly downstream of these binding sites (Nuijten *et al.*, 1990; Guerry *et al.*, 1990; 1991; Wassenaar *et al.*, 1994; Kinsella *et al.*, 1997). In *C. jejuni*, *flgE* is located 3 bp downstream of *flgD*, encoding a putative hook-associated assembly protein, and appears to lack a promoter (Parkhill *et al.*, 2000). The promoter of *flgD* has a typical $-24/-12$ σ^{54} binding site, indicating that, like *flaB*, it may be transcribed by σ^{54}. As *flgE* appears to lack a promoter element, we hypothesized that *flgE* may be co-transcribed with *flgD* by σ^{54}. Despite previous studies on the transcription of *flaA*, *flaB* and *flgE* in *Campylobacter*, mutants lacking *fliA* or *rpoN* have not been reported in any *Campylobacter* species, and conclusive evidence demonstrating that these σ-factors are required for the transcription of flagellar genes is therefore lacking.

To verify the non-motile phenotype of the 81-176 *fliA* and *rpoN solo* mutants, we developed a method for creating site-specific, non-polar deletions on the chromosome of *C. jejuni* (see *Experimental procedures*). This technique allowed us to delete a large portion of the coding region of *fliA* and *rpoN* in the chromosome of DRH212, a streptomycin-resistant (Sm^R) mutant of *C. jejuni* 81-176. For *fliA*, we made an in frame fusion deleting codons 17–223, creating a non-functional fusion protein of 32 amino acids to generate strain DRH311. For *rpoN*, we deleted a portion of the gene including all codons except the start and stop codons to generate strain DRH321. These deletions did not affect expression of the respective genes immediately downstream of *fliA* or *rpoN*, as determined by reverse transcription–polymerase chain reaction (RT–PCR; data not shown), demonstrating that the mutations are non-polar. In MH motility agar, the Δ*fliA* mutant displayed dramatically reduced motility, and the Δ*rpoN* mutant was completely non-motile (Fig. 2A), verifying the results with the *solo* mutants demonstrating
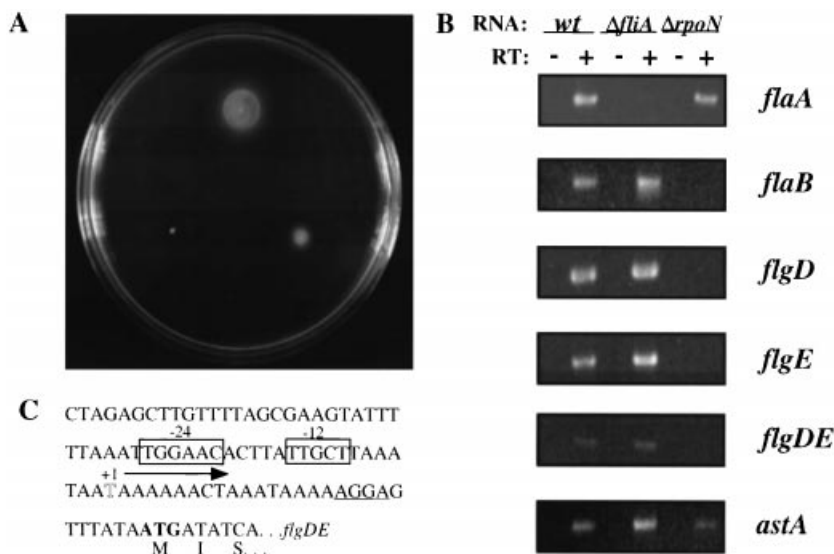
Fig. 2. Analysis of σ^{28} and σ^{54} in motility and transcription of flagellar genes.
A. Motility phenotypes of 81-176 Sm^R (top), DRH321 ($\Delta rpoN$; bottom left) and DRH311 ($\Delta fliA$; bottom right) in MH motility media.
B. RT–PCR analysis of transcription of flagellar genes in 81-176 Sm^R, DRH311 ($\Delta fliA$) and DRH321 ($\Delta rpoN$). RT–PCR analyses were performed using *astA* as a positive control. cDNA synthesis reactions were performed in the presence and absence of reverse transcriptase (RT) to ensure that the amplified DNA in the PCR was derived originally from RNA and not from chromosomal DNA.
C. Promoter structure of *flgD*. The putative $-24/-12$ σ^{54} binding sites are boxed, and the start of transcription is outlined. The putative ribosomal binding site is underlined, and the start codon is in bold.

that these genes are required for motility in 81-176. These mutants have no apparent growth defects in MH agar (data not shown).

To determine whether these two σ-factors are required for transcription of specific flagellar genes in *C. jejuni* 81-176, we performed RT–PCR with RNA isolated from 81-176 Sm^R, DRH311 ($\Delta fliA$) and DRH321 ($\Delta rpoN$). Transcription of *flaA* was greatly decreased in the $\Delta fliA$ mutant (Fig. 2B), implicating σ^{28} in the production of the major flagellin in *C. jejuni*. A *flaB* transcript was detected in wild-type 81-176 and DRH311, but not in DRH321, demonstrating that transcription of *flaB* requires σ^{54} (Fig. 2B). In the *rpoN* mutant, transcription of *flgD* was decreased considerably, and transcription of *flgE* was undetectable (Fig. 2B). Using primers that would amplify a *flgDE* transcript if these two genes were co-transcribed, a *flgDE* transcript was detected using RNA from the wild-type strain, but not with RNA from the $\Delta rpoN$ mutant (Fig. 2B). The transcription start site of *flgDE* was mapped by primer extension to a position immediately downstream of a typical $-24/-12$ σ^{54} binding site in the promoter of *flgD* (Fig. 2C). A transcription start site for *flgE* could not be detected, further suggesting that this gene lacks a promoter and is transcribed with *flgD* by σ^{54}. Taken together, these results strongly suggest that σ^{28} and σ^{54} transcribe multiple flagellar genes and demonstrate the requirement of σ-factors for transcription of a *C. jejuni* virulence determinant.

Identification of Cj1189c *and* Cj1190c *in the energy taxis response of* C. jejuni *81-176*

Three mutants identified in our motility screen had *solo* insertions in two adjacent open reading frames (ORFs) in

the *C. jejuni* genome: two were found in *Cj1189c* and one was found in *Cj1190c* (Table 1). The predicted proteins encoded by *Cj1189c* and *Cj1190c* suggest that they may function in energy taxis and aerotaxis. Energy taxis is the behavioural response of motile organisms to migrate towards energy-producing environments, whereas aerotaxis is a specific type of energy taxis in which bacteria migrate towards O$_2$ (Taylor *et al.*, 1999). In *E. coli* and *P. putida*, both responses are mediated by the aerotaxis and energy response protein, Aer (Bibikov *et al.*, 1997; Rebbapragada *et al.*, 1997; Nichols and Harwood, 2000). Aer is an inner membrane protein with two cytoplasmic domains. One domain is a PAS domain that senses the redox state of flavin adenine dinucleotide (FAD) as a way of monitoring electron transport. The other domain is a highly conserved domain (HCD) common to chemotaxis receptors that interacts with the chemotaxis proteins CheA and CheW to transduce signals to CheY to change the direction of rotation of the flagellum. Aer thus regulates directed motility towards environments promoting optimal electron transport, which provides high levels of ATP generation (reviewed by Taylor *et al.*, 1999).

Both Cj1190c and Cj1189c contain regions with homology to the domains of Aer. *Cj1190c* is predicted to encode a protein of 459 amino acids and *Cj1189c*, located 18 bp downstream of *Cj1190c*, is predicted to encode a protein of 165 amino acids (Parkhill *et al.*, 2000). Cj1190c is predicted to insert into the membrane at its N-terminus; the C-terminal portion is predicted to be cytoplasmic and contains an HCD similar to that of the *E. coli and P. putida* Aer proteins (Fig. 3A). However, unlike Aer, Cj1190c does not have a PAS domain at its N-terminus. Cj1189c contains a PAS domain similar to that found in Aer (Fig. 3A), which comprises nearly 70% of the protein, but no predicted membrane-spanning
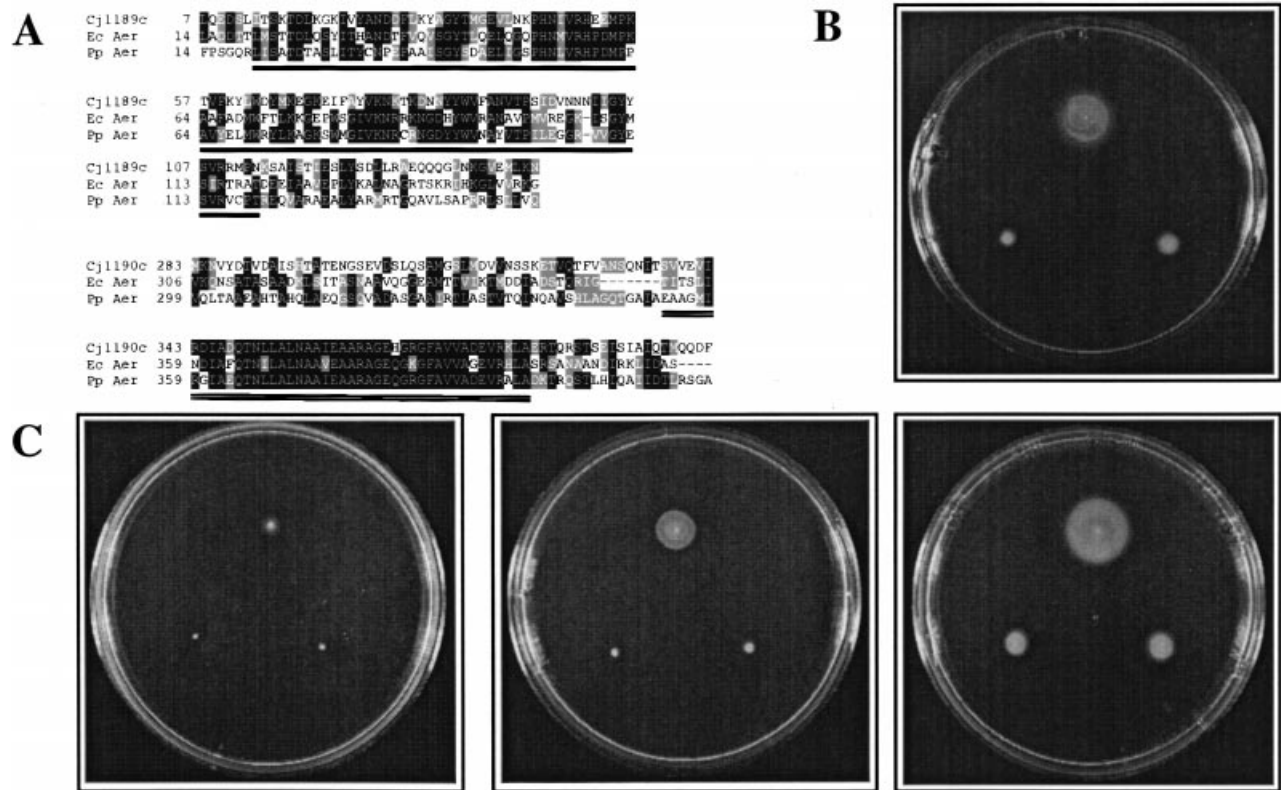
Fig. 3. Analysis of Cj1189c and Cj1190c in motility and energy taxis.
A. Alignment of portions Cj1189c and Cj1190c to the Aer proteins of *E. coli* (Ec Aer) and *P. putida* (Pp Aer). The PAS domain of Cj1189c and the Aer proteins are underlined. The HCDs of Cj1190c and the Aer proteins are indicated with a double underline. Identical residues are shaded in black, and similar residues are shaded in grey.
B and C. Motility phenotypes of 81-176 Sm^R, DRH307 (ΔCj1189c) and DRH333 (ΔCj1190c) in MH motility agar or CDM motility agar in the presence or absence of supplements.
B. MH motility agar.
C. CDM motility agar alone (left), CDM motility agar with 0.91 M sodium pyruvate (middle) and CDM motility agar with 30 mM fumarate (right). For (B) and (C), 81-176 Sm^R is at the top, DRH307 (ΔCj1189c) is at the bottom left, and DRH333 (ΔCj1190) is at the bottom right.

domains, suggesting that the putative protein is cytoplasmic.

As Cj1189c and Cj1190c contain Aer-like domains, we hypothesized that these proteins are involved in motility and in mediating Aer-like energy taxis in *C. jejuni*. To test this hypothesis, we made mutant strains by deleting portions of *Cj1189c* or *Cj1190c* from the chromosome of strain 81-176 (see *Experimental procedures*). For *Cj1189c*, we deleted the entire coding sequence (strain DRH307) and, for *Cj1190c*, we made an in frame fusion deleting codons 10–447 (strain DRH333). As shown in Fig. 3B, both mutants displayed an altered motility phenotype consisting of reduced motility without the characteristic concentric ring pattern common to wild-type motility, confirming the results obtained with our originally isolated *solo* mutants.

To determine whether Cj1189c and Cj1190c mediate energy taxis in *C. jejuni*, the corresponding mutants were inoculated into *Campylobacter* defined motility agar (CDM) in the absence or presence of sodium pyruvate

or fumarate. These substances are critical substrates in *C. jejuni* metabolism. Sodium pyruvate is rapidly metabolized by *C. jejuni* (Mendz *et al.*, 1997), causing an increase in electron transport and ATP generation but depletion of O$_2$, which can serve as the final electron acceptor (Hazeleger *et al.*, 1998). Owing to the microaerophilic, mixed acid fermentative characteristics of *C. jejuni* metabolism, fumarate is the preferred terminal electron acceptor even in the presence of O$_2$ (Smith *et al.*, 1999).

When wild-type 81-176 Sm^R and its mutant derivatives were inoculated into CDM lacking supplements, all strains showed minimal migration from the point of inoculation (Fig. 3C). However, in CDM containing 0.91 M sodium pyruvate or 30 mM fumarate, the wild-type strain displayed energy taxis in the form of a highly motile phenotype analogous to Aer-mediated energy taxis in *E. coli* and *P. putida* (Fig. 3C; Bibikov *et al.*, 1997; Nichols and Harwood, 2000). These findings suggest that, in CDM with sodium pyruvate, *C. jejuni* migrates rapidly from the point of inoculation to new environments providing

increased pyruvate and O_2 concentrations, maintaining high levels of electron transport and ATP generation. Likewise, in CDM with fumarate, wild-type *C. jejuni* migrated from the point of inoculation, presumably towards higher concentrations of fumarate to achieve optimal electron transport and maximal ATP generation. In contrast, the Δ*Cj1189c* and Δ*Cj1190c* mutants showed dramatically reduced motility compared with the wild-type strain in the presence of supplements (Fig. 3C), suggesting that they are defective in energy taxis, thus hindering their ability to migrate to new environments and maintain maximal electron transport and ATP generation. The defects in motility are unlikely to be related to overall growth defects in the mutants, as all strains showed similar growth rates in these media (data not shown). As *Cj1189c and Cj1190c* appear to mediate energy taxis in *C. jejuni*, we propose to name these genes *cetA* (*Cj1190c*) and *cetB* (*Cj1189c*) for *Campylobacter* energy taxis response.

Discussion

We have developed a system for *in vitro* transposition of the *C. jejuni* genome with a *mariner*-based transposable element and constructed large mutant libraries consisting of thousands of individual random mutants. These libraries were used to perform the first genetic screen of *C. jejuni* with a random transposon-generated mutant library to identify genes required for a virulence determinant. Among the required components identified for flagellar motility were two σ-factors, σ^{28} and σ^{54}, and two previously unidentified proteins, Cj1190c (CetA) and Cj1189c (CetB). Using a method we developed to make defined deletions in the *C. jejuni* chromosome, we deleted the coding regions of σ^{28} and σ^{54} and provided direct evidence that these σ-factors are responsible for the transcription of specific flagellar genes. In addition, we provided evidence that CetA and CetB may together mediate Aer-like energy taxis responses in *C. jejuni*.

Our method of *in vitro* transposition of the *C. jejuni* genome allows for the efficient generation of random transposon mutant libraries. Previously, an analogous *mariner*-based transposon was shown to mediate *in vivo* transposition of the *C. jejuni* genome (Golden *et al.*, 2000). Although this method generated random transposon insertions, only ≈ 37 mutants were created per transposition reaction, severely restricting the ability of this method to generate large transposon mutant libraries in *C. jejuni*. Our method takes advantage of the efficient mechanism of natural transformation by *C. jejuni* to recombine *in vitro*-transposed chromosomal DNA. In combination with the genome sequence of *C. jejuni* (Parkhill *et al.*, 2000), we can now begin to identify factors required for colonization and pathogenesis of this

important human pathogen using random mutagenesis systems that have been successful with other pathogens (Hensel *et al.*, 1995).

We developed an additional method for creating non-polar mutations in *C. jejuni*, a very useful tool for genetic analysis of an organism in which 94% of the genome consists of ORFs (Parkhill *et al.*, 2000). Many genes in *C. jejuni* appear to lack promoter regions and, thus, there is probably a high degree of co-transcription of these genes. The current accepted method for generating mutants in *C. jejuni* is insertional inactivation of genes by cassettes conferring antibiotic resistance, which may have polar effects on the transcription of downstream genes. Our method allows for the generation of deletion mutants lacking antibiotic resistance cassettes, reducing the possibility that the mutations have polar effects on transcription and providing greater flexibility in future genetic manipulation using plasmid constructs. By first generating a streptomycin-resistant *Campylobacter* isolate either spontaneously or using our cloned *rpsL*^{Sm} (pDRH173), this system of defined deletion mutagenesis should be easily adapted to other *Campylobacter* strains.

In order to test the utility of our mutagenesis system, we screened our 81-176 *solo* library for defects in motility and identified two σ-factors, σ^{28} and σ^{54}, encoded by *fliA* and *rpoN* respectively. With σ^{70}, these three σ-factors are the only identified σ-factors in the *C. jejuni* genome (Parkhill *et al.*, 2000), suggesting that this bacterium may depend more on transcriptional activators rather than using a myriad of σ-factors for specific control of gene expression. Previously, σ^{28} and σ^{54} were proposed to direct *flaA* and *flaB* transcription, respectively, in both *C. jejuni* and *C. coli* (Guerry *et al.*, 1990; 1991; Nuijten *et al.*, 1990; Wassenaar *et al.*, 1994), and σ^{54} had been proposed to be required for the transcription of *flgE* in *C. coli* (Kinsella *et al.*, 1997). However, direct evidence to support these hypotheses was lacking because a *fliA* or an *rpoN* mutant was not constructed. By constructing these mutants, we showed that σ^{28} is required for the transcription of *flaA* and that σ^{54} is required for the transcription of *flaB*. The residual motility of the *fliA* mutant, which does not express *flaA*, suggests that functional flagella can be assembled with only FlaB. This observation indicates that *fliA* may not regulate other flagellar biosynthesis or structural genes. In addition, we found that, unlike in *C. coli*, σ^{54}-mediated transcription of *flgE* is coupled to transcription of *flgD* in *C. jejuni*. As σ^{54} is dependent upon transcriptional activators for initiating transcription (Shingler, 1996), we are currently using *solo*-based mutagenesis to identify candidate activators for transcription of *flaB* and *flgDE*.

We identified CetA (Cj1190c) and CetB (Cj1189c) as two proteins in *C. jejuni* that may mediate an energy taxis response. The predicted protein sequence of CetA contains an N-terminal transmembrane region that would
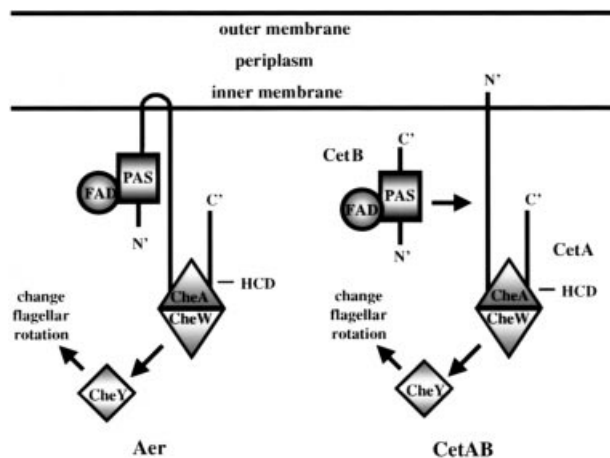
Fig. 4. Model of Aer and the Cet proteins in mediating energy taxis responses. Comparison of the bipartite Cet system of *C. jejuni* with the unipartite Aer system of *E. coli* and *P. putida* is shown. See *Discussion* for details.

localize the protein to the bacterial inner membrane, followed by a large C-terminal cytoplasmic domain with an HCD to interact with the CheA–CheW complex, similar to the C-terminal region of Aer (Fig. 4; [Bibikov *et al.*, 1997; Rebbapragada *et al.*, 1997; Nichols and Harwood, 2000]). CetA lacks any region resembling a PAS domain, but CetB is predicted to be a cytoplasmic protein containing a PAS domain similar to that found in the N-terminus of Aer (Bibikov *et al.*, 1997; Rebbapragada *et al.*, 1997; Nichols and Harwood, 2000). We hypothesize that *C. jejuni* lacks a typical Aer protein and, instead, has the Aer-like PAS and HCD domains divided among CetA and CetB. We propose that, like the PAS domain of Aer (Bibikov *et al.*, 1997; 2000; Repik *et al.*, 2000), the PAS domain of CetB may sense environments providing high electron transport and ATP generation (e.g. environments containing appropriate carbon sources or favoured terminal electron acceptors), possibly using FAD as a redox sensor to monitor electron transport. CetB may recognize energy-poor environments by monitoring the redox state of FAD and then interacting with CetA to transduce signals via the HCD to the chemotaxis proteins, altering flagellar rotation and directing motility towards new environments with higher energy-producing capabilities. The bipartite nature of this proposed signalling apparatus would be an unusual departure from the structure of PAS-containing motility regulators in other bacteria (Taylor and Zhulin, 1999). Interaction between CetA and CetB (if it is demonstrated) would then raise the question of whether either protein interacts with other partners to mediate different signalling responses. Relevant to this question is the fact that immediately upstream of *cetA* is *Cj1191c* (Parkhill *et al.*, 2000), which has a coding sequence that is 63% identical to CetB and is also predicted to contain a PAS domain. Preliminary evidence indicates that Cj1191c is not

required for motility or energy taxis in our assays (D. R. Hendrixson and V. J. DiRita, unpublished data). However, it is possible that the PAS domain in Cj1191c may sense other environmental signals to alter other energy-dependent behaviours.

Even though *C. jejuni* has been established as the leading cause of bacterial diarrhoea in humans, by comparison with other diarrhoeal pathogens little is known about pathogenic mechanisms in this organism, mainly because of the paucity of genetic tools to manipulate the bacterium. This report describes two new techniques for genetic analyses in *C. jejuni*. Application of these tools coupled with the recent completion of the genome sequence of *C. jejuni* has enabled us to identify genes required for motility and to generate new knowledge regarding gene regulation and tactic responses in *C. jejuni*. We anticipate adapting these tools to identify factors required for colonization and virulence of this important pathogen.

Experimental procedures

Bacterial strains and plasmids

All bacterial strains and plasmids used in this study are listed in Table 2. *Campylobacter jejuni* 81-176 is a clinical isolate that has been shown to cause diarrhoeal disease in human volunteers (Korlath *et al.*, 1985; Black *et al.*, 1988). NCTC 11168 is another *C. jejuni* clinical isolate whose genome sequence was reported recently (Parkhill *et al.*, 2000). *C. jejuni* was routinely grown at 37°C on Mueller–Hinton (MH) agar with 10 μg ml^{-1} trimethoprim in microaerophilic conditions. Microaerophilic conditions were generated using the BBL CampyPak Plus (Becton Dickinson) gas pack in an enclosed chamber or by inflating plastic sealed bags with a gas mixture containing 85% N_2, 10% CO_2 and 5% O_2. For *C. jejuni*, antibiotics were used in the following concentrations: kanamycin, 50 or 100 μg ml^{-1}; chloramphenicol, 10 or 20 μg ml^{-1}; and streptomycin, 0.1, 1 or 2 mg ml^{-1}. All *C. jejuni* strains were stored at −80°C in MH broth containing 15% glycerol. *E. coli* DH5α and DH5$\alpha\lambda$pir were grown in Luria–Bertani (LB) agar or broth. For *E. coli*, antibiotics were used in the following concentrations: ampicillin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; and chloramphenicol, 30 μg ml^{-1}. All *E. coli* strains were stored at −80°C in LB containing 20% glycerol.

Plasmid pFD1 contains the *Himar1* transposase under the control of the *lac* promoter and the *Himar1*-based minitransposon (Rubin *et al.*, 1999). Plasmids pILL600 (Labigne-Roussel *et al.*, 1988) and pRY109 (Yao *et al.*, 1993) were the sources of the *Campylobacter aphA-3* (encoding kanamycin resistance) and *cat* (encoding chloramphenicol resistance) cassettes respectively.

Random transposon mutagenesis of C. jejuni

To construct *Himar1*-based minitransposons for *C. jejuni*, pFD1 was digested with *MluI* to release the Tn*903* kanamycin

Table 2. Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristics	Reference
Bacteria		
E. coli		
DH5α	*supE*44 Δ*lac*U169 (φ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Hanahan (1983)
DH5αλpir	DH5α with λ(*pir*)	Laboratory collection
C. jejuni		
NCTC 11168	Clinical isolate used for genome sequencing	Parkhill *et al.* (2000)
81-176	Clinical isolate	Korlath *et al.* (1985)
DRH178	NCTC 11168 Sm^R; spontaneous mutant	This study
DRH212	81-176 Sm^R	This study
DRH307	DRH212 Δ*cetB*; contains deletion of entire coding sequence for *cetB*	This study
DRH311	DRH212 Δ*fliA*; contains in frame deletion of codons 18−222 in the coding sequence of *fliA*	This study
DRH321	DRH212 Δ*rpoN*; contains in frame fusion of the start and stop codons deleting intervening coding sequence	This study
DRH333	DRH212 Δ*cetA*; contains in frame deletion of codons 11−446 in the coding sequence of *cetA*	This study
Plasmids		
pUC19	Amp^R	New England Biolabs
pBR322	Amp^R; Tc^R	New England Biolabs
pFD1	Contains gene for the *Himar1* transposase and the *Himar1* minitransposon	Rubin *et al.* (1999)
pRY109	Source of the *Campylobacter cat* cassette	Yao *et al.* (1993)
pILL600	Source of the *Campylobacter aphA-3* cassette	Labigne-Roussel *et al.* (1988)
pFalcon	pFD1 derivative containing the *solo* transposon	This study
pEnterprise	pFD1 derivative containing the *picard* transposon	This study
pDRH172	pUC19 with 0.7 kb fragment harbouring *rpsL* from *C. jejuni* NCTC 11168 cloned into the *Hind*III site	This study
pDRH173	pUC19 with 0.7 kb fragment harbouring *rpsL*^{Sm} from DRH178 cloned into the *Hind*III site	This study
pDRH181	pDRH172 with *cat* cloned into the *Sph*I site	This study
pDRH263	pUC19 with 1.7 kb fragment harbouring *fliA* from 81-176 cloned into the *Bam*HI site	This study
pDRH265	pUC19 with 1.4 kb *cat-rpsL* cassette from pDRH181 cloned into the *Sma*I site	This study
pDRH276	pBR322 with 2.5 kb fragment harbouring *rpoN* from 81-176 cloned into the *Bam*HI site	This study
pDRH277	pBR322 with 1.5 kb fragment harbouring *cetB* from 81-176 cloned into the *Eco*RV site	This study
pDRH281	pBR322 with 1.5 kb fragment harbouring *cetA* coding sequence cloned into the *Eco*RI site	This study

resistance cassette from the *magellan3* minitransposon (Rubin *et al.*, 1999). After pILL600 was digested with *Sma*I to release *aphA-3* and pRY109 was digested with *Pvu*II to release *cat*, the antibiotic resistance cassettes were ligated to *Mlu*I linkers, digested with *Mlu*I and then ligated to *Mlu*I-digested pFD1 to create the plasmids pFalcon and pEnterprise. pFalcon contains the minitransposon *solo* (containing *aphA-3*), and pEnterprise contains the minitransposon *picard* (containing *cat*).

In vitro transposition reactions were performed with purified chromosomal DNA from strain 81-176 by modifying the method of Lampe *et al.* (1999). 81-176 chromosomal DNA (2 μg) was incubated with pFalcon or pEnterprise (1 μg) and ≈ 250 ng of purified *Himar1* MarC9 transposase in a final volume of 80 μl of transposition buffer (Lampe *et al.*, 1999) containing 10% glycerol, 25 mM HEPES (pH 7.9), 250 μg ml^{-1} BSA, 1 mM dithiothreitol (DTT), 100 mM NaCl and 5 mM MgCl$_2$ for 4 h at 30°C. Chromosomal DNA was extracted with phenol–chloroform and precipitated with ethanol. To repair small gaps at transposon–chromosomal DNA junctions, the DNA was first treated with 1.5 units of T4 DNA polymerase (Gibco BRL) for 20 min at 11°C. The enzyme was heat inactivated by incubating the reaction for 15 min at 75°C. To complete repair, the DNA was treated with 600 units of T4 DNA ligase (New England Biolabs) for 16 h at 16°C. The transposed chromosomal DNA was dialysed against dH$_2$O and then introduced into 81-176 by biphasic

natural transformation (van Vliet *et al.*, 1997). Transposon mutants were recovered after growth in microaerophilic conditions for 48 h on MH agar containing appropriate antibiotics. Typically, 600−1450 *solo* or *picard* mutants were obtained per transposition reaction. To create a large transposon library, 15 individual transposition reactions were performed with *solo*, and mutants obtained after transformation of each transposition reaction were combined to create a library of ≈ 16 000 individual random transposon mutants.

Isolation and identification of mutants defective in motility

Individual mutants from two libraries consisting of approximately 900 and 1450 individual 81-176 *solo* mutants were picked with a needle and stabbed into MH motility medium containing 0.4% agar. Plates were incubated for 36 h at 37°C in microaerophilic conditions. Transposon mutants were scored against the motility phenotype of wild-type 81-176 to identify mutants that were non-motile or expressed an altered or reduced motility phenotype. To verify that the defect in motility was linked to the insertion of *solo*, chromosomal DNA from each mutant was purified and introduced into wild-type 81-176 by biphasic natural transformation (van Vliet *et al.*, 1997), and the transformants were tested for motility. The sequence surrounding the *solo* insertion in each motility mutant was determined by inverse PCR (Ochman *et al.*,

1988; Tchetina and Newman, 1995) and semi-exponential cycle sequencing (Sarkar and Bolander, 1997). The sequences were then compared with the genome sequence of *C. jejuni* NCTC 11168 to identify the location of *solo*.

Defined deletion mutagenesis of C. jejuni

To construct defined chromosomal deletion mutants of *fliA*, *rpoN*, *Cj1189c* and *Cj1190c* in 81-176, we modified the method of Skorupski and Taylor (1996). A diagram depicting our defined deletion mutagenesis system is shown in Fig. 5. We first isolated a streptomycin-resistant (Sm^R) mutant *C. jejuni* NCTC 11168 strain by plating a lawn of bacteria ($\approx 10^9 - 10^{10}$) on MH plates containing a gradient of 0–1 mg ml^{-1} streptomycin. A single 11168 Sm^R colony was isolated (DRH178), and a 0.7 kb fragment containing *rpsL*^{Sm} from this strain was amplified by PCR with 5′ *Hin*dIII restriction sites. The gene was cloned into pUC19 to create pDRH173. This suicide plasmid was electroporated into 81-176 to replace wild-type *rpsL*, thereby creating DRH212 (81-176 *rpsL*^{Sm}).

To create a *cat–rpsL* cassette, a 0.7 kb fragment containing wild-type *rpsL* from *C. jejuni* NCTC 11168 was amplified by PCR with primers adding 5′ *Hin*dIII restriction sites and ligated into the *Hin*dIII-digested pUC19 to create pDRH172. A

*Pvu*II fragment containing the *cat* cassette was purified from pRY109 (Yao *et al.*, 1993) and ligated into the *Sph*I site that had been filled in by T4 DNA polymerase (Gibco BRL) in pDRH172 to create pDRH181. The *cat–rpsL* cassette from pDRH181 was then amplified by PCR with primers adding 5′ *Sma*I restriction sites and ligated into *Sma*I-digested pUC19 to create pDRH265.

In the first step for defined deletion mutagenesis, the genes to be deleted from the chromosome of 81-176 were amplified with at least 500 bp of upstream and downstream flanking DNA using primers based on the corresponding sequence in *C. jejuni* NCTC 11168 (Parkhill *et al.*, 2000). The primers added specific 5′ restriction sites, and the PCR products were cloned into either pUC19 or pBR322. The following plasmids were constructed: pDRH263 (containing a 1.7 kb fragment harbouring *fliA* in the *Bam*HI site of pUC19); pDRH276 (containing a 2.5 kb fragment harbouring *rpoN* in the *Bam*HI site of pBR322); and pDRH277 (containing a 1.5 kb fragment harbouring *cetB* cloned into the *Eco*RV site of pBR322). Because of difficulties in cloning a fragment harbouring *cetA* with upstream and downstream DNA, we amplified a 1.5 kb fragment containing the coding sequence of the gene into the *Eco*RI site of pBR322 to create pDRH281. The genes were then interrupted by insertion of a *Sma*I fragment containing the *cat–rpsL* cassette from pDRH265. Next, these suicide plasmids harbouring the insertional mutations containing the *cat–rpsL* cassette were electroporated (van Vliet *et al.*, 1997) into 81-176 Sm^R (DRH212), and the insertional mutations replaced the chromosomal copy of the respective wild-type gene through homologous recombination. Transformants were selected for chloramphenicol resistance (Cam^R) and scored for streptomycin sensitivity (Sm^S). These intermediate strains are Sm^S as a result of the recessive nature of the *rpsL*^{Sm} mutation.

SOEing reactions (Higuchi, 1990) were performed to generate fusions of the upstream and downstream DNA segments surrounding each gene, creating the following in frame deletions: *fliA* (in frame fusion of codon 16 to codon 223); *rpoN* (in frame fusion of the start and stop codons); *Cj1189c* (in frame fusion of the entire coding sequence); and *Cj1190c* (in frame fusion of codon 9 to codon 448). The products of the SOEing reactions were cloned into pUC19 to create suicide plasmids. These plasmids containing an in frame deletion were electroporated (van Vliet *et al.*, 1997) into the respective 81-176 intermediate mutant. Transformants were initially selected on MH agar containing 1 or 2 mg ml^{-1} streptomycin and then selected for chloramphenicol sensitivity (Cam^S) on MH agar containing 10 μg ml^{-1} chloramphenicol. Selection for Sm^R and scoring for Cam^S identifies transformants in which the insertional mutation is crossed out of the chromosome, removing the *cat–rpsL* cassette containing the wild-type *rpsL* Sm^S allele and leaving behind the deletion allele on the chromosome of 81-176 Sm^R. Mutants were verified for deletion of appropriate genes by colony PCR.
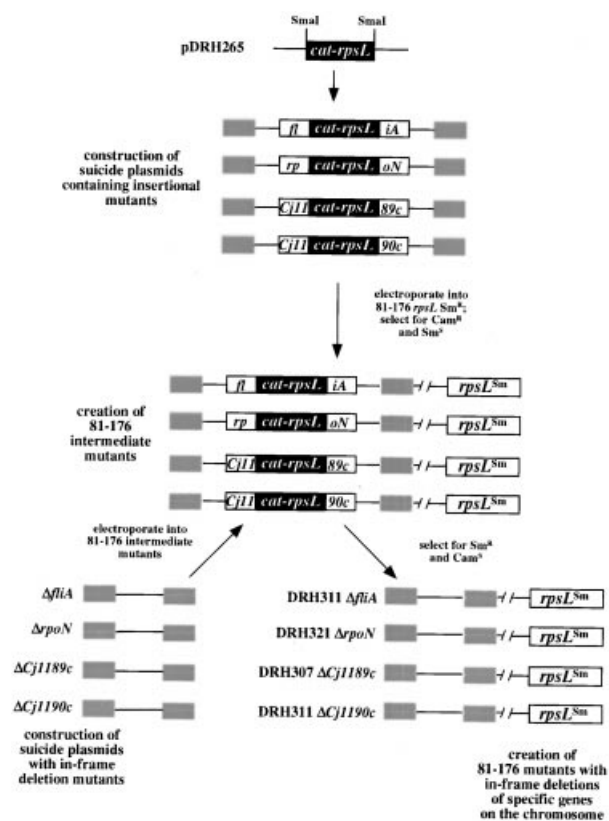

Fig. 5. Diagram of the method for constructing defined chromosomal deletion mutants in *C. jejuni*. This method is a variation of the method developed by Skorupski and Taylor (1996). See *Experimental procedures* for details.

RT–PCR and primer extension analysis

Trizol reagent (Gibco BRL) was used to extract RNA from wild-type 81-176 Sm^R, DRH311 and DRH321 that had been grown on MH agar for 16 h at 37°C in microaerophilic

conditions. RT–PCR reactions were performed with Super-script II reverse transcriptase (Gibco BRL) according to the manufacturer's instructions. RNA (5 μg) from each strain was used along with 250 nmol of random primers (Gibco BRL) to synthesize cDNA. Equal amounts of cDNA products were used in a PCR reaction with appropriate primers to amplify a portion of the coding sequence of *astA* (encoding aryl-sulphatase; Yao and Guerry, 1996), *flaB*, *flgD* and *flgE*. To amplify the coding sequence of *flaA*, primers were used as described previously (Alm *et al.*, 1993) in a PCR reaction with an annealing temperature of 44°C. Control reactions lacking reverse transcriptase were performed as described above. For primer extension reactions, primers that bound ≈ 30–50 bp downstream of the start codons on the coding strands of *flgD* and *flgE* were end labelled with [γ-^{32}P]-ATP by T4 DNA kinase (Gibco BRL). Reactions were performed as described previously (Higgins and DiRita, 1994). Products from each set of reactions were run on a 6% polyacrylamide sequencing gel alongside a sequencing ladder of the respec-tive gene using the same end-labelled primer for the primer extension reactions. The sequencing ladder was generated by semi-exponential cycle of a PCR product containing portions of the promoter and coding sequence of each gene.

Energy taxis analysis of C. jejuni mutants

To determine the ability of the strains to demonstrate an energy taxis response, bacteria from MH agar grown for 16 h at 37°C in microaerophilic conditions were resuspended in MH broth to an OD$_{600}$ of 0.7 and then stabbed with a needle into a *Campylobacter* defined motility (CDM) media. We modified the medium described by Leach *et al.* (1997) by omitting the sodium pyruvate and adding agar to a final concentration of 0.4%, forming our basic defined motility media. Motility medium was supplemented with 0.91 M sodium pyruvate or 30 mM fumarate. Motility phenotypes were examined after incubation of the plates at 37°C for 48 h in microaerophilic conditions.

Acknowledgements

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