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 (KmR) 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 KmR cassette in the chromosome but lacking the vector. In order to evaluate this approach for the construction of a mutant bank, the KmR insertion 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 KmR cassette inserted in genes involved in flagella biosynthesis, namely flaAB, neuB and fliGK, and produced incomplete or no flagella. Four mutants had the KmR cassette inserted in genes possibly involved in flagella motor function: pflA, fliM and orf1 downstream of the fliN gene. Three mutants had the KmR 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* : KmR) have been described previously (Wassenaar et al., 1991). *Escherichia coli* strain DH5α (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). pILLS50 (Labigne-Roussel et al., 1987) was the original source of the kanamycin-resistance (KmR) cassette. pBF14 is a pUOA18 vector containing the KmR cassette flanked by two polylinkers and used to obtain KmR cassettes with BamHI, PstI or PsuI (blunt) ends. pBF6 is a pBluescript vector containing the flaA and flaB genes of *C. jejuni* 81116 separated by a KmR 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 Pharmacia Biotech Benelux. DNA fragments were isolated from modified Eagle's medium (DMEM; Gibco) supplemented with 1% 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 Geneclean kit (Biolol). The hybridizations were performed at 62 °C and the DIG-labeled nucleic acid hybrids were detected using the DIG Luminescent Detection kit (Boehringer Mannheim). Hybridizations were performed at 62 °C and the DIG-labeled 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 Sau3A1. 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 SalI 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 15000 transformants.

Secondly, plasmid DNA was isolated from this pool of transformants and 10 µg was digested with BgIII, BclI, BstEII, NsiI or EcoRV. The KmR 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 BgIII, BclI, BstEII, NsiI or EcoRV sites were not restored by using KmR cassettes with BamHI, PstI or PsuI 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 KmR cassette in the chromosome and the absence of the shuttle vector pUOA18.

**DNA sequence analysis of the insertional mutants.** Circularized BgIII–BclI, Asel or HinfI 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 KmR cassette were used to determine the point of insertion of the KmR cassette. Primer 1 (5'-TATCCCTCATGTCCGGCAGG-3') and primer 2 (5'-GGGATCAAGCCTGATTGGGAGA-3') respectively. Inverse PCR was performed in a GeneAmp 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 BLAST.
Table 1. Characterization of C. jejuni strain 81116 non-motile mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Site of Km(^+) insertion</th>
<th>Flagella</th>
<th>Mutated gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>BG1</td>
<td>BglII</td>
<td>Normal</td>
<td>flaA, C. jejuni</td>
</tr>
<tr>
<td>BG2</td>
<td>BglII</td>
<td>None</td>
<td>mbrl, C. jejuni</td>
</tr>
<tr>
<td>BG3</td>
<td>BglII</td>
<td>None</td>
<td>flaA, C. jejuni</td>
</tr>
<tr>
<td>BG4</td>
<td>BglII</td>
<td>Normal</td>
<td>flaA, C. jejuni</td>
</tr>
<tr>
<td>BG5</td>
<td>BglII</td>
<td>Normal</td>
<td>flaA, H. pylori</td>
</tr>
<tr>
<td>BG6</td>
<td>BglII</td>
<td>Normal</td>
<td>np</td>
</tr>
<tr>
<td>BC7</td>
<td>BglII</td>
<td>Normal</td>
<td>flaA, H. pylori</td>
</tr>
<tr>
<td>BS8</td>
<td>BglII</td>
<td>Normal</td>
<td>satf, C. jejuni</td>
</tr>
<tr>
<td>BS9</td>
<td>BglII</td>
<td>Normal</td>
<td>satf, C. jejuni</td>
</tr>
<tr>
<td>NS10</td>
<td>Nil</td>
<td>Unknown</td>
<td>flaA, H. pylori</td>
</tr>
<tr>
<td>EC11</td>
<td>EcorV</td>
<td>None</td>
<td>Unknown, H. pylori</td>
</tr>
</tbody>
</table>

Similarity

- Percentage identity:
  - 1: 99% over 282 bases
  - 2: 99% over 423 bases

Function or structure

- Flagella movement
- Sulphate acid synthase
- Flaggin A
- Flaggin B
- Hypothetical integral membrane protein
- Hypothetical protein
- Flaggin
- Flaggin motor switch
- Hypothetical protein

Database entry

- CJU09019
- AJ000683
- J06e15
- M64e71
- AE100612 gene HP1044
- AE100618 gene HP1119
- AE100064 gene HP0245
- AE100154 gene HP0131
- AE1000611 gene HP1031
- AE100053 gene HP0114

**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 originating from E. coli if at least 10 \(\mu\)g DNA was used. However, the cloning of the same flaA and flaB genes in the pUOA18 shuttle vector resulted in 80 transformants \((\mu\)g DNA\(^{-1}\) when the plasmid was isolated from E. coli, and \(1.6 \times 10^5\) 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 μm.
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 Sau3A1. 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 KmR 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 KmR transformants lacked the shuttle vector and contained the KmR cassette in the chromosome.

A total of 1300 KmR 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 KmR 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 KmR transformants were non-motile, showing that the acquisition of KmR 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 KmR cassette (Fig. 1a). Since a complete library should contain at least a mutant with a KmR 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::KmR) (Wassenaar et al., 1991), indicating the presence of a KmR cassette in the BglII site of the flaA gene (Fig. 1b, lanes 3 and 14). By sequencing the flanking regions of the KmR cassette, we could demonstrate that a 3.4 kb BglII 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 KmR cassette were amplified by inverse PCR on circularized genomic BglII–BclI, AseI or Hinfl 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 KmR insertion. The position of the KmR insertions in the non-motile mutants and their phenotypes are summarized in Table 1. A KmR insertion was found in the pflA gene (Yao et al., 1994) for mutant BG1 and the newB gene (A. Karlyshev, database entry AJ000855) for mutant BG2. The KmR 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 KmR cassette was inserted in a gene that showed homology with the orfI located downstream of the fliN gene of C. jejuni (A. Karlyshev, database entry AJ000400). The KmR cassette in mutant BS9 was flanked by the orfI 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 KmR 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 KmR 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 KmR cassette in mutant BG5 showed no homology with known genes. We were unable to determine the sequence of the flanking regions of the KmR 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\textsuperscript{R} cassette within the inserts of the mutant library are not random. They are based on the presence of Bgl\textsuperscript{II}, Bcl\textsuperscript{I}, Bst\textsuperscript{II}, Nsi\textsuperscript{I} or EcoRV 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\textsuperscript{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\textsuperscript{R} cassette a double-crossover event will take place, resulting in the insertion of the Km\textsuperscript{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\textsuperscript{R} cassette. In mutant BG1 a Bgl\textsuperscript{II} fragment of 513 bp from the pfl\textsuperscript{A} gene was replaced by the Km\textsuperscript{R} cassette. In mutant BG3 a 3-4 kb Bgl\textsuperscript{II} fragment containing the 3' part of the fla\textsuperscript{A} gene and the complete fla\textsuperscript{B} gene was replaced by the Km\textsuperscript{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: pfl\textsuperscript{A}/B, neu\textsuperscript{B} and flg\textsuperscript{K}. An insertion of the Km\textsuperscript{R} cassette in the major flagellin subunit genes, fla\textsuperscript{A} and fla\textsuperscript{B}, 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\textsuperscript{R} insertion was also detected in the neu\textsuperscript{B} 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\textsuperscript{+} phenotype of mutant BG2. Our data also demonstrate an insertion in the putative flg\textsuperscript{K} gene, encoding the HAPI protein. In Salmonella the HAPI protein 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 flg\textsuperscript{K} gene of H. pylori flanking the Km\textsuperscript{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 flg\textsuperscript{K} gene since this is the maximum size of the inserts in our chromosomal library before introduction of the Km\textsuperscript{R} cassette. Mutant BC7 had stump-like flagella, which is in line with the mutated flg\textsuperscript{K} gene and the non-motile phenotype. However, mutant BG4 had long flagella. Apparently it still contains a functional HAPI.

Insertions were also detected in three genes possibly involved in the flagellar motor function: pfl\textsuperscript{A}, fli\textsuperscript{M} and orf\textsuperscript{I} downstream of flt\textsuperscript{N}. The flagellated mutant BG1 with the Km\textsuperscript{R} cassette insertion in the pfl\textsuperscript{A} 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\textsuperscript{R} cassette in the fli\textsuperscript{M} gene or in the orf\textsuperscript{I} located downstream of the flt\textsuperscript{N} gene may lead to a switching defect since the Flt\textsuperscript{N} 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\textsuperscript{R} insertions were also found in genes with no homology to known genes. The observed phenotypes of these mutants (Mot\textsuperscript{−}, Inv\textsuperscript{−} and Flaf\textsuperscript{−}/−) 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 ssp. 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\textsuperscript{R} cassette this system could be used to identify virulence genes involved in colonization and invasion in vivo (Hensel et al., 1995).

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