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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 M_r 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*.

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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% CO_2–85% N_2-5% O_2. *E. coli* was grown on Luria agar (45) 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 PMP1 and REV3 were 40 cycles of denaturation at 94°C for 1 min, annealling at 22°C for 1 min, and extension at 72°C for 1 min. Conditions for primers Rec1 and Rec2 were 30 cycles of denaturation at 94°C for 1 min, annealling 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-^{32}P]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 dideoxy-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 Assemblylign 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% CO_2–95% air instead of air was used (1, 21, 53). *C. jejuni* cells were harvested from overnight MH plates and resuspended in MH broth to an approximate optical density at 600 nm of 0.5. Two-hundred-fifty-microliter aliquots were dispensed into polypropylene tubes (12 by 75 mm) containing 1.5 ml of MH agar. The tubes were incubated in an atmosphere of 5% CO_2–95% air for 2 h at 37°C, at which time 1 to 2 μg of DNA was added. After 4 to 5 h of incubation at 37°C in 5% CO_2–95% air, the cells were plated to selective media and grown in an atmosphere of 10% CO_2–85% N_2–5% O_2.

UV sensitivity measurements. Cells were harvested after 18 h of growth on MH agar and resuspended in 0.1 M $MgSO_4$ to an optical density at 600 nm of approximately 0.5. The cells were transferred to a petri dish and exposed to UV light in a UV cross-linker (Hoeffer Scientific Instruments, San Francisco, Calif.). After increasing doses of UV exposure, aliquots were removed, serially diluted in MH broth, and plated to MH agar.

Rabbit experiments. Female New Zealand White rabbits (Hazelton Research Products, Denver, Pa.), weighing 1.0 to 2.0 kg, were fed either strain 81-176, strain 81-176 *recA* mutant, or sterile broth via a nasogastric tube after neutralization of gastric acidity as described previously (10, 40). One month later, all three groups were challenged by the removable intestinal tie adult rabbit diarrhea (RITARD) procedure (10, 40) with strain 81-176.

Frozen stocks of 81-176 and 81-176 *recA* were thawed and inoculated on MH or MH supplemented with kanamycin, respectively, and incubated at 37°C microaerobically. After 18 h of incubation, cells were suspended in brucella broth to a concentration giving an optical density at 625 nm of approximately 0.1. Eight milliliters of the suspension was overlaid in 25-cm^2 tissue culture flasks (Becton Dickinson, Oxnard, Calif.) containing 5 ml of brucella blood agar, and these biphasic cultures were incubated ungassed at 37°C (10, 40). After 10 h of incubation, the broth phase of the biphasic culture system was collected and used for animal challenge. All challenge doses were monitored by plate counts. Oral feeding doses were approximately 10^{10} per animal. For the RITARD challenge, the pooled culture was diluted 10^2-fold in brucella broth, resulting in a challenge dose of approximately 10^6 per animal.

After oral and RITARD challenge, the colonization of rabbits was monitored by daily rectal swabs. The swabs were cultured for *C. jejuni* by direct plating on *Campylobacter*-selective agar (Remel, Lenexa, Kans.). *Campylobacter* spp. were identified by a positive oxidase test (Oxidase Reagent Droppers; Marion Scientific, Kansas City, Kans.) and by microscopic examination. Presumed 81-176 *recA* colonies were confirmed by testing for kanamycin resistance (Km^r). An animal was considered to be no longer colonized by *Campylobacter* spp. after 3 consecutive days of negative cultures.

Experiments reported herein were conducted according to the principles set forth in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, U.S. Department of Health and Human Services).

Nucleotide sequence accession number. The DNA sequence shown in Fig. 2 has been deposited with GenBank under accession number UO3121.

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 (3, 16, 27, 33, 37, 39, 43, 46, 47). These primers were PMP1 (GAAAT[AT]TATGGTCCTGA) and REV3 (TTCACC[AT]GTATC[AT]GGTT). PMP1 encodes amino acids EIYGP (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 RecA protein of *E. coli* within this region, as can be seen in Fig. 3. This PCR product was labelled with ^{32}P by nick translation and used as a probe to clone a 2.7-kb *Hin*dIII fragment into pUC18. This plasmid was termed pPMP100. Sequence analysis of the insert in pPMP100 indicated that it lacked the final 153 bp of the RecA open reading frame (ORF1; see below). pPMP100 was used as a probe to clone an overlapping 1.8-kb *Xba*I-*Pst*I fragment, and this plasmid was termed pPMP101. The plasmids are shown diagrammatically in Fig. 1.

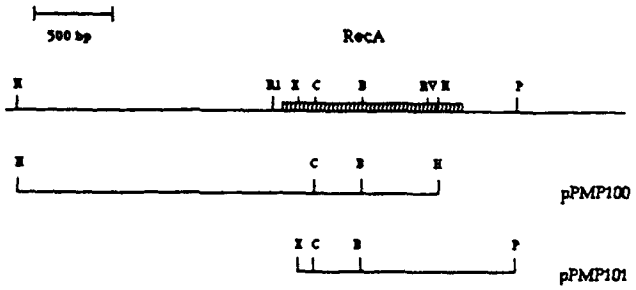

FIG. 1. Restriction map of the *recA*$^+$ region. The top line represents the chromosome of strain 81-176. The position of the inserts in plasmids pPMP100 and pPMP101 are indicated. Restriction site abbreviations: B, *Bgl*II; C, *Cla*I; H, *Hin*dIII; R1, *Eco*RI; RV, *Eco*RV; P, *Pst*I; X, *Xba*I. The kanamycin resistance cassette was inserted into the *Cla*I site on pPMP100 to generate the plasmid pPMP102 which was used to generate the *recA* mutation.

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 M_r of 37,012. The G+C content within the coding region is 37%. There is a putative ribosome binding site (shown by overlining in Fig. 2) 5 bp upstream from the translational start of the ORF. The protein 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 gonorrheae* (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 sequence similarity with the N-terminal sequences of enolases from several sources. The predicted protein is 53% identical to the N terminus of enolase of *E. coli* (MSKIVKIIGREIIDSRGNP TVEAEVHLEGGFVG [55]) and 47% identical to rat enolase (MSIQKIWAREILDSRGNPTVEVDLHTAKG) [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, 28). Several plasmids were constructed in which a Km^r cassette (28) was inserted into either the *Cla*I site or the unique *Bgl*II site in pPMP100 (Fig. 1). The inserts were transferred into the suicide vector pGK2003 (22) and mobilized from DH5 (RK212.2) hosts into strain 81-176 selecting for Km^r. Transconjugants were screened by hybridization to pUC18 DNA to determine whether the suicide vector had integrated into the chromosome by a single crossover event. All transconjugants examined from numerous crosses contained vector DNA. In an effort to circumvent this unexpected result, we introduced the mutated *recA* allele into *C. jejuni* by natural transformation. Plasmid pPMP102 is the construction in which the Km^r cassette was inserted into the *Cla*I site in pPMP100 (Fig. 1). To increase the likelihood of a double crossover event, this plasmid was linearized with *Bam*HI and used to transform 81-176 and VC83 by the biphasic method described by Wang and Taylor (53). VC83 is a strain of *C. jejuni* which has been shown previously to be highly transformable (1, 21). No transformants were obtained from 81-176, but a single VC83 transformant was obtained, and colony blot hybridization with the pUC18 probe indicated that no vector sequences were present in this transformant (data not shown). DNA was

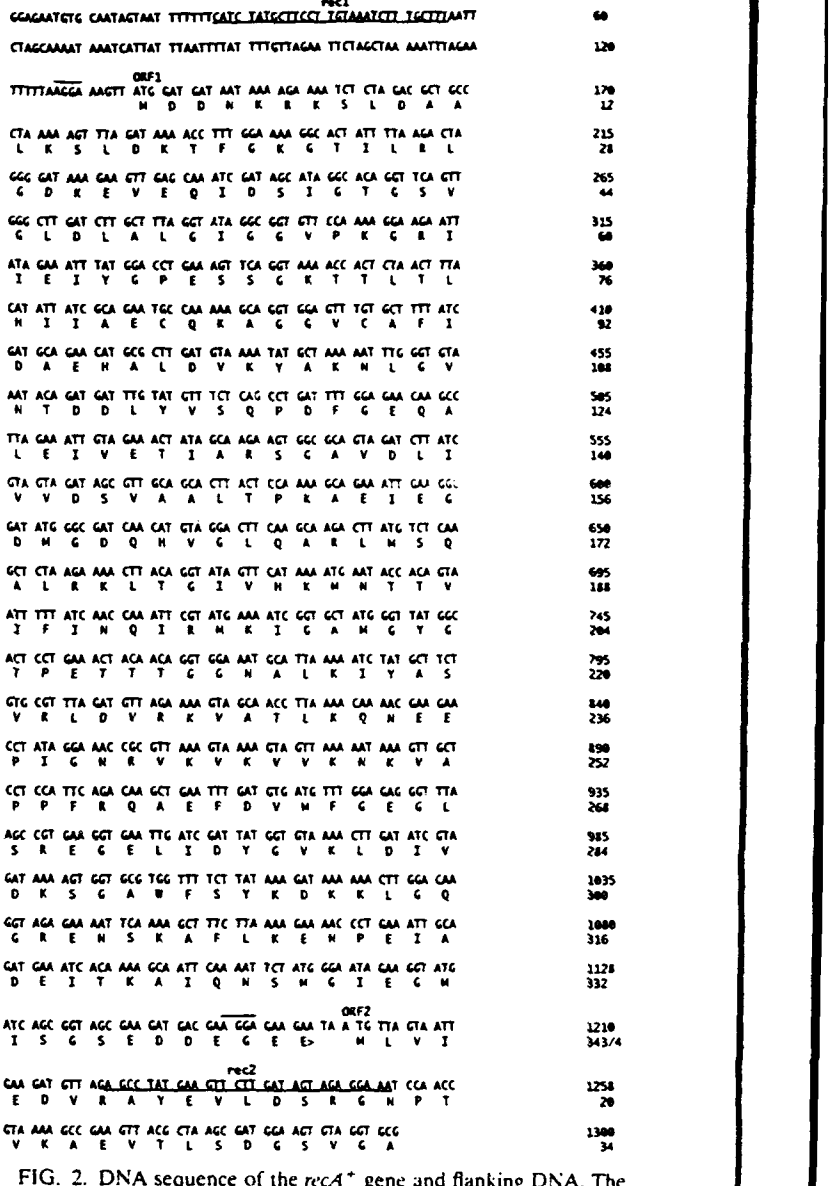

FIG. 2. DNA sequence of the *recA*$^+$ gene and flanking DNA. The predicted amino acid residues are shown in the single-letter code. The numbers on the right refer to the nucleotide numbers (top) and predicted amino acid residues (bottom). ORF1 marks the beginning of the *recA*$^+$ gene, and ORF2 marks the beginning of the putative enolase gene. Putative ribosome binding sites are indicated by overlining. The positions of the 2 PCR primers, Rec1 and Rec2, are shown by underlining.

purified from this transformant and characterized by Southern blot hybridization to pPMP101. The results, shown in Fig. 4, indicate that when digested with *Xba*I and *Pst*I, the mutant *recA* gene has increased in molecular mass by 1.4 kb (the size of the Km^r cassette; lane B) to 3.2 kb compared with the corresponding 1.8-kb fragment from wild-type VC83 (lane A). When the Km^r cassette was used as the probe, it hybridized to the same 3.2-kb fragment in the mutant (data not shown) but did not hybridize to the wild type. UV kill curves on VC83 and VC83 *recA* confirmed that the mutant was more sensitive to UV damage than the parent (Fig. 5), and subsequent DNA

```
                10        20        30        40        50        60
                *         *         *         *         *         *
Cj      MDDNK RKSLD AALKS LDKTF GKGTI LRLGD KEVEQ IDSIG TGSVG LDLAL GIGGV PKGRI
Ng      MaDdK sKaLe AALaq leKsF GKGaI mkmGs qaaEn levIs TGSIG LDLAL GvGGl rrGRI
Mf      MDeNr sKaLe AALsq leKqF GKGsI mRmGD tdVad Iqevs TGSIG LDiAL GIGGl PrGRI
Pa      MDeNK kraLe AALgq leraF GKGav mRmGD hErqa IpaIs TGSIG LDiAL GIGGl PKGRI
Tf      MDeqr sKgLs AALsq lDKqF GKGav mRlGD naikd Ievys TGSIG LDLAL GvGGl PrGRv
Ec      lDeNK qKaLe AALgq leKqF GKGsI mRlGe drsmn vetIs TGSIs LDvAL GaGGl PrGRI

                70        80        90        100       110       120
                *         *         *         *         *         *
Cj       IEIYG PESSG KTTLT LNIIA ECQKA GGVCA FIDAE HALDV KYAKN LGVNT DDLYV SQPDF
Ng      vEIFG PESSG KTTLc LeqvA qCQKn GGVCA FvDAE HAfDp vVArk LGVkv eeLYl SQPDt
Mf      vEIYG PESSG KTTLT LsvIA qmQKl GGtaA FIDAE HALDp vVAqk LGVRv sDLli SQPDt
Pa      vEIYG PESSG KTTLT LsvIA EqQKq GatCA FvDAE HALDp dVAgk LGVRv DDLlV SQPDt
Tf      vEIYG PESSG KTTLT LNaIA sCQaA GGtaA FIDAE HALDp gYAhk LGVdl enLli SQPDt
Ec      vEIYG PESSG KTTLT LqvIA aaQre GktCA FIDAE HALDp iYArk LGVdi DnLlc SQPDt

                130       140       150       160       170       180
                *         *         *         *         *         *
Cj      GEQAL EIVET IARSG AVDLI VVDSV AALTP KAEIE GDMGD QMVGL QARLM SQALR KLTGI
Ng      GEQAL EIcdT lvRSG giDmv VVDSV AALvP KAEIE GDMGD sHVGL QARLM SQALR KLTGh
Mf      GEQAL EIadm lvRSG sVDvv VVDSV AALTP KAEIE GeMGD sHmGL QARLM SQALR KLTan
Pa      GEQAL EItdm lvRSn AVDvI iVDSV AALvP KAEIE GeMGD aHVGL QARLM SQALR KiTGn
Tf      GEQAL EIadm lvRSG AVDLI ViDSV AALTP KAEIE GeMGD sHVGk QARLM SQALR nLTan
Ec      GEQAL EIcdm lARSG AVDvI VVDSV AALTP KAEIE GeiGD sHmGL aARmM SQAmR KLaGn

                190       200       210       220       230       240
                *         *         *         *         *         *
Cj      VHKMH TTVIF INQIR MKIGA MGYGT PETTT GGNAL KfYAS VRLDV RKVAT LKQME EPIGN
Ng      ikKtN TlVvF INQIR MKIGv M-fGs PETTT GGNAL KfYsS VRLDi Rrtgs iKkgE EvlGN
Mf      ikrtN TlVIF INQIR MKIGl M-fGn PETTT GGNAL KfYAS VRLDi Rrtgo iKkgd EvtGs
Pa      iknaN clVIF INQIR MKIGv M-fGn PETTT GGNAL KfYAS VRLDi Rrtgo vKegd EvvGs
Tf      isrsN TlVIF INQIR MKIGv M-YGs PETTT GGNAL KfYAS VRLDi Rrigo iKksd EvvGN
Ec      lkqsN TlliF INQIR MKIGv M-fGn PETTT GGNAL KfYAS VRLDi Rrigo vKegE nvvGs

                250       260       270       280       290       300
                *         *         *         *         *         *
Cj      RVKVK VVKNK VAPPF RQAEF DVMFG EGLSR EGELI DYGVK LDIVD KSGAW FSYKD KKLGQ
Ng      etrVK ViKNK VAPPF RQAEF DilyG EGiSm EGELI DiGVK nDIin KSGAW ySYng aKiGQ
Mf      etrVK VVKNn VAPPF klAEF DilyG EGiSR EGELI elGVn Lklie KaGAW ySYKg eKiGQ
Pa      etrVK VVKNK VsPPF RQAEF qilyG kGiyR tGELI DlGVq LglVe KSGAW ySYqg sKiGQ
Tf      dtrVK VVKNK VAPPF RaAEF qiyyG EGiSR lsELv DlGVl fDIVe KSGAW ySYqg hriGQ
Ec      etrVK VVKNK iAaPF kQAEF qilyG EGinf yGELv DlGVK eklie KaGAW ySYKg eKiGQ

                310       320       330       340
                *         *         *         *
Cj      GRENS KAFLR ENPEI ADEIT KAIQN SMGIE GMISG SEDDE GEE
Ng      GkdNv rvwLK ENPEI sDEId akIra lnGvE mhIte gtqDE td
Mf      GkdNa reFLr EhPEI AnEId akIre hsnln aamtt apDeE sdE
Pa      GkaNa akyLe dNPEI gsvle KtIrd qlaks Gpvka doeev ad
Tf      GkdNa rqyLK vhPEl AanIe qrIre aaagh plafa aEves pq
Ec      GkaNa tAwLK dNPEt AkEIe Kkvre ll
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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 flagellatum* (19); Pa, *Pseudomonas aeruginosa* (47); Tf, *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.

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 pPMP102 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^r cassette in the mutant gene. The PCR product from VC83 *recA* was purified from agarose and used to transform 81-176 to Km^r. Approximately 50 transformants were obtained, and one was further characterized. Southern blot analysis of strain 81-176 (Fig. 4, lane C) and this Km^r 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

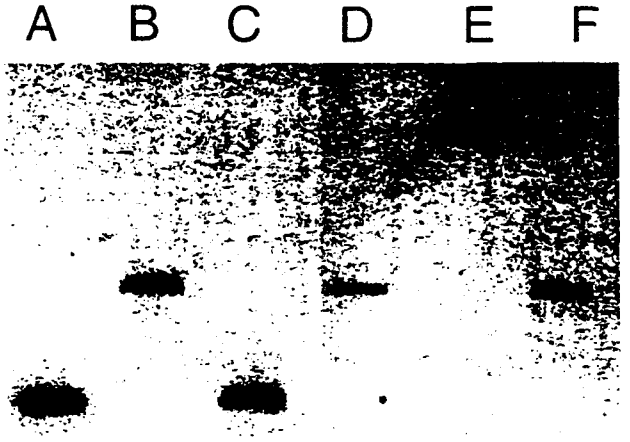

FIG. 4. Southern blot analysis of *Campylobacter* DNAs probed with pPMP101. DNAs were digested with *Xba*I and *Pst*I, electrophoresed on a 0.7% agarose gel, and transferred to nitrocellulose. Lanes: A, VC83; B, VC83 *recA*; C, 81-176; D, 81-176 *recA*; E, 81-116; F, 81-116 *recA*. The bands in lanes A and C correspond to the 1.8-kb insert in pPMP101, and those in lanes B and D are 3.2 kb, corresponding to an increase in 1.4 kb due to addition of the Km^r cassette.

(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-

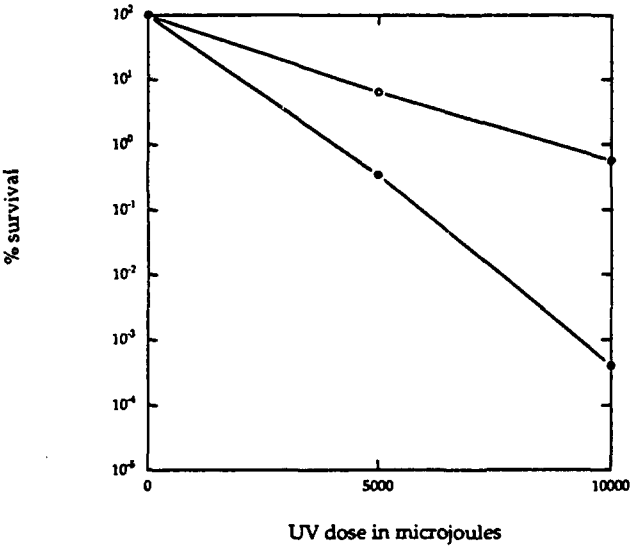

FIG. 5. UV kill curves of strain 81-176 (open circles) and 81-176 *recA* (closed circles). Cultures were exposed to increasing doses of UV light and serially diluted as described in Materials and Methods.

TABLE 1. Transformation of wild-type and *recA* mutants with Str^r marker from various strains[a]

Recipient	No. of Str^r transformants/100 μl[b]		
	C. coli VC167 Str^r[c]	*C. jejuni* VC83 Str^r	*C. jejuni* 81-176 Str^r
VC83	1.1×10^4	1.25×10^4	1.5×10^3
VC83 *recA*	0	0	0
81-176	1.4×10^2	22	2.3×10^2
81-176 *recA*	0	0	0
81-116	2.1×10^2	5×10^2	2.2×10^3
81-116 *recA*	0	0	0

[a] Results represent the average of two experiments.
[b] Viable counts were approximately 5×10^6 to 2×10^7/100 μl.
[c] Source of DNA.

resistant (Str^r) 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^r 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 VC167 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^r with VC167 DNA and used this DNA to transform the *C. jejuni* strains to Str^r. VC83 Str^r DNA transformed VC83 at approximately the same frequency as VC167 Str^r, indicating that VC83 does not appreciably restrict incoming *C. coli* DNA. 81-176 Str^r DNA, however, transforms VC83 at a lower frequency than either VC83 Str^r or VC167 Str^r DNA. Transformation of VC167, VC83, and 81-176 Str^r 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^r DNA. This reduction in transformation frequency was not due to restriction of VC83 DNA, however, since 6.9×10^2 transformants were obtained when VC83 *recA* DNA was used to transform 81-176 to Km^r.

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 *recA* 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 *recA* mutants were 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 *recA*, 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 *recA*-immunized rabbits and in two of five of the 81-176-immunized animals.

TABLE 2. Colonization of rabbits after oral immunization with 10^{10} CFU of strain 81-176 or 81-176 *recA* and RITARD challenge with 10^6 CFU of strain 81-176

Immunization strain	No. of days colonized post-feeding (mean ± SD)[a]	No. of days colonized post-RITARD challenge (mean ± SD)[b]
81-176	19.5 ± 8.9	1.6 ± 1.8
81-176 *recA*	15.0 ± 7.4	1.5 ± 1.9
None		7.0 ± 0.8

[a] The number of animals per group was six.
[b] Number of animals per group: 81-176, five; 81-176 *recA*, four; control, four.

DISCUSSION

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 cloned *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 mutagenesis 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 *lcrD* 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. (53). If this is the case, such an uptake site must be present on the DNA between the RecI and

Rec2 primers used to generate the PCR product. Comparison of this sequence with other clones of *C. jejuni* DNA capable of natural transformation should help to elucidate this uptake sequence.

Natural transformation of *C. jejuni* is clearly dependent on a functional RecA gene product. No transformation was detectable when a standard assay system was used in any of the three *recA* mutant strains. Both Wang and Taylor (53) and Alm et al. (1) reported considerable variation in the ability of different strains to be transformed, and similar observations were made in this study. VC83 transformed consistently better than strain 81-176 or 81-116, even with *C. coli* donor DNA. The highest numbers of transformants were seen when VC83 was transformed with DNA from Str^r VC83, and this level of transformation is approximately 10-fold higher than the highest reported by Wang and Taylor (53). Our transformation data do not suggest any significant degree of restriction of DNA, including *C. coli* DNA. The very low level of transformation seen with 81-176 when Str^r VC83 DNA was used was surprising but was not due to restriction since the kanamycin marker from VC83 *recA* transformed 81-176 at a high frequency. The genomic map may vary between 81-176 and VC83 in such a way that the crossover of the Str^r marker is markedly reduced in 81-176.

The introduction of a *recA* allele into the live oral cholera vaccine strain CVD103 diminished colonization ability and immunogenicity in human feeding studies (26). However, the introduction of a *recA* allele into another *V. cholerae* strain resulted in minimal reduction in colonization ability (41). In this study, 81-176 *recA* colonized rabbits almost as well as the wild type and was capable of protecting against subsequent homologous wild-type and was capable of protecting against subsequent homologous wild-type challenge. The absence of detectable natural transformability without loss of immunogenicity would indicate that *recA* mutations would be useful for inclusion into vaccine strains. Further characterization will be needed to determine what, if any, effect the introduction of a *recA* allele has on virulence in in vivo and in vitro models. Ultimately, it could be envisioned that antibiotic-sensitive *recA* mutations could be combined with mutations in virulence genes to generate living attenuated vaccine strains of *Campylobacter* spp. expressing the full complement of surface antigens.

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