Gene Transfer from *Escherichia coli* to *Campylobacter* Species: Development of Shuttle Vectors for Genetic Analysis of *Campylobacter jejuni*

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A shuttle cloning vector (pILL550) has been constructed which can be mobilized from *Escherichia coli* to *Campylobacter jejuni*, *Campylobacter coli*, and *Campylobacter fetus* by complementation with the transfer functions of an IncP plasmid in trans, with a frequency of 10^{-4} transconjugants per donor. We also present evidence for a DNA modification system in *C. jejuni*.

Molecular genetic analyses and recombinant DNA techniques have proved to be powerful methods in exploring mechanisms of pathogenesis in many bacteria species. Genetic approaches to elucidating the pathogenesis of *Campylobacter* infections have been hampered in two ways. The first impediment was the absence of a genetic exchange system permitting the introduction of genes into *Campylobacter* cells. No methods of transformation or general transduction have been previously described for these species. Moreover, systematic efforts to transfer plasmids from different incompatibility groups from *Escherichia coli* to *Campylobacter* species via conjugation have been entirely unsuccessful (16). The second major barrier to the genetic analysis of *Campylobacter* species lies in the difficulties encountered in attempts to clone, maintain, and express *Campylobacter* genes in *E. coli* host strains (16). To elucidate the molecular basis of virulence in *Campylobacter* species, we embarked on the in vitro construction of a shuttle cloning vector.

We constructed a chimeric plasmid derived from *E. coli* plasmid pBR322 into which *Campylobacter* replication functions, a kanamycin resistance gene, and the origin of transfer (*oriT*) from a broad-host-range plasmid were inserted. We chose the kanamycin resistance gene encoding a 3' aminoglycoside phosphotransferase type III as the selectable marker because it is self-expressed in both *E. coli* and *Campylobacter* species. This sequence is present on a *Campylobacter coli* plasmid (pIP1433, Tra^+ Km Tc) (13) and is contained within a 1.427-kilobase (kb) *ClaI*-*HindIII* DNA restriction fragment. Therefore, to construct a plasmid which can express a selectable marker in both *E. coli* and *Campylobacter* species, we first cloned this fragment into the *ClaI*-*HindIII* sites of pBR322, creating pILL502 (Table 1). In the next step, we introduced *Campylobacter* replication functions by incorporating the entire cryptic *C. coli* plasmid pIP1455 (10). This replicon was selected because of its small size (4.57 kb) and its apparent high copy number. Although we detected no cutting with restriction endonucleases *Bam*HI, *Bgl*II, *Cla*I, *Eco*RI, *Sal*I, or *Pst*I, digestion of the cryptic plasmid with *Hind*III produced five restriction fragments. The pILL505 hybrid plasmid resulted from the insertion of the linearized cryptic plasmid, partially

digested by *Hind*III, into the unique *Hind*III site of pILL502 (Table 1). To ensure plasmid mobilization from *E. coli* to *Campylobacter* species, we inserted the 760-base-pair (bp) *Bam*HI *oriT* sequence isolated from pEYDG1 plasmid (17), encoding the origin of transfer from the broad-host-range IncP plasmid (7), into the *Bam*HI site of pILL505, thus creating pILL512 (Table 1) and other recombinants not presented here. The precise restriction map of the chimeric plasmid and the relative order of the six *Hind*III fragments were determined by *Bal*31 nuclease digestion (12) (Fig. 1).

The pILL512 chimeric plasmid was introduced by transformation (3) into *E. coli* HB101 (2) harboring a helper plasmid pRK212.1 (5), and mating experiments between *E. coli* and *Campylobacter jejuni* C31 were performed as follows. *E. coli* cells were grown in L broth without antibiotic with gentle shaking to a density of 10^8 bacteria per ml. *Campylobacter* recipient cells were grown overnight in heart infusion broth supplemented with vancomycin (10 mg/liter), cephalothin (15 mg/liter), polymyxin B (5 mg/liter), and trimethoprim (5 mg/liter) at 37°C with vigorous shaking under microaerophilic conditions in an aerobic jar with a carbon dioxide generator envelope (BBL 70304). Ten milliliters of the overnight recipient cultures (10^8 bacteria per ml) were centrifuged, washed in sterile water, pelleted again, and suspended in 1 ml of donor *E. coli* cells. The donor and recipient strains were therefore mixed in a 1:10 ratio (10^8 : 10^9 bacteria per ml). The mating mix (1 ml) was spread on the surface of a Mueller-Hinton plate and incubated in microaerophilic conditions for 5 h at 37°C. The bacteria were harvested and suspended in a final volume of 1 ml, and aliquots of 100 μ l were plated on Mueller-Hinton medium containing vancomycin, polymyxin, cephalothin, trimethoprim, and kanamycin. Plates were incubated at 42°C in microaerophilic conditions for 48 h.

Mating between donor *E. coli* cells and *C. jejuni* C31 recipients resulted in chimeric plasmid transfer and the production of stable transconjugants. Several lines of evidence demonstrated that Km^r transconjugants were, in fact, derived from *C. jejuni* C31. Bacterial cells from colonies which developed after conjugation had typical *Campylobacter* morphology after Gram stain and appropriate biochemical markers (oxidase positive, microaerophilic dependent growth at 37 or 42°C, and no growth at 25°C). Total chromosomal DNA was extracted from the transconjugants and from the recipient *C. jejuni* C31 by adapting the technique described by Hull et al. (8) to a 10-fold-reduced scale;

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TABLE 1. Properties of plasmids constructed for this study

Plasmid ^a	Phenotypic characteristics	Size (kb)	Origin
pILL502	Ap Tc Km	5.8	pBR322 Ω (1.4-kb Km fragment from pIP1433)
pILL505	Ap Tc Km	10.4	pILL502 Ω pIP1445
pILL512	Ap Km Mob ⁺	11.1	pILL505 Ω oriT
pILL518	Ap Km Mob ⁺	6.5	pILL512 without pIP1445
pILL512- Δ 1 to - Δ 5	Ap Km Mob ⁺	Variable	pILL512 variants ^b
pILL512- Δ 6	Ap Km Mob ⁺	9.8	pILL512 variant ^b
pILL512- Δ 7 to - Δ 9	Ap Km Mob ⁺	Variable	pILL512 variants ^b
pILL550	Km Mob ⁺	8.5	Shuttle cloning vector ^b

^a Plasmid nomenclature was according to E. Lederberg (11).

^b According to Fig. 2.

digestion of total DNA with *Bgl*II, *Cla*I, and *Hind*III (data not shown) produced identical chromosomal restriction patterns. In addition, analysis of plasmid DNA extracted from Km^r transconjugants (12) revealed that pILL512 had been transferred and remained intact in *C. jejuni* recipients, showing no detectable deletions or rearrangements. Significantly, we noted that whereas digestion with *Hind*III, *Cfo*I, and *Sau*3A of the shuttle vector plasmids extracted from *E. coli* donors and *Campylobacter* recipients produced identical restriction fragment patterns, pILL512 DNA isolated from *E. coli* was resistant to *Mbo*I digestion, while shuttle plasmid DNA extracted from *Campylobacter* recipients was completely digested (Fig. 2). These data suggested that DNA was not methylated in *C. jejuni* C31 at the adenosine residue of the GATC recognition sequence. Reciprocally, shuttle plasmids isolated from *C. jejuni* C31 were resistant to *Eco*RI digestion (Fig. 2). Transformation and propagation of plasmids into *E. coli* HB101 resulted in recovery of the *Eco*RI site. These observations have led us to conclude that *C. jejuni* DNA may be modified at one or more sites of the *Eco*RI recognition sequence, GAATTC.

To reduce the size of pILL512 and locate the region essential for *C. jejuni* replication, we isolated the deleted

plasmids (Fig. 1). pILL512 variants Δ 1 to Δ 5 were obtained by subcloning into pILL518 (Table 1) each of the five *Hind*III fragments from pIP1445 (Table 2). None of these could be successfully mobilized and maintained in *C. jejuni* C31. pILL512- Δ 6 was obtained spontaneously after *E. coli* transformation; it had undergone a 1,300-bp deletion overlapping the 1,200- and 800-bp *Hind*III fragments of pILL512 (Table 1 and Fig. 1). pILL512- Δ 7 to - Δ 9 were obtained by partial *Hind*III digestion of pILL512- Δ 6 and ligation of the generated fragments. The lack of successful mobilization of pILL512- Δ 7 to - Δ 9 showed that deletion of either the 700- (Δ 9) or 340- (Δ 7) or 1,600-bp (Δ 8) *Hind*III sequences leads to nonreplicative plasmids. The contribution of the 600-bp *Hind*III fragment to replication is not yet known. Altogether, these results suggested that discrete loci on the cryptic plasmid are involved in its replication and maintenance in *C. jejuni*.

pILL512- Δ 6 shuttle vector was subsequently modified *in vitro* to delete nonessential regions from pBR322 and to add a sequence containing multiple cloning sites. This was accomplished in the following manner. The kanamycin resistance gene from Tn903, as contained in pUC71K (15), was selected as a convenient source of polylinker sequences. The

FIG. 1. Restriction endonuclease-generated maps of the chimeric plasmid *Ava*I (A), *Bam*HI (B), *Eco*RI (E), *Cla*I (C), *Pst*I (P), *Sal*I (S), and *Sma*I (Sm) recognition sites. ■, *Campylobacter* kanamycin gene; □, DNA sequences from the cryptic pIP1445 plasmid; ◀, *Ori*T DNA sequence; ▢, Tn903 flanked by polylinker sequences. —, pBR322 DNA; ---, deleted DNA sequences. Numbers (in base pairs) indicated the size of the *C. coli* cryptic plasmid *Hind*III (H) fragments.

FIG. 2. Comparison of the restriction patterns generated from pILL512 plasmid DNA when isolated from *E. coli* HB101 (a) or from *C. jejuni* ALR101 (b). L, 1-kb ladder used as molecular size standard.

Tn903 sequence was purified after digestion of the pUC71K plasmid with *EcoRI* and ligated into the unique *EcoRI* site of pILL512-Δ6. The resulting plasmid was digested with *PstI* and religated to generate pILL535 (data not shown). The *BamHI*, *Sall*, and *PstI* restriction sites flanking or internal to the *oriT* sequence were eliminated by filling the recessed ends after partial digestion of the pILL535 plasmid with the respective endonucleases and self-ligating the linearized plasmid, creating the pILL550 cloning shuttle vector (Table 1 and Fig. 1). The unique sites for *BamHI*, *ClaI*, *EcoRI*, *PstI*, *Sall*, and *SmaI* make this a useful general vector to clone *Campylobacter* DNA sequences in *E. coli* and to return them to *Campylobacter* species for analysis of expression.

Transfer of pILL550 was achieved by using various clinical isolates of *C. jejuni*, *C. coli* (strain 79 K), and *C. fetus* (strain 5396) as recipients. Transconjugants were obtained with a frequency of 10^{-4} transconjugants per donor present at the beginning of the mating.

We have been able to demonstrate, for the first time, gene transfer between *E. coli* and *Campylobacter* species by recombinant plasmid vectors. Analysis of the results of these conjugation experiments illustrate mechanisms which have previously prevented successful genetic exchange between *E. coli* and *Campylobacter* species. Three independent natural barriers may account for the failure of these bacterial species to exchange genes even though they share similar ecological niches. First, our results have established that conjugative transfer of a chimeric plasmid containing the *oriT* sequences from an IncP plasmid occurs between *E. coli* and *Campylobacter* cells. Therefore, other naturally occurring IncP plasmids might be transferred in vivo in nature, but they presumably cannot either replicate or express antibiotic resistance in *Campylobacter* cells. A second significant barrier to successful genetic exchange between *E. coli* and *Campylobacter* cells was further illustrated by our finding that *Campylobacter* cells harboring the chimeric plasmid pILL512 did not express the TEM beta-lactamase gene contained on pBR322. This gene can be fully expressed in diverse gram-negative species, including all members of the family *Enterobacteriaceae*, *Haemophilus* species, and *Neisseria* species. The TEM gene is not expressed in gram-positive bacilli (*Bacillus subtilis* [9]) nor in *Bacteroides fragilis* (6). Therefore, it would appear that these results, and the difficulties encountered by ourselves and others to clone

TABLE 2. Bacterial strains and plasmids used in this study

Bacteria or plasmids	Description	Reference
<i>Campylobacter</i> strains		
BM2509	<i>C. coli</i> Ap Cm Em Km Sm Sp Tc, pIL1433, pIP1445	10
C31	<i>C. jejuni</i>	Guerrant et al. ^a
79K	<i>C. coli</i>	4
5396	<i>C. fetus</i>	14
ALR101	C31(pILL512)	This work
Plasmids		
pBR322	Ap Tc Mob ⁻	1
pRK212.1	IncP Tra ⁺ Tc Ap	5
pEYDG1	Km Mob ⁺	17
pUC71K	Km, polylinker-flanked kanamycin resis- tance gene from Tn903	15

^a R. L. Guerrant, R. A. Pennie, L. J. Barrett, and A. O'Brien, 3rd Int. Workshop *Campylobacter* Infect., abstr. no. 093, 1985.

and express *Campylobacter* genes in *E. coli*, suggest that a fundamental difference in gene expression exists between *Enterobacteriaceae* and *Campylobacter* species. Finally, we noted that a *Campylobacter*-specific modification system operates to modify one or more residues in the *EcoRI* recognition sequence. The in vitro construction of the shuttle cloning vector pILL550 was designed to overcome these natural barriers and has now provided a general approach to the selection of *Campylobacter* genes and mutants and the opportunity to study gene function and gene expression in *Campylobacter* cells.

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