A novel approach for the construction of a *Campylobacter* mutant library

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Given the lack of functional transposons for use in *Campylobacter* spp., an alternative method of insertional mutagenesis using natural transformation was developed. High efficiencies of transformation were only obtained with species-specific DNA. This feature was a key element in the construction of mutant libraries of this bacterium. A chromosomal library of *Campylobacter jejuni* 81116 DNA was made in shuttle vector pUOA18. Next, a kanamycin-resistance (Km^R) cassette was ligated into the inserts of the plasmids. *C. jejuni* 81116 was then transformed with the resulting products to allow homologous recombination between genomic fragments present in the shuttle vector and the chromosome. Transformants were pooled and chromosomal DNA from these transformants was used to retransform *C. jejuni* 81116. This resulted in transformants containing the Km^R cassette in the chromosome but lacking the vector. In order to evaluate this approach for the construction of a mutant bank, the Km^R insertional mutants were screened for loss of motility. Partial characterization of 11 non-motile mutants indicated that the inserted genes are involved in motility. Four mutants had the Km^R cassette inserted in genes involved in flagella biosynthesis, namely *flaA/B*, *neuB* and *flgK*, and produced incomplete or no flagella. Four mutants had the Km^R cassette inserted in genes possibly involved in flagella motor function: *pflA*, *fliM* and *orf1* downstream of the *fliN* gene. Three mutants had the Km^R cassette inserted in genes that are homologous to genes encoding hypothetical proteins of *Helicobacter pylori*.

Keywords: *Campylobacter jejuni*, mutant library, motility mutants, genetics

INTRODUCTION

Campylobacter jejuni is a Gram-negative bacterium which is widespread in nature. It is one of the major causes of human enteritis both in developed and developing countries (Tauxe, 1992; Taylor, 1992), but it is also a commensal of many animal species (Skirrow & Blaser, 1992). How *C. jejuni* causes disease is only partially known. So far, the flagella have been proven to play an important role in pathogenicity (Grant *et al.*, 1993; Ruiz-Palacios *et al.*, 1992; Wassenaar *et al.*, 1991; Yao *et al.*, 1994). The identification and characterization of *C. jejuni* virulence factors have been hampered by the

lack of an animal model and the sparsity of useful genetic tools. Given the lack of functional transposons for use in *Campylobacter* spp., alternative methods to obtain mutants are needed.

Some strains of *C. jejuni* are naturally transformable (Wassenaar *et al.*, 1993). This has been shown to be the most efficient way for DNA uptake in *C. jejuni* strain 81116 (Wassenaar *et al.*, 1993). Methods for insertional mutagenesis have been described for several bacteria which, like *Campylobacter* spp., are naturally transformable (Labarre *et al.*, 1989; Sharetzsky *et al.*, 1991). Also for *Campylobacter* spp. several attempts have been made to construct a mutant library; Yao *et al.* (1994) used an adaptation of a method described originally by Sharetzsky *et al.* (1991) for *Haemophilus influenzae*, while Dickinson *et al.* (1995) have used integrational plasmid vectors. However, complete libraries could not be constructed, mainly due to low frequencies of transformation.

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With the knowledge obtained on natural transformation of *C. jejuni* 81116 we have developed a novel approach for the construction of a mutant library of this bacterium. This new approach is an alternative for transposon mutagenesis. To evaluate the mutagenesis procedure the mutants were screened for non-motility, which is an easily detectable phenotype. We have isolated and partially characterized non-motile mutants from this library.

METHODS

Bacterial strains and plasmids. The origin of *C. jejuni* strain 81116, its Fla⁻ phase variant and its flagellin mutant R1 (*flaA*::Km^R) have been described previously (Wassenaar *et al.*, 1991). *Escherichia coli* strain DH5 α (Sambrook *et al.*, 1989) was used as a host for plasmid pUOA18 in the experiments for optimizing natural transformation.

The shuttle vector pUOA18 containing the chloramphenicol-resistance gene has been described previously (Wang & Taylor, 1990). pILL550 (Labigne-Roussel *et al.*, 1987) was the original source of the kanamycin-resistance (Km^R) cassette. pBF14 is a pUOA18 vector containing the Km^R cassette flanked by two polylinkers and used to obtain Km^R cassettes with *Bam*HI, *Pst*I or *Pvu*II (blunt) ends. pBF6 is a pBluescript vector containing the *flaA* and *flaB* genes of *C. jejuni* 81116 separated by a Km^R cassette. pBF10 is the pUOA18 vector containing the insert from pBF6.

Media and growth conditions. *C. jejuni* strain 81116 was grown under microaerophilic conditions on Skirrow agar medium (Skirrow, 1977) at 42 °C for 48 h. *E. coli* was grown in LB medium at 37 °C. Antibiotic concentrations used were as follows: chloramphenicol (Sigma), 20 µg ml⁻¹; kanamycin (Sigma), 30 µg ml⁻¹; ampicillin (Centrafarm), 100 µg ml⁻¹. For motility tests bacteria were grown in thioglycollate medium (BBL) containing 0.4% agar (Caldwell *et al.*, 1985). The human embryonic intestine cell line (INT407) was obtained from Flow Laboratories and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 5% heat-inactivated foetal calf serum (Gibco), penicillin (Gibco; 100 U ml⁻¹) and streptomycin (Gibco; 10 µg ml⁻¹).

Recombinant DNA techniques. DNA isolation, restriction enzyme digestion and DNA ligation were performed as described by Ausubel *et al.* (1992). Restriction enzymes were obtained from Pharmacia Biotech Benelux. DNA fragments for subcloning and for use as probes were isolated from agarose gels using the GeneCleanII kit (Bio101). The *flaA* gene of *C. jejuni* 81116 was amplified by PCR using primers CGTATTAACACAAATGTTGCAGCA and CTGCTGCTTTAACATTAACCTTGAG (nucleotides 92–115 and 1653–1675 of database entry J05635) and used as probe. Probes for hybridization experiments were labelled with digoxigenin-dUTP using the DIG DNA labelling kit (Boehringer Mannheim). Hybridizations were performed at 62 °C and the DIG-labelled nucleic acid hybrids were detected using the DIG Luminescent Detection kit (Boehringer Mannheim). Plasmid DNA used for sequencing reactions was purified by using the Qiagen plasmid kit.

Natural transformation. For natural transformation of *C. jejuni* 81116 with plasmid or chromosomal DNA the bacteria were cultured overnight and harvested in heart infusion (HI; Difco) broth and diluted to a concentration of 10⁸ c.f.u. ml⁻¹. To induce competence, 200 µl cells was incubated for 3 h at

37 °C under microaerophilic conditions on 1 ml HI agar in a 2.0 ml micro test tube (Eppendorf), hereby creating a biphasic medium. Then, DNA was added and incubated for 3 h at 37 °C under microaerophilic conditions. Cells were harvested and plated on Skirrow plates supplemented with the appropriate antibiotics and incubated for 48 h at 42 °C.

Construction of the mutant library. Firstly, a genomic library of *C. jejuni* 81116 was made by partial digestion of chromosomal DNA with *Sau*3AI. After fractionation by centrifugation in 10–40% sucrose gradients, fragments of 0.5–5 kb were isolated. To prevent self-ligation the sticky ends of 10 µg DNA were partially filled in with 1 µl 30 mM dATP and dGTP and 3 U Klenow fragment in a volume of 100 µl for 15 min at 37 °C. The vector pUOA18, isolated from *C. jejuni* 81116, was digested with *Sall* and partially filled in with dCTP and dTTP as described above. After extraction with phenol/chloroform and ethanol precipitation, fragments and vector were ligated with T4 DNA ligase for 48 h at 16 °C. The ligation mixture was used to transform *C. jejuni* 81116 wild-type cells by natural transformation and plated onto Skirrow plates with chloramphenicol. The resulting library contained 15 000 transformants.

Secondly, plasmid DNA was isolated from this pool of transformants and 10 µg was digested with *Bgl*II, *Bcl*I, *Bst*EII, *Nsi*I or *Eco*RV. The Km^R cassette, also isolated from *C. jejuni* 81116, was ligated into these restriction sites in a volume of 20 µl for 48 h at 16 °C. The *Bgl*II, *Bcl*I, *Bst*EII, *Nsi*I or *Eco*RV sites were not restored by using Km^R cassettes with *Bam*HI, *Pst*I or *Pvu*II ends. These ligation mixtures were used to transform *C. jejuni* 81116 wild-type cells and resulted in five different batches of > 1000 transformants.

Finally, chromosomal DNA was isolated from the batches of pooled transformants and used to transform the wild-type strain 81116 by a second round of natural transformation. The resulting transformants were grown on Skirrow plates with kanamycin and Skirrow plates with chloramphenicol to confirm the presence of the Km^R cassette in the chromosome and the absence of the shuttle vector pUOA18.

DNA sequence analysis of the insertional mutants. Circularized *Bgl*II–*Bcl*I, *Asel* or *Hinf*I chromosomal DNA fragments from the *C. jejuni* non-motile mutants were used as template in the inverse PCR. Two primers reaching outward from within the Km^R cassette were used to determine the point of insertion of the Km^R cassette. Primer 1 (TATCACCTC-AAATGGTTCGCTGGG) corresponds to nucleotides 36–50 and primer 2 (GGGGATCAAGCCTGATTGGGAGA) contains the nucleotides 1368–1390 of the Km^R cassette (nucleotides 7–21 and 1339–1361 of database entry M26832, respectively). Inverse PCR was performed in a Gene Amp 9600 Thermocycler (Perkin-Elmer Cetus). The cycling conditions were as follows: 35 cycles of 15 s of denaturation at 94 °C; 10 s of primer annealing at 65 °C; and 2 min of extension at 72 °C; followed by a final extension of 5 min at 72 °C. The inverse PCR product was cloned in the pGEM-T Easy vector (Promega). The sequence of the cloned DNA was determined by the dideoxy chain-termination method (Sanger *et al.*, 1977) with an Automated Laser Fluorescent DNA Sequencer (Pharmacia), the autoread sequencing kit using T7 DNA polymerase (Pharmacia) and the fluorescein-labelled nucleotide primers T7 and SP6 (Pharmacia).

The 2.0 version of the BLAST program (Altschul *et al.*, 1990) was used to compare nucleotide sequences with those in

Table 1. Characterization of *C. jejuni* strain 81116 non-motile mutants

Mutant	Site of Km ^R insertion	Flagella	Mutated gene				
			Similarity	Percentage identity*		Function or structure	Database entry
				1	2		
BG1	<i>Bgl</i> II	Normal	<i>pflA</i> , <i>C. jejuni</i>	99% over 282 bases	99% over 423 bases	Flagella movement	CJU09019
BG2	<i>Bgl</i> II	None	<i>neuB</i> , <i>C. jejuni</i>	96% over 194 bases	95% over 150 bases	Sialic acid synthase	AJ000855
BG3	<i>Bgl</i> II	None	<i>flaA</i> , <i>C. jejuni</i>	100% over 224 bases		Flagellin A	J05635
			<i>flaB</i> , <i>C. coli</i>		89% over 166 bases	Flagellin B	M64671
BG4	<i>Bgl</i> III	Normal	Unknown, <i>H. pylori</i>	66% over 43 aa		Hypothetical integral membrane protein	AE000612 gene HP1044
			<i>flgK</i> , <i>H. pylori</i>		61% over 80 aa	HAP1	AE000618 gene HP1119
BG5	<i>Bgl</i> II	Normal	Unknown				
BG6	<i>Bgl</i> II	Normal	NP				
BC7	<i>Bcl</i> I	Stump	<i>flgK</i> , <i>H. pylori</i>	85% over 20 aa		HAP1	AE000618 gene HP1119
			Unknown, <i>H. pylori</i>		52% over 98 aa	Hypothetical protein	AE000544 gene HP0245
B58	<i>Bst</i> EII	Normal	<i>orf1-fltN</i> , <i>C. jejuni</i>	98% over 220 bases	88% over 153 bases	Hypothetical protein	AJ000400
BS9	<i>Bst</i> EII	Normal	<i>orf1-fltN</i> , <i>C. jejuni</i>	56% over 61 aa		Hypothetical protein	AJ000400
			<i>flaC</i> , <i>S. marcescens</i>		67% over 35 aa	Flagellin	P13713
NS10	<i>Nsi</i> I	Normal	<i>flaM</i> , <i>H. pylori</i>	56% over 243 bases		Flagellar motor switch	AE000611 gene HP1031
EC11	<i>EcoRV</i>	None	Unknown, <i>H. pylori</i>	53% over 111 aa		Hypothetical protein	AE000533 gene HP0114

NP, Not possible to determine because inverse PCR failed.

* The two columns refer to the sides flanking the kanamycin insertion.

databases available at Genome Net (www.ncbi.nlm.nih.gov/BLAST/). The database entry codes are given in Table 1.

Invasion assay. To roughly estimate the invasive capacity of the non-motile mutants we adapted the invasion assay of Wassenaar *et al.* (1991). Briefly, approximately 10^7 bacteria were added to a confluent monolayer of 10^5 INT407 cells per well in a 24-well plate. After an incubation period of 1 h at 37 °C and 5% CO₂, the monolayers were washed five times with DMEM and reincubated for another 3 h with DMEM containing 250 µg gentamicin ml⁻¹. Following this second incubation, the monolayers were washed three times with PBS and lysed in 0.5 ml PBS containing 0.5% Triton X-100. To determine the number of intracellular bacteria, 50 µl of the 10⁻¹ dilution was plated on Skirrow agar plates. We observed 500 or more colonies when *C. jejuni* 81116 wild-type was used in this assay, while no colonies were observed with the non-motile Fla⁻ variant.

Electron microscopy. Bacteria were grown on Skirrow agar plates for 24 h at 42 °C and suspended in 2% bovine serum albumin in PBS. Next, the bacteria were incubated on 200-mesh copper grids coated with Formvar, stained in 2% tungstophosphoric acid (pH 6.8) for 2 min and examined in a Philips CM 10 electron microscope.

RESULTS

Optimizing the efficiency of natural transformation

Three different suicide and shuttle vector constructs have been used to optimize natural transformation of *C. jejuni* strain 81116. No transformants were obtained when the shuttle vector pUOA18 originating from either *C. jejuni* strain 81116 or *E. coli* was used. *C. jejuni* could be transformed with the suicide vector pBluescript containing the *flaA* and *flaB* genes of *C. jejuni* 81116 originating from *E. coli* if at least 10 µg DNA was used. However, the cloning of the same *flaA* and *flaB* genes in the pUOA18 shuttle vector resulted in 80 transformants (µg DNA)⁻¹ when the plasmid was isolated from *E. coli*,

Fig. 1. Southern blot analysis of DNAs from non-motile mutants of *C. jejuni* 81116 digested with *Bgl*II and probed with the Km^R cassette (a) and the *flaA* gene (b). Lanes: 1, BG1; 2, BG2; 3, BG3; 4, BG4; 5, BG5; 6, BG6; 7, BC7; 8, B58; 9, B59; 10, NS10; 11, EC11; 12, wild-type; 13, pUOA18; 14, R1. Molecular size markers are indicated on the right.

and 1.6×10^5 transformants when isolated from *C. jejuni* strain 81116. These results confirm the high species-specificity for DNA in natural transformation of strain 81116. To obtain a high transformation efficiency of *C. jejuni* strain 81116, in subsequent experiments plasmid DNA was always isolated from *C. jejuni* strain 81116.

Fig. 2. Electron micrographs of the *C. jejuni* 81116 wild-type (a) and non-motile mutants BG2 (b), BG3 (c), BC7 (d), BS8 (e) and NS10 (f). Bars, 0.5 μ m.

Construction and screening of the insertional mutant library

The mutants were constructed as described in Methods. Briefly, chromosomal DNA of strain 81116 was partially digested with *Sau*3AI. A chromosomal library was made by ligating the resulting fragments of 0.5–5 kb in shuttle vector pUOA18, isolated from *C. jejuni* 81116. Next, a Km^R cassette, also isolated from *C. jejuni* 81116, was inserted in several restriction sites present in the inserts of the plasmids from this library. The *C. jejuni* wild-type strain 81116 was then transformed with this library by natural transformation. The transformants were cultured for 48 h to enable recombination between the DNA inserts of the shuttle vectors and the chromosomal DNA.

Chromosomal DNA was isolated from the pooled transformants and used to transform the wild-type strain 81116. This second round of natural transformation was performed to ensure that the resulting Km^R transformants lacked the shuttle vector and contained the Km^R cassette in the chromosome.

A total of 1300 Km^R transformants was screened for motility in semi-solid thioglycollate medium. Eleven non-motile mutants were identified and their non-motile phenotype was confirmed microscopically. In order to be certain that the observed phenotype resulted from insertion of the Km^R cassette and not from a motility phase switch or a secondary mutation, total DNA was extracted from each mutant and used to retransform the wild-type strain 81116. All resulting Km^R transformants were non-motile, showing that the acquisition of Km^R correlated with acquisition of the non-motile phenotype. All mutants were susceptible to chloramphenicol, indicating the absence of the pUOA18 shuttle vector.

Characterization of the non-motile mutants

The mutants showing motility defects (listed in Table 1) were chosen to study the molecular events associated with the mutagenesis procedure. Chromosomal DNA from these mutants was analysed in a Southern blot and probed with the Km^R cassette (Fig. 1a). Since a complete library should contain at least a mutant with a Km^R cassette in one of the flagella genes (*flaA* or *flaB*), all mutants were also hybridized to a *flaA* probe (Fig. 1b). One mutant (BG3) showed a hybridization pattern similar to the flagellin A mutant R1 (*flaA*:: Km^R) (Wassenaar *et al.*, 1991), indicating the presence of a Km^R cassette in the *Bgl*II site of the *flaA* gene (Fig. 1b, lanes 3 and 14). By sequencing the flanking regions of the Km^R cassette, we could demonstrate that a 3.4 kb *Bgl*II fragment containing a part of the *flaA* gene and the complete *flaB* gene was deleted.

To determine the morphology of the mutants, they were examined by electron microscopy. One mutant had truncated flagella (BC7), three mutants completely lacked flagella (BG2, BG3 and EC11) and seven mutants had flagellar filaments with the same length as the wild-type strain (Fig. 2).

When tested in an invasion assay, all 11 mutants showed a more than 100-fold reduction of invasiveness, compared to the wild-type strain (results not shown).

DNA sequence analysis of the non-motile insertional mutants

The flanking regions of the Km^R cassette were amplified by inverse PCR on circularized genomic *Bgl*II–*Bcl*II, *Ase*I or *Hin*fI fragments. The resulting PCR products were cloned in the pGEM-T Easy vector and their sequences were determined. A search in the databases available at Genome Net (www.ncbi.nlm.nih.gov/BLAST/) indicated the position of the Km^R insertion. The position of the Km^R insertions in the non-motile mutants and their phenotypes are summarized in Table 1. A Km^R insertion was found in the *pflA* gene (Yao *et al.*, 1994) for mutant BG1 and the *neuB* gene (A. Karlyshev, database entry AJ000855) for mutant BG2. The Km^R insertion in mutant BG3 was flanked on one side by the *flaA* gene (Nuijten *et al.*, 1990) while the other side was located downstream of the *flaB* gene (Guerry *et al.*, 1991). In mutant BS8 the Km^R cassette was inserted in a gene that showed homology with the *orf1* located downstream of the *fliN* gene of *C. jejuni* (A. Karlyshev, database entry AJ000400). The Km^R cassette in mutant BS9 was flanked by the *orf1* located downstream of the *fliN* gene and sequences which corresponded at the amino acid level with the *fliC* gene of *Serratia marcescens* (Harshey *et al.*, 1989). In mutant NS10 we have identified one flanking region which was homologous to the *fliM* gene of *Helicobacter pylori* (Tomb *et al.*, 1997).

The flanking regions of the Km^R insertions in mutants BG4, BC7 and EC11 were only at the amino acid level homologous with genes of *H. pylori*. In mutants BG4 and BC7 one flanking region showed homology to the flagellar hook-associated protein (HAP1; Tomb *et al.*, 1997) while the other flanking regions were homologous to genes encoding different hypothetical proteins. The flanking regions of the Km^R cassette in mutant EC11 corresponded also at the amino acid level with a gene encoding a hypothetical protein of *H. pylori* (Tomb *et al.*, 1997).

The flanking regions of the Km^R cassette in mutant BG5 showed no homology with known genes. We were unable to determine the sequence of the flanking regions of the Km^R cassette in mutant BG6.

DISCUSSION

We describe here a novel approach for the construction of mutant libraries of *C. jejuni* strain 81116 using natural transformation. In *C. jejuni* DNA uptake by means of natural transformation is highly efficient when DNA sequences identical to the incoming DNA are already present in the acceptor cell (Wang & Taylor, 1990; Wassenaar *et al.*, 1993). Additionally, the use of DNA isolated from the same bacterial strain as the acceptor cell, thus carrying the same restriction modifications, greatly enhances the efficiency of transform-

ation. These two features are key elements in the construction of the mutant library described here. Furthermore, recombinations between the incoming plasmids and the chromosome were facilitated by the use of a multi-copy shuttle vector. To our knowledge this is the first report on recombinations occurring between a shuttle vector and chromosomal DNA. When given enough time by using a shuttle vector as opposed to a suicide vector the frequency of crossover events is high in *C. jejuni*.

The positions of the Km^R cassette within the inserts of the mutant library are not random. They are based on the presence of *Bgl*II, *Bcl*II, *Bst*EII, *Nsi*I or *Eco*RV restriction sites in the inserts of the chromosomal library. These restriction enzymes were selected since their sites are absent in the pUOA18 vector. The position of the restriction site in the inserts determines the length of homologous DNA flanking the Km^R cassette and thus the efficiency of the homologous recombination. A stretch of 200 bp of homologous DNA is sufficient for recombinational events, but the frequency of recombination increases with longer homologous stretches (Wassenaar *et al.*, 1993). If homologous sequences of sufficient length are present on both sides of the Km^R cassette a double-crossover event will take place, resulting in the insertion of the Km^R cassette into the chromosome.

If two or more restriction sites of the same enzyme are present in the insert, a deletion of a particular fragment can be followed by an insertion of the Km^R cassette. In mutant BG1 a *Bgl*II fragment of 513 bp from the *pflA* gene was replaced by the Km^R cassette. In mutant BG3 a 3.4 kb *Bgl*II fragment containing the 3' part of the *flaA* gene and the complete *flaB* gene was replaced by the Km^R cassette.

The mutants were tested for motility and partially characterized in order to evaluate the mutagenesis procedure. The characterization of the non-motile mutants supports the concept that the inserted genes are involved in motility.

Insertions were detected in three genes involved in flagella formation: *flaA/B*, *neuB* and *flgK*. An insertion of the Km^R cassette in the major flagellin subunit genes, *flaA* and *flaB*, prevented the synthesis of flagellin A and flagellin B, and consequently the formation of flagella, thus explaining the non-motile phenotype of mutant BG3. A Km^R insertion was also detected in the *neuB* gene, encoding a sialic acid synthase. The post-translational modification of *Campylobacter* flagellin includes sialic acid (Doig *et al.*, 1996), and may explain the Fla⁻ phenotype of mutant BG2. Our data also demonstrate an insertion in the putative *flgK* gene, encoding the HAP1 protein. In *Salmonella* the HAP1 displaces the hook cap to form the first hook-filament junction zone, followed by distal assembly of the HAP3 protein, HAP2 protein and flagellin subunits (Macnab, 1996). We characterized two different mutants (BG4, BC7) with one region homologous to the *flgK* gene of *H. pylori* flanking the Km^R cassette and one region homologous

to different hypothetical proteins of *H. pylori* (Tomb *et al.*, 1997). The genes encoding these different hypothetical proteins must be located within 5 kb of the putative *flgK* gene since this is the maximum size of the inserts in our chromosomal library before introduction of the Km^R cassette. Mutant BC7 had stump-like flagella, which is in line with the mutated *flgK* gene and the non-motile phenotype. However, mutant BG4 had long flagella. Apparently it still contains a functional HAP1.

Insertions were also detected in three genes possibly involved in the flagellar motor function: *pflA*, *fliM* and *orf1* downstream of *fliN*. The flagellated mutant BG1 with the Km^R cassette insertion in the *pflA* gene has paralysed flagella and was the same as the mutant generated from plasmid RY302 as described by Yao *et al.* (1994). An insertion of the Km^R cassette in the *fliM* gene or in the *orf1* located downstream of the *fliN* gene may lead to a switching defect since the FliN protein presumably interacts with the FliG and the FliM protein to form the switch complex in a similar way as described for *Salmonella* (Toker & Macnab, 1997).

Km^R insertions were also found in genes with no homology to known genes. The observed phenotypes of these mutants (Mot⁻, Inv⁻ and Fla^{+/-}) confirm that the genes must be involved in motility. Further characterization will be needed to reveal their function.

In *E. coli* and *Salmonella* more than 40 genes, organized in 15 (*E. coli*) or 17 (*Salmonella typhimurium*) operons, are involved in flagellar assembly, structure and function (Macnab, 1996). The size of the genome of *Campylobacter* is only 37% of that of these two bacteria. If the number of motility genes would be proportionally lower, additional genes involved in the motility of *C. jejuni* remain to be recovered.

So despite the limitation that the mutants are not completely random, the method described here to construct a mutant library in *C. jejuni* is the best that is presently available. The method may also be applicable for exploring the genetics of *Campylobacter* spp. and related organisms which are also naturally transformable. The method has potential for improvement. The construction of *Campylobacter* vectors with fewer restriction sites will enable the creation of more 'random' mutant libraries. Furthermore, by adding unique DNA sequence tags to the Km^R cassette this system could be used to identify virulence genes involved in colonization and invasion *in vivo* (Hensel *et al.*, 1995).

ACKNOWLEDGEMENTS

We thank Ton Ultee from the Department of Pathology for making the electron micrographs.

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Received 6 January 1999; revised 6 April 1999; accepted 13 April 1999.