JOURNAL OF BACTERIOLOGY, Apr. 1988, p. 1704–1708
0021-9193/88/041704-05$02.00/0
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Vol. 170, No. 4

Gene Disruption and Replacement as a Feasible Approach for Mutagenesis of *Campylobacter jejuni*

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Received 9 September 1987/Accepted 21 January 1988

Campylobacter jejuni and _Campylobacter coli_ are important causes of human enteric infections. Several determinants of pathogenicity have been proposed based on the clinical features of diarrheal disease and on the phenotypic properties of _Campylobacter_ strains. To facilitate an understanding of the genetic determinants of _Campylobacter_ virulence, we have developed a method for constructing _C. jejuni_ mutants by shuttle mutagenesis. In the example described here, a kanamycin resistance gene was inserted into _Campylobacter_ DNA fragments encoding 16S rRNA cloned in _Escherichia coli_. These disrupted, modified sequences were returned to _C. jejuni_ via conjugation. Through the apparent process of homologous recombination, the kanamycin resistance-encoding sequences were rescued by chromosomal integration, resulting in the simultaneous gene replacement of one of the 16S sequences of _C. jejuni_ and the loss of the vector. We propose that _Campylobacter_ isogenic mutants could be developed by using this system of shuttle mutagenesis.

Campylobacter species are now recognized as important enteric pathogens in human diarrheal illness throughout the world (1, 2, 4), and many laboratories are investigating the mechanisms by which these species cause disease. Potential determinants of virulence attributed to *Campylobacter* spp. include colonization (15), adhesion (7, 16, 17), invasion (1, 6), and cytotoxin and enterotoxin production (10, 11, 18). However, the precise role and the importance of each of these putative determinants in the pathogenic process remain unknown, mainly because it has not been possible to construct isogenic mutants, each modified in a single determinant. We therefore designed a genetic approach that would allow us to mutagenize the *Campylobacter* genome at a precise locus.

We recently described the construction of a shuttle vector (pILL550) which can be mobilized from *Escherichia coli* to *Campylobacter* recipients when complemented in *trans* by an IncP plasmid (12). This hybrid plasmid was constructed by ligating pBR322 DNA sequences and part of a *Campylobacter* cryptic plasmid, thus ensuring replication of the plasmid in both species. A *Campylobacter* kanamycin resistance gene (11, 23) and the "mob" region of the IncP plasmid RK2 (9) were inserted into the plasmid to create a recombinant shuttle vector, which for the first time permitted the introduction of foreign DNA into *Campylobacter* cells. Using this new genetic tool, we subsequently attempted to mutagenize the *Campylobacter* genome by transconjugation of a suicide shuttle vector carrying typical gram-negative (Tn*5* [21]) or gram-positive (Tn*917* [20]) transposable elements. Unfortunately, all of our attempts to create transposon-mediated mutants remained unsuccessful (unpublished data). We have therefore turned our attention toward a shuttle mutagenesis approach (19) to create precise insertional mutations, using a modified genetic sequence encoding *Campylobacter* 16S rRNA as a suitable gene for testing the feasibility of this approach. This sequence was selected because we reasoned that inactivation of one of the three to five copies encoding 16S rRNA in the *Campylobacter* chromosome would not be lethal. As described below, the transfer of a disrupted copy of the 16S rRNA gene into *C. jejuni* results in the replacement of a chromosomal 16S rRNA allele by the mutagenized copy of the gene, an effect most likely due to homologous recombination.

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MATERIALS AND METHODS

Bacterial strains and plasmids. *C. jejuni* INN73-83 and C31 were kindly provided by G. M. Ruiz-Palacios (Institute Nacional de la Nutrición, Tlalpan, Mexico) and R. L. Guerrant (R. L. Guerrant, R. A. Pennie, L. J. Barrett, and A. O'Brien, 3rd International Workshop on *Campylobacter* Infections, abstr. no. O93), respectively. *E. coli* HB101 (3) (*hsdR hsdM recA supE44 lacZ4 leuB6 proA2 thi-1* Sm) was used as the host in all transformation experiments and as a host for plasmid DNA analyses. Cells competent for transformation were prepared by the method of Davis et al. (5). Plasmid pHSS6 (19) was kindly provided by H. S. Seifert.

Culture conditions. *E. coli* strains were grown in L broth without glucose, containing (per liter) 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl (pH 7.0), or they were grown on L agar plates (1.5% agar). *Campylobacter* strains were grown on Columbia agar base (Difco Laboratories, Inc.) or heart infusion broth (Difco) supplemented or not with vancomycin (10 mg/liter), cephalothin (15 mg/liter), polymyxin B (2,500 U/liter), trimethoprim (5 mg/liter) at 37 or 42°C under microaerophilic conditions in an anaerobic jar with a carbon dioxide generator envelope (model no. 70304; BBL Microbiology Systems). Antibiotic concentrations for the selection of transformants or transconjugants were as follows (in milligrams per liter): kanamycin, 20; tetracycline, 8; and ampicillin, 100.

Mating experiments between *E. coli* and *C. jejuni* were performed as previously described (12) by mixing 10^8 donor cells with 10^9 recipient cells for 5 h at 37°C under microaerophilic conditions.

Preparation of DNA. Plasmid DNA was isolated from *E. coli* or *Campylobacter* strains by an alkaline lysis procedure (14). Whole-cell DNA from *Campylobacter* strains was prepared as follows. Portions (10 ml) of *Campylobacter*

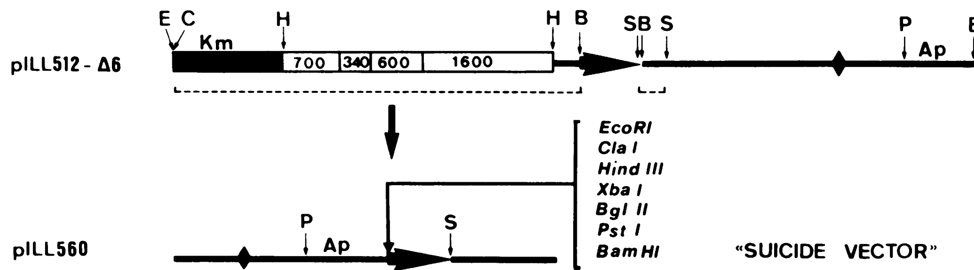

FIG. 1. pILL560 suicide vector construction. Recognition sites: *Bam*HI (B), *Bgl*II (G), *Cla*I (C), *Eco*RI (E), *Pst*I (P), *Sal*I (S), *Sma*I (Sm), and *Xho*I (X). ----, Deletion of DNA sequences; ——, vector DNA; □, DNA sequences from the *Campylobacter* cryptic plasmid; ▶, OriT DNA sequence; ■, *Campylobacter* kanamycin resistance gene. Numbers (in base pairs) indicate the size of the restriction fragments.

liquid growth medium were centrifuged, washed once in TES buffer (30 mM Tris, 5 mM EDTA, 50 mM NaCl; pH 8.0), suspended in 0.2 ml of 25% sucrose in 50 mM Tris–1 mM EDTA (pH 8.0) containing lysozyme (5 mg/ml), and transferred to a VTi65 polyallomer quick seal tube. After 5 min on ice, 2 μl of proteinase K (20 mg/ml) was added. Cells were lysed by adding 65 μl of EDTA-Sarkosyl (0.5 M EDTA [3.25 ml], 10% Sarkosyl [1.25 ml]), and incubated at 65°C for 3 h. CsCl solution (126 g of CsCl in 99 ml of TE buffer [Tris, 10 mM; EDTA, 1 mM; pH 7.5) and 1 ml of aprotinine (Sigma)] was added to fill up the tube. Lysates were centrifuged overnight (45,000 rpm, 18°C) in a VTi65 rotor (Beckman Instruments, Inc.), and total DNA was collected and dialyzed extensively at 4°C against TE buffer.

DNA analysis. Restriction endonucleases were purchased from Bethesda Research Laboratories or Amersham Corp. Enzymatic reaction conditions were as recommended by the manufacturers. DNA fragments were separated by electrophoresis in horizontal slab gels containing 0.7 or 1.4% agarose and run in Tris-acetate buffer (14). The 1-kilobase (kb) ladder from Bethesda Research Laboratories was used as a molecular weight standard.

Hybridization. DNA restriction fragments fractionated by agarose gel electrophoresis were transferred to nitrocellulose sheets (0.45 μm pore size; Schleicher & Schuell, Inc.) by the Southern technique (22) and hybridized with ^{32}P-labeled deoxyribonucleotide probes (Amersham) by nick translation (14). Hybridization was revealed by autoradiography with XAR-Omat Kodak film in the presence of an intensifying screen for various periods of time at −70°C.

Cloning methodologies. All of the hybrid plasmids described in this study were constructed as described by Maniatis et al. (14). Electroelution of DNA fragments from agarose gels was performed as previously described (13). DNA polymerase I large fragment and T4 DNA polymerase (used to make blunt-end fragments), calf intestine phosphatase, and T4 DNA ligase were purchased from Amersham, Pharmacia, and Bethesda Research Laboratories, respectively.

RESULTS

Construction of the suicide vector. pILL560, a suicide vector with the same transfer functions as the shuttle vector, pILL550 (12), but which is unable to replicate in *Campylobacter* spp., was constructed as follows. The shuttle plasmid pILL512-Δ6 (Fig. 1) (12) was digested with *Sal*I and religated to eliminate one of the two *Bam*HI sites bordering the OriT fragment. The resulting plasmid was then simultaneously digested with *Eco*RI and *Bam*HI to eliminate *Campylobacter* replication sequences, and the smallest generated *Eco*RI-*Bam*HI fragment (4.5 kb) was electroeluted and ligated to the *Eco*RI-*Bam*HI polylinker fragment containing multiple unique restriction sites derived from pHSS6, thus generating the conjugative suicide plasmid pILL560 (Fig. 1).

Insertion into the suicide vector and disruption of the 16S rRNA gene. pILL560 suicide vector was used as a vector for subcloning a portion of the *C. jejuni* gene encoding 16S rRNA (M. Krajden, L. Palmer, and L. S. Tompkins, Program abstr. 25th Intersci. Conf. Antimicrob. Agents Chemother., abstr no. 1131, 1985; unpublished data). The sequence encoding 16S rRNA had been cloned into a pEMBL8 vector as a 2.5-kb *Bgl*II restriction fragment derived from *C. jejuni* INN73-83 and encompassed the 16S rRNA gene and flanking sequences (pMK11, Fig. 2). On the basis of Southern hybridization and DNA sequencing analyses (J. Harel and L. Tomkins, manuscript in preparation) it has been shown that the 1.65-kb *Eco*RI fragment derived from pMK11 contains

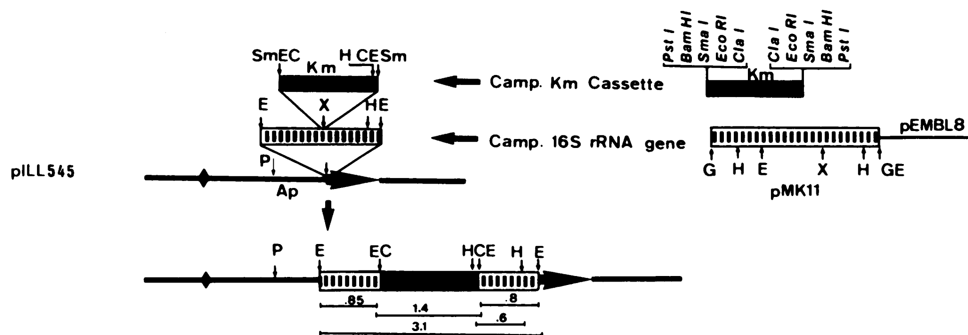

FIG. 2. pILL545 recombinant plasmid construction. ▩, *Campylobacter* chromosomal DNA. All other symbols are as described in the legend to Fig. 1. Numbers (in kilobase pairs) indicate the size of the restriction fragments.

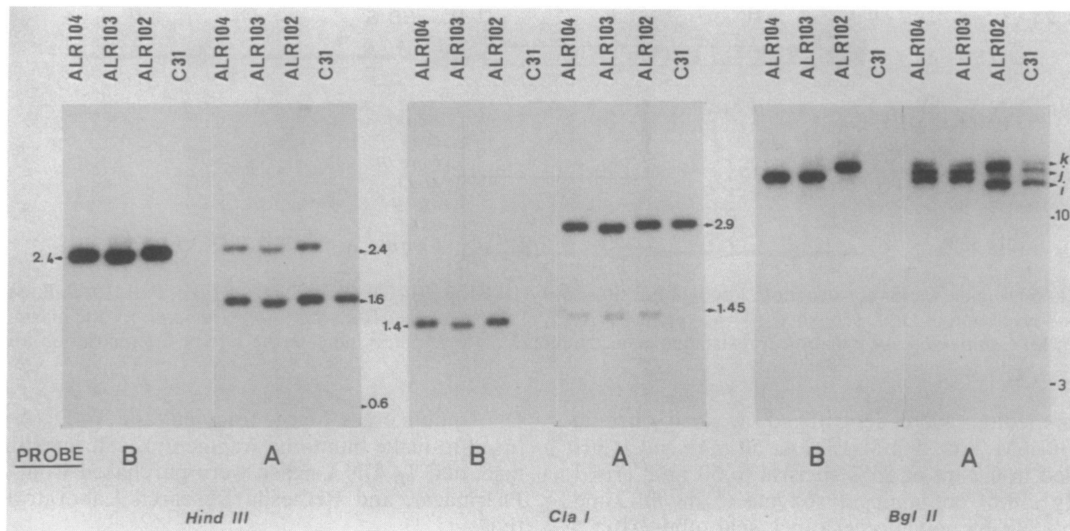

FIG. 3. Total DNA from C31 and three kanamycin-resistant transconjugants (ALR102, ALR103, and ALR104) were digested with *BglII*, *ClaI*, or *HindIII*, fractionated by agarose gel electrophoresis, and transferred to nitrocellulose sheets. *BglII* digests were migrated on a separated agarose gel. Each filter was hybridized to a nick-translated ^{32}P-labeled probe consisting of the 2.5-kb *BglII* fragment from pMK11 (A) or the 1.4-kb *ClaI-HindIII* kanamycin gene fragment from pILL512-Δ6 (B). Numbers either refer to the size (in kilobase pairs) of the generated fragments hybridizing to the 16S rRNA probe or the kanamycin resistance-encoding gene (*HindIII* and *ClaI* digests) or refer to size standards (*BglII* digests). Bands i, j, and k indicate the *BglII* fragments discussed in the text and are larger than 20 kb.

sequences of the 16S rRNA gene and that the *XhoI* site is internal to the 16S rRNA gene. This fragment was cloned into pILL560 in the orientation depicted in Fig. 2. The 16S rRNA gene was then disrupted by inserting a 1.4-kb *SmaI* "kanamycin cassette module" into the *XhoI* site of the 16S rRNA gene treated with DNA polymerase Klenow fragment. The kanamycin cassette module was constructed in vitro and consists of the *Campylobacter* kanamycin resistance gene, expressed both in *E. coli* and *Campylobacter* supp. (12, 23), flanked by inverted multiple restriction sites (Fig. 2).

Transfer of pILL545 from *E. coli* into *C. jejuni*. The resulting plasmid, pILL545, was introduced by transformation into HB101 harboring the pRK212.1 IncP helper plasmid (8) and mobilized into *C. jejuni* C31 by conjugation by using previously described mating conditions (12). A total of 10^8 *E. coli* cells were mixed with 10^9 *C. jejuni* cells in a 1-ml suspension, spread on the surface of a Mueller-Hinton plate, and incubated for 5 h at 37°C under microaerophilic conditions. Cells were harvested and suspended in a final volume of 1 ml, which was then plated as aliquots (100 μl) onto Mueller-Hinton medium containing vancomycin, polymyxin, cephalothin, trimethoprim, and kanamycin. An average of one kanamycin-resistant transconjugant per plate was obtained (transfer frequency, 10^{-7} transconjugants per donor); these transconjugants had typical *Campylobacter* morphology as indicated by Gram stain and appropriate biochemical markers (oxidase-positive, microaerophilic growth at 37 or 42°C, with no growth at 25°C).

Analysis of *Campylobacter* transconjugants. To examine the nature of the transconjugants, total DNA was extracted from three *Campylobacter* transconjugants (ALR102, ALR103, and ALR104) obtained from independent transfers. After digestion with *BglII*, *ClaI*, and *HindIII*, the restriction fragments were transferred from agarose gels to nitrocellulose and probed with the following three nick-translated, ^{32}P-labeled fragments: the 2.5-kb *BglII* fragment of pMK11, the kanamycin sequence (*ClaI-HindIII* 1.4-kb fragment from pILL512-Δ6; Fig. 1), and the pILL560 plasmid vector.

The results of these experiments (Fig. 3) showed that the labeled 2.5-kb *BglII* fragment probe hybridized to a 2.9-kb *ClaI* fragment, a 1.6-kb *HindIII* fragment, and three *BglII* fragments (bands i, j, and k; all larger than 20 kb) of the *C. jejuni* C31 chromosomal DNA. These results suggest that at least three loci of ribosomal DNA clusters exist in *C. jejuni* C31 and that the 2.9-kb *ClaI* and 1.6-kb *HindIII* fragments are internal to the conserved and repeated rRNA encoding sequences. No homology was detected between the kanamycin resistance gene probe and *C. jejuni* C31 chromosomal DNA (Fig. 3) or between pILL560 and *C. jejuni* C31 total DNA (data not shown).

The 2.5-kb *BglII* probe hybridized to the 1.45- and 2.9-kb *ClaI* fragments and to the 0.6-, 1.6-, and 2.4-kb *HindIII* fragments of ALR102, ALR103, and ALR104 (Fig. 3). These results demonstrate the insertion into one of the 16S rRNA gene clusters of a 1.4-kb DNA fragment flanked by two *ClaI* restriction sites. Comparison of the *BglII* patterns of ALR102, ALR103, and ALR104 revealed that integration did not always occur in the same 16S rRNA gene (as seen in the changes in size of C31 fragment j in ALR102 and of C31 fragment i in ALR103 and ALR104).

The kanamycin gene probe hybridized to the 1.4-kb *ClaI* and 2.4-kb *HindIII* fragments and to the *BglII* fragments in ALR102, ALR103, and ALR104, which displayed an increased size when compared to the respective *BglII* fragments of *C. jejuni* C31 chromosomal DNA. No hybridization occurred between the labeled pILL560 vector DNA and DNA extracted from any of the transconjugants (data not shown).

DISCUSSION

The results of the present study show that a *Campylobacter* DNA sequence, when cloned into a conjugative suicide vector in *E. coli* and disrupted by insertion of a kanamycin resistance gene in vitro, can be rescued after its transfer from *E. coli* to *C. jejuni* by chromosomal integration. This

integration event occurs at a frequency of about 10^{-7} transconjugants per donor and can be selected for by virtue of the kanamycin resistance of the integrants. Integration of the resistance determinant appears to involve a double crossover between (i) the *Campylobacter* sequences flanking the kanamycin resistance gene on either side and (ii) the corresponding regions of homology in the *Campylobacter* genome (Fig. 4). Consequently, the chromosomal copy of the cloned *Campylobacter* gene is replaced by the kanamycin gene-disrupted allele carried on the plasmid. At the same time, the suicide vector is lost from the integrants due to the lack of a suitable origin of replication.

The *Campylobacter* DNA sequence which was used as a model system in the present study encodes the 16S rRNA and represents one of the very few chromosomal *Campylobacter* genes cloned to date. Unfortunately, none of these cloned genes determines a known phenotype, e.g., a metabolic trait. Therefore, rather than mutagenizing a randomly cloned *Campylobacter* sequence which might turn out to be essential for the survival of this organism, we chose to disrupt one of the three to five copies of the 16S rRNA gene which had been cloned and sequenced in our laboratory (Harel and Tompkins, in preparation). Since we could not expect that mutagenesis of one of the 16S rRNA genes would lead to an altered phenotype, we monitored its insertional inactivation by Southern hybridization.

By using the precise restriction map of plasmid pILL545 containing the 16S rRNA gene, we were able to follow the fate of the plasmid after its mobilization into *C. jejuni*. Depending on how the kanamycin resistance marker was maintained in the cells, different fragment sizes would be expected when genomic restriction digests of the transconjugants were hybridized with a given set of probes. Analysis of the Southern data obtained with three different probes (i.e., the original cloned *Campylobacter* sequences, the suicide vector, and the kanamycin gene) clearly demonstrates that the kanamycin resistance determinant was integrated into one of the 16S rRNA loci of the *Campylobacter* chromosome rather than being maintained on an independent replicon. Statistically, the integration event most likely to occur would be the result of a single crossover between plasmid and genomic sequences and lead to the rescue of the entire mobilized plasmid into the chromosome. However, no vector sequences or vestige of the pILL560 plasmid could be found in any of the three transconjugants examined. Furthermore, none of the restriction fragments visualized by Southern hybridization fit the sizes predicted for such an integration mechanism. Our data suggest instead that a double crossover occurred between the *Campylobacter* sequences flanking the kanamycin resistance gene on either side and the corresponding regions of homology in the *Campylobacter* genome (Fig. 4). This recombination mechanism would be expected if the mobilized plasmid remained linear upon entering the *Campylobacter* cells, thus requiring two crossovers for the rescue of the kanamycin resistance gene. Alternatively, the entering plasmid might be circularized in *Campylobacter* cells but integration via a single crossover might lead to an unstable intermediate comprising a large stretch of exogenous *E. coli* DNA which might then be rapidly excised via a second crossover. Whatever the precise mechanism of integration might be, it is clearly possible to use plasmid pILL545 and its derivatives to direct foreign DNA to one of the 16S rRNA loci of *C. jejuni* without deleterious effects for the cell. Plasmids carrying the 16S rRNA sequence and the kanamycin resistance gene as a selectable marker might therefore be used as a vehicle for integrating foreign DNA into the *Campylobacter* genome.

As described above, the lack of a genetic system allowing the delivery of transposable elements for insertional inactivation of genes in *Campylobacter* species in vivo can now be overcome by using a shuttle mutagenesis approach involving *E. coli* as an intermediate host. Even though this study focused on the introduction of a specific insertional mutation into a defined *Campylobacter* gene, the technique described above should open the way for a random mutagenesis of randomly cloned *Campylobacter* DNA fragments in vivo. This might be achieved by replacing the in vitro step of gene disruption described here by a random transposition process using the *E. coli* host cells as a vehicle. Preliminary data obtained in our laboratory indicate that this is indeed the

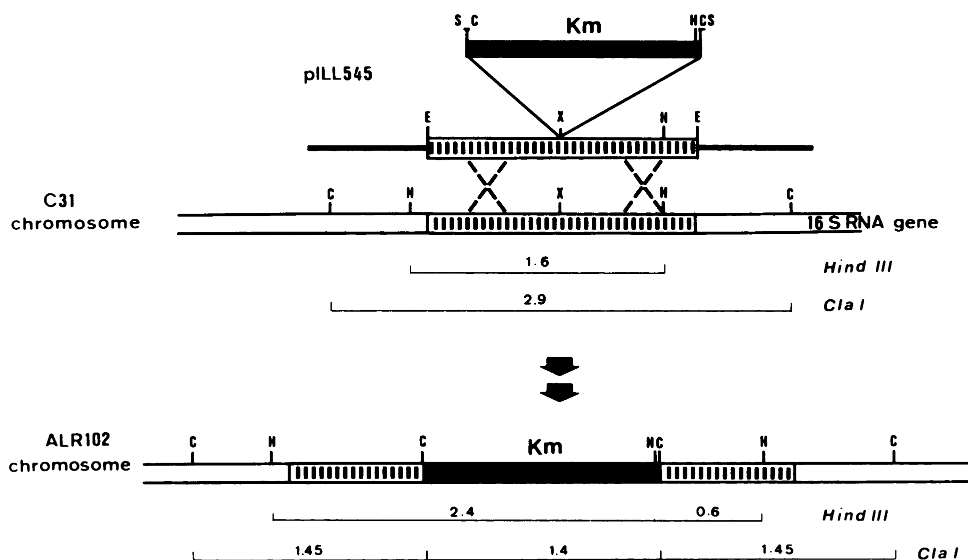

FIG. 4. Incorporation of the disrupted 16S rRNA allele carried on the suicide vector into the chromosomal C31 16S rRNA encoding region. The suicide hybrid plasmid pILL545 was introduced into strain C31 by conjugation; the double-crossover event depicts the formation of strain ALR102. Symbols are as described in the legends to Fig. 1 and 2.

way to generalize the shuttle mutagenesis approach described above, and work is in progress to create a bank of isogenic *Campylobacter* mutants.

The isolation of such mutants, deficient in a single potentially pathogenic determinant (e.g., adhesion, invasion, or the production of toxin) would be extremely useful in clarifying the contribution of each of these determinants to the pathogenicity of *Campylobacter* spp. Other investigators have attempted to correlate clinical syndromes such as watery diarrhea and dysentery with the expression of phenotypic properties, including invasiveness and the production of cytotoxins and enterotoxins (11). However, the results of such studies have been equivocal, at best, since the *Campylobacter* strains examined were not isogenic and might have expressed more than a single putative virulence determinant. Therefore, the availability of defined, isogenic *Campylobacter* mutants which could be tested in relevant animal models should allow a better understanding of the contribution of each putative determinant to the pathogenic process.

ACKNOWLEDGMENTS

We gratefully acknowledge the assistance of R. Fleer in the preparation of this manuscript.

This work was supported by Public Health Service grant AI23796 from The National Institutes of Health and by a gift from Life Technologies, Inc.

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