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Development and Characterization of recA Mutants of Campylobacter jejuni for Inclusion in Attenuated Vaccines

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Isogenic recA mutants of Campylobacter jejuni have been constructed for evaluation of their usefulness in attenuated vaccines against this major worldwide cause of diarrhea. The recA+ gene of C. jejuni 81-176 was cloned by using degenerate primers to conserved regions of other RecA proteins in a PCR. The C. jejuni recA+ gene encodes a predicted protein with an Mr of 37,012 with high sequence similarity to other RecA proteins. The termination codon of the recA+ gene overlaps with the initiation codon of another open reading frame which encodes a predicted protein which has >50% identity with the N terminus of the Escherichia coli enolase protein. A kanamycin resistance gene was inserted into the cloned recA+ gene in E. coli and returned to C. jejuni VC83 by natural transformation, resulting in allelic replacement of the wild-type recA gene. The resulting VC83 recA mutant displayed increased sensitivity to UV light and a defect in generalized recombination as determined by natural transformation frequencies. The mutated recA gene was amplified from VC83 recA by PCR, and the product was used to transfer the mutation by natural transformation into C. jejuni 81-176 and 81-116, resulting in isogenic recA mutants with phenotypes similar to VC83 recA. After oral feeding, strain 81-176 recA colonized rabbits at levels comparable to wild-type 81-176 and was capable of eliciting the same degree of protection as wild-type 81-176 against subsequent homologous challenge in the RITARD (removable intestinal tie adult rabbit diarrhea) model.

Campylobacter spp., particularly Campylobacter jejuni, are a major cause of enteric disease worldwide. Acute symptoms most often include dysentery, fever, and abdominal pain (5). Sequelae can include colitis, reactive arthritis, and Guillain-Barré syndrome (5, 36). An estimated 400 million cases occur annually, with 2.4 million occurring in the United States (48). Campylobacters have recently been recognized as a significant cause of travelers diarrhea, with attack rates approaching 25 to 37% per year among individuals traveling to highly endemic areas (15, 34, 50). Outbreaks and sporadic cases of Campylobacter gastroenteritis are also well documented among U.S. military personnel (6, 7, 14, 50, 52). While the need for a vaccine against Campylobacter spp. for the general population in developed nations is arguable, the emergence of these organisms as a major cause of diarrhea has led to interest in development of a vaccine for selected high-risk populations.

Vaccine strategies against Campylobacter spp. to date have focused largely on identification of potential protective antigens for inclusion into carrier vaccine strains (42). One of the most promising of such antigens is flagellin, the subunit of the flagella filament. Although development of antibodies against flagellin seems to correlate with acquisition of immunity to infection (32), it remains to be shown that flagellin is protective. Moreover, since a nonflagellated mutant strain (22) was able to fully protect against subsequent challenge in a rabbit model (40), other antigens are capable of eliciting protective responses. Even if flagellin does prove to be a protective antigen, there exist other problems in its inclusion in a carrier vaccine, including antigenic diversity among serotypes (30), phase and antigenic variability (11, 25), and the presence of posttranslational modifications on the surface (2, 31). Similar uncertainties exist for other individual candidate antigens (42).

An alternative approach to enteric vaccines is the development of living attenuated strains expressing the full complement of native surface antigens, such as Salmonella typhi Ty21a (17) and several living candidate vaccine strains of Vibrio cholerae (29, 51). Some of the V. cholerae candidates have included a recA mutation for two reasons. First, amplification of cholera toxin genes, a process which enhances virulence, has been shown to be recA-dependent (18), and second, the presence of a recA allele would reduce concerns about reversion of virulence defects in vaccine strains once introduced into endemic areas (26, 51). Furthermore, it has recently been shown that recA mutants of Salmonella typhimurium are avirulent (8).

Campylobacter spp. are unique among enteric pathogens in that they are naturally transformable (53). This ability to take up and incorporate exogenous DNA may increase the possibility of reversion of attenuated strains of C. jejuni in the environment. One way of precluding this problem, and at the same time achieving some degree of attenuation, would be the incorporation of recA mutations into vaccine strains. In this study, we report the cloning, sequencing, and site-specific mutagenesis of the recA+ gene of C. jejuni and demonstrate that a C. jejuni recA mutant is still capable of colonizing and eliciting protection in a rabbit model. In addition, we have also demonstrated that natural transformation can be exploited to generate primary mutations in C. jejuni without the need for conjugative transfer of suicide vectors from Escherichia coli and used a combination of PCR and natural transformation to transfer the isogenic recA mutation among other strains of C. jejuni.
**MATERIALS AND METHODS**

Bacterial strains and growth conditions. *C. jejuni* 81-176 is a clinical isolate which has been described previously (4). *C. jejuni* 81-116 was the gracious gift of Diane Newell and has been described previously (38). *C. jejuni* VC83 is a clinical isolate from Canada (1). *E. coli* DH5α was used as the host for molecular cloning experiments. *C. jejuni* was routinely cultivated on Mueller-Hinton (MH) agar supplemented when indicated with kanamycin (50 μg/ml) at 37°C in an atmosphere of 10% CO₂-85% N₂-5% O₂. *E. coli* was grown on Luria agar (35) supplemented as indicated with ampicillin (50 μg/ml) or kanamycin (50 μg/ml).

Recombinant DNA techniques and plasmids. Restriction enzymes and T4 DNA ligase were purchased from Boehringer Mannheim (Indianapolis, Ind.) and used as recommended by the supplier. pUC18 (GIBCO, Gaithersburg, Md.) was used for cloning.

PCR conditions. PCRs were run in a Perkin-Elmer Cetus thermal cycler with reagents supplied by Perkin-Elmer Cetus (Norwalk, Conn.) at the concentrations recommended by the supplier. The conditions for primers PMPI and REV3 were 40 cycles of denaturation at 94°C for 1 min, annealing at 22°C for 1 min, and extension at 72°C for 2 min. Conditions for primers Rec1 and Rec2 were 30 cycles of denaturation at 94°C for 1 min, annealing at 42°C for 1 min, and extension at 72°C for 2 min.

DNA hybridizations. DNA restriction fragments separated in agarose gels were transferred to nitrocellulose sheets as described by Sambrook et al. (45) and hybridized under conditions previously described (23). Probes were labelled with [α-32P]dCTP by nick translation performed with a commercial kit (DuPont-New England Nuclear, Wilmington, Del.).

DNA sequencing. DNA sequencing reactions were performed on double-stranded plasmid templates with commercially available dyeoxy-terminator cycle sequencing kits from Applied Biosystems, Inc. (Foster City, Calif.). Sequencing reactions were run on an Applied Biosystems model 373 automated DNA sequencer. Custom oligonucleotide primers were synthesized on an Applied Biosystems model 392 DNA synthesizer. Sequences were analyzed by using MacVector and Assemblylin software packages (IBI, New Haven, Conn.).

Natural transformation. A slight modification of the biphasic method of natural transformation of *C. jejuni* in which the biphasic cultures are incubated in 5% CO₂-95% air instead of 10% CO₂-95% N₂-5% O₂. Two degenerate PCR primers were designed on the basis of previously described (38). These primers were PMPI (GAAAT[ATITATGGTCCTGA]) and REV3 (TTI [ATITATGGGTACTGTGTT]). PMPI encodes amino acids E1YGP (Fig. 3, residues 62 to 66) which are highly conserved in *E. coli* recA proteins. The inverse of REV3 encodes amino acids PDTGE corresponding to residues 118 to 122, another highly conserved region. When these primers were used in a PCR with strain 81-176 DNA, they produced a product of the predicted 180-bp size. This product was cloned into pUC18, and the insert was sequenced by using forward and reverse primers. The DNA sequence encoded a predicted protein with 70% sequence identity with the *E. coli* recA protein.

**RESULTS**

Cloning and sequence analysis of the *recA* gene of *C. jejuni*. Two degenerate PCR primers were designed on the basis of the known G+C bias of *Campylobacter* spp., which would encode two highly conserved regions of RecA proteins from several species (5, 16, 27, 33, 37, 39, 43, 46, 47). These primers were PMPI (GAAAAT[ATITATGGTCCTGA]) and REV3 (TT CACC[AT]GTATC[AT]GGT). PMPI encodes amino acids E1YGP (Fig. 3, residues 62 to 66) which are highly conserved in RecA proteins. The inverse of REV3 encodes amino acids PDTGE corresponding to residues 118 to 122, another highly conserved region. When these primers were used in a PCR with strain 81-176 DNA, they produced a product of the predicted 180-bp size. This product was cloned into pUC18, and the insert was sequenced by using forward and reverse primers. The DNA sequence encoded a predicted protein with 70% sequence identity with the *E. coli* recA protein. This PCR product was labelled with 32P by nick translation and used as a probe to clone a 2.7-kb HindIII fragment into pUC18. This plasmid was termed pMP100. Sequence analysis of the insert in pMP100 indicated that it lacked the final 153 bp of the RecA open reading frame (ORF1; see below). pMP100 was used as a probe to clone an overlapping 1.8-kb *XbaI* fragment, and this plasmid was termed pMP101. The plasmids are shown diagrammatically in Fig. 1.
The DNA sequence of the recA* information on pPMP100 and pPMP101 is shown in Fig. 2. There is an ORF (ORF1) of 1,029 nucleotides encoding a predicted protein of 343 amino acids with a predicted Mr. of 37,012. The G+C content within the coding region is 37%. There is a putative ribosome binding site (shown by underlining in Fig. 2) 5 bp upstream from the translational start of the ORF. The predicted amino acid sequence encoded by ORF1 shows significant sequence identity with other RecA-like proteins, as shown in Fig. 3, with the best match (66% identity overall) being to the recA* gene of Neisseria gonorrhoeae (16).

A second ORF begins in the sequence at the second A of the TAA termination codon of the recA* gene, as shown in Fig. 2. The predicted N terminus of this ORF shows similarity with the N-terminal sequences of enolases from several sources. The predicted protein is 55% identical to the N terminus of enolase of Escherichia coli (MSKIVKGGREIDSGRGNPTVEAEVHLEGGFVG [55]) and 47% identical to rat enolase (MSIQKIKAREIDSGRGNPTVEVDLHTAKG [44]). A putative ribosome binding site is found 8 bp upstream from the translational initiation site of ORF2 and within the recA* gene.

Generation of a primary recA mutation in C. jejuni. Initial strategies to generate a site-specific mutation in the recA* gene utilized standard methods of shuttle mutagenesis (20, 22, 26). Several plasmids were constructed in which a Kmr cassette (28) was inserted into either the CiaI site or the unique BglII site in pPMP100 (Fig. 1). The inserts were transferred into the suicide vector pGK2003 (22) and mobilized from DH5α strains into strain 81-176 selecting for am5, am6. The am5 and am6 mutations were used to generate the recA* gene.

The predicted amino acid residues are shown in the single-letter code. The numbers on the right refer to the nucleotide numbers (top) and predicted amino acid residues (bottom). ORF1 marks the beginning of the recA* gene. ORF2 marks the beginning of the putative enolase gene. Putative ribosome binding sites are indicated by underlining.
transformation experiments revealed a defect in generalized recombination (see below).

Transfer of the recA mutant allele among C. jejuni strains by natural transformation of a PCR product. Since all attempts to introduce a mutation into 81-176 by using plasmid pPMP10I by conjugation or natural transformation were unsuccessful, an alternate mutational strategy was developed. Two PCR primers were designed, Rec1 and Rec2, which were capable of amplifying the entire recA gene. The Rec2 primer also includes the beginning of the putative enolase gene. The position of these primers is indicated in Fig. 2. These primers amplified a 1.2-kb fragment from VC83 and a 2.6-kb fragment from VC83 recA, further indication of the presence of the Km' cassette in the mutant gene. The PCR product from VC83 recA was purified from agarose and used to transform 81-176 to Km'.

Approximately 50 transformants were obtained, and one was further characterized. Southern blot analysis of strain 81-176 (Fig. 4, lane C) and this Km' transformant (lane D) indicated that the patterns were identical to those of VC83 wild type (lane A) and VC83 recA (lane B). UV sensitivity experiments indicated that the 81-176 mutant showed an increase in UV sensitivity similar to that seen in Fig. 5 for the VC83 mutant (data not shown). The recA allele was similarly transformed by using the same PCR product in C. jejuni 81-116. Southern blot analysis of one transformant from this experiment is also shown in Fig. 4 (lane F) in comparison with 81-116 (lane E). The recA' gene of 81-116 shows restriction polymorphisms from the recA' genes of VC83 and 81-116, but the pattern of the transformant (Fig. 4, lane D) includes the 3.2-kb band of VC83 recA and 81-176 recA (lanes B and D, respectively). The UV sensitivity of the 81-116 mutant was also confirmed (data not shown).

Effect of recA mutations on natural transformation in C. jejuni. To determine the effect of recA mutations on natural transformation, we initially used DNA from a streptomycin-resistant C. jejuni strain 81-116. The recA gene of strain 81-116 shows restriction polymorphisms from the recA' genes of VC83 and 81-116, but the pattern of the transformant (Fig. 4, lane D) includes the 3.2-kb band of VC83 recA and 81-176 recA (lanes B and D, respectively). The UV sensitivity of the 81-116 mutant was also confirmed (data not shown).

FIG. 3. Amino acid alignments of the C. jejuni RecA protein with other bacterial RecA proteins. Abbreviations: Cj, C. jejuni; Ng, N. gonorrhoeae (16); Mf, Methylobacillus flagellatus (19); Pa, Pseudomonas aeruginosa (47); Ti, Thiobacillus ferrooxidans (43); Ec, E. coli (46). The C. jejuni sequence is shown in capital letters; residues in the other RecA proteins which are identical to the C. jejuni RecA are capitalized. A dash indicates a gap introduced to maximize alignment. The numbers refer to the amino acid residue of the C. jejuni protein.
resistant (Str') strain of Campylobacter coli, VC167 (25).

Cross-species transformations between C. coli and C. jejuni have been reported at different frequencies (1, 49). The results, shown in Table 1, indicate that the Str' marker from C. coli VC167 can be transformed into all three strains of C. jejuni, although VC83 shows the highest frequency. recA mutants in all three strains showed no detectable transformation with VC67 DNA.

The differences in transformation frequencies among the three strains could be due to differences in inherent transformability or to differences in restriction of incoming DNA. To distinguish these possibilities, and to try to maximize the transformation frequencies in the wild-type strains, we purified DNA from cells of VC83 and 81-176 which had been transformed to Str' with VC167 DNA and used this DNA to transform the C. jejuni strains to Str'. VC83 Str' DNA transformed VC83 at approximately the same frequency as VC167 Str', indicating that VC83 does not appreciably restrict incoming C. coli DNA. 81-176 Str' DNA, however, transforms VC83 at a lower frequency than either VC83 Str' or VC167 Str' DNA. Transformation of VC167, VC83, and 81-176 Str' DNAs into strain 81-116 occurs at approximately the same frequency. Transformation into 81-176 was consistently the lowest with all DNAs used but was particularly low with VC83 Str' DNA. This reduction in transformation frequency was not due to restriction of VC83 DNA, however, since 6.9 x 10^7 transformants were obtained when VC83 recA DNA was used to transform 81-176 to Km'.

Evaluation of ability of 81-176 recA to immunize rabbits against intestinal colonization. Similar to previously studied strains of C. jejuni (10, 40), rabbits fed either 81-176 or 81-176 rec4 for the first time were colonized without signs of diarrhea for 1 to 3 weeks. The mean duration of primary colonization for 81-176 and 81-176 rec4 mutants was 19.5 ± 8.9 and 15.0 ± 7.4 days, respectively (Table 2). One month after oral immunization, both immunized and control rabbits were challenged by the RITARD procedure with 10^6 CFU of strain 81-176 per animal. This dose is approximately 4 logs higher than the 100% infectious dose (9). The mean number of days that rabbits were colonized after RITARD challenge is shown in Table 2. For both 81-176 and 81-176 rec4, the duration of colonization after challenge was significantly shorter than in control animals fed only sterile broth. In both immunized groups, the majority of rabbits had negative fecal swabs by 2 days postchallenge. By day 1 postchallenge, fecal cultures were negative in two of four of the 81-176 rec4-immunized rabbits and in two of five of the 81-176-immunized animals.

The predicted RecA protein of C. jejuni is highly conserved compared with other RecA proteins, showing the highest similarity to that of N. gonorrhoeae (16). The genomic organization is interesting in that the recA' gene apparently overlaps the enolase gene. Overlapping, unrelated genes have been described in C. jejuni before. The ATG start of the glyA gene overlaps the TGA stop codon of lysS (12, 13), and the transcriptional start of the other glyA gene has been mapped in E. coli and shown to be within the lysS gene (12, 13), suggesting that the two genes are transcribed independently in C. jejuni also. Transcriptional units remain to be determined for C. jejuni recA and enolase genes. It also remains to be determined what, if any, effect the insertional inactivation of recA has on the expression of the downstream enolase gene. The rather unusual occurrence of overlapping genes may offer a mechanism by which C. jejuni selects against deleterious mutations. Thus, mutations affecting the carboxy terminus of the RecA protein would also affect either the promoter or N terminus of enolase. Such a mechanism may be particularly important in a naturally transformable organism like C. jejuni.

It is not clear why conventional methods of conjugative suicide vector muagenesis were unsuccessful in generating a recA mutation. The technique has been used in one strain of C. coli (20, 22) and several strains of C. jejuni (28, 54), including the generation of an IcrD mutant in 81-176 (35). The original recA mutant in VC83 was ultimately generated by introducing linearized pPMP102 into VC83 by natural transformation, a method which presumably forced a double crossover event. Subsequent analysis has indicated that circular plasmid DNA is also capable of generating the mutation (data not shown). This method of using natural transformation to introduce mutated alleles into Campylobacter spp. greatly simplifies mutant construction and should have general application. A similar method has been reported in the related organism, Helicobacter pylori (24), and we have constructed mutations in other genes by using this approach in both C. jejuni and C. coli (21, 56). The recA mutation was moved into other strains by using a PCR product from VC83 recA as the source of DNA. The use of VC83 recA DNA as the template allowed for synthesis of a full-length mutated recA allele without any adjacent DNA sequences. This is particularly significant in terms of transferring isogenic mutations among different strains without affecting adjacent markers. While the frequency of transformants using the PCR product was generally low (<50 transformants), it was reproducible and a suitable method for introducing isogenic mutations into other strains. In Haemophilus influenzae and N. gonorrhoeae, there exist specific DNA uptake sequences which are involved in the early steps of natural transformation. It has been suggested that similar uptake sites exist for Campylobacter spp. (55). If this is the case, such an uptake site must be present on the DNA between the RecI and

### TABLE 1. Transformation of wild-type and recA mutants with Str' marker from various strains

<table>
<thead>
<tr>
<th>Recipient</th>
<th>C. coli VC167 Str'</th>
<th>C. jejuni VC83 Str'</th>
<th>C. jejuni 81-176 Str'</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC83</td>
<td>1.1 x 10⁴</td>
<td>1.25 x 10⁴</td>
<td>1.5 x 10³</td>
</tr>
<tr>
<td>VC83 recA</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>81-176</td>
<td>1.4 x 10²</td>
<td>22</td>
<td>2.3 x 10²</td>
</tr>
<tr>
<td>81-176 recA</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>81-116</td>
<td>2.1 x 10²</td>
<td>5 x 10²</td>
<td>2.2 x 10³</td>
</tr>
<tr>
<td>81-116 recA</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Results represent the average of two experiments.

** Source of DNA.

### TABLE 2. Colonization of rabbits after oral immunization with 10⁶ CFU of strain 81-176 or 81-176 rec4 and RITARD challenge with 10⁶ CFU of strain 81-176

<table>
<thead>
<tr>
<th>Immunization strain</th>
<th>No. of days colonized post-feeding (mean ± SD)</th>
<th>No. of days colonized post-RITARD challenge (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>81-176</td>
<td>19.5 ± 8.9</td>
<td>1.6 ± 1.8</td>
</tr>
<tr>
<td>81-176 recA</td>
<td>15.0 ± 7.4</td>
<td>1.5 ± 1.9</td>
</tr>
<tr>
<td>None</td>
<td>7.0 ± 0.8</td>
<td>1.5 ± 0.8</td>
</tr>
</tbody>
</table>

* Number of animals per group: 81-176, five; 81-176 recA, four; control, four.
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