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Nested DNA Inversion of *Campylobacter fetus* S-Layer Genes Is *recA* Dependent

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Wild-type strains of *Campylobacter fetus* are covered by a monomolecular array of surface layer proteins (SLPs) critical for virulence. Each cell possesses eight SLP gene cassettes, tightly clustered in the genome, that encode SLPs of 97 to 149 kDa. Variation of SLP expression occurs by a mechanism of nested DNA rearrangement that involves the inversion of a 6.2-kb *sapA* promoter-containing element alone or together with one or more flanking SLP gene cassettes. The presence of extensive regions of identity flanking the 5′ and 3′ ends of each SLP gene cassette and of a Chi-like recognition sequence within the 5′ region of identity suggests that rearrangement of SLP gene cassettes may occur by a generalized (RecA-dependent) homologous recombination pathway. To explore this possibility, we cloned *C. fetus recA* and created mutant strains by marker rescue, in which *recA* is disrupted in either S^+ or S^- strains. These mutants then were assessed for their abilities to alter SLP expression either in the presence or absence of a complementary shuttle plasmid harboring native *recA*. In contrast to all previously reported programmed DNA inversion systems, inversion in *C. fetus* is *recA* dependent.

DNA inversion is often used by microorganisms to permit the regulation of surface-exposed proteins, resulting in phase (on/off) variation (8, 19). Such inversions are programmed (nonrandom) and involve site-specific recombinases that recognize an inverted repeat (IR) sequence bracketing the invertible element (9, 19, 41). In these inversions, a segment of DNA is cleaved at two sites, inverted, and rejoined by a site-specific recombinase. The inverting DNA segment may contain either the promoter that directs expression of fixed bracketing structural genes or two divergent structural genes controlled by a fixed promoter (14, 19, 59). RecA is a protein that promotes general homologous recombination by mediating homologous base pairing and single-strand DNA exchange. All of the previously recognized inversion systems are independent of RecA activity (8, 22, 26, 34, 41, 43).

Campylobacter fetus cells produce an outermost crystalline surface layer composed of a monomolecular protein species (S-layer protein; SLP) (11a, 17) that is critical for resistance to host immune defenses and thus for virulence (4–6, 38, 63). Each cell possesses eight SLP gene cassettes that encode SLPs of 97 kDa or larger (57) and that have two regions of identity. The first (5′ conserved region) begins 74 bp upstream of the open reading frame (ORF) and extends into the ORF to encode the N-terminal 184 amino acids, and the second (3′ conserved region) begins downstream of the ORF (3, 12, 58). In the noncoding portion of the 5′ conserved region is a sequence (GCTGGTGA) sharing seven of eight bases with the *E. coli* RecBCD recognition (Chi) site (GCTGGTGG); this sequence is followed by three pentameric (ATTTT) repeats, then by 7-bp IRs with a single nucleotide spacer, ending at the ATG initiation codon (12). Beyond the 5′ conserved region, the SLP gene cassettes possess a variable region that diverges 5′ to 3′ in a semiconservative manner (12).

At high frequency, *C. fetus* strains shift the predominant SLP produced, resulting in size and antigenic variation (18, 60). Variation occurs by a novel mechanism of nested DNA rearrangement that involves inversion of the 6.2-kb promoter-containing element alone or in concert with one or more flanking SLP gene cassettes (10, 11) that are tightly clustered on the chromosome (12). Inversion of the 6.2-kb element positions the *sapA* promoter upstream of one of two bracketing SLP gene cassettes and results in its exclusive expression (10, 11), an arrangement similar to that described for the invertible promoters of *Escherichia coli* (1) and *Salmonella typhimurium* (50), among others (19). *C. fetus* has the unique characteristic that inversion of the promoter-containing element plus one or more SLP gene cassettes potentially allows expression of each of the eight cassettes which can be positioned downstream of the *sapA* promoter (11). The IR that may serve as a recombinase recognition site in *C. fetus* is located within the 5′ conserved region common to all SLP gene cassettes (12), consistent with the general mechanism of site-specific DNA inversion described for all other microbes (14, 46).

However, the presence of regions of extensive identity and similarity among SLP gene cassettes (12, 13, 58), permitting possible homologous base pairing, and of a potential Chi-like site suggests that generalized (RecA-dependent) homologous recombination also may have a role in *C. fetus sapA* inversion. To test this hypothesis, we established the presence of *recA* in *C. fetus*, cloned *recA*, and created mutant strains by marker rescue in which *recA* is disrupted in either S^+ or S^- strains. These mutants then were assessed for their abilities to alter SLP expression either in the presence or absence of a complementary shuttle plasmid harboring native *recA*. In contrast to what was found in all previously reported programmed DNA inversion systems, inversion in *C. fetus* is *recA* dependent. Thus, programmed DNA rearrangements, previously attributed to dedicated enzymatic machinery, can be mediated by generalized recombination proteins.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. Strains and plasmids used in this study are listed in Tables 1 and 2. Wild-type S^+ *C. fetus* 23D and spontaneous S^-

TABLE 1. Strains of *C. fetus* used in this study

Strain	Relevant phenotype				Source
	Sensitivity[a] to			Molecular mass (kDa) SLP on immunoblot	
	Chloramphenicol[b]	Kanamycin[c]	Serum[d]		
23D	S	S	R	97	Wild-type strain
23B	S	S	S	—[e]	Spontaneous mutant
23D:AC100	R	S	S	—	Insertion of *cm* into *sapA*
23D:RK100	S	R	R	127	Insertion of *km* into *recA*
23D:RKAC100	R	R	S	—	Insertion of *cm* into *sapA* followed by insertion of *km* into *recA*

[a] S, sensitive; R, resistant.
[b] Selection was performed by plating to media supplemented with 15 μg of chlorampenicol per ml.
[c] Selection was performed by plating to media supplemented with 30 μg of kanamycin per ml.
[d] Selection was performed by incubating cells in 10% normal human serum for 60 min at 37°C.
[e] —, no SLP detected.

mutant strain 23B have been extensively characterized (5, 6, 57, 60). Other *C. fetus* strains used were mutants derived from strain 23D, including S^- mutant 23D:AC100 with the chloramphenicol resistance gene (*cm*) inserted into the *sapA* ORF (11). Stock cultures were stored and grown as described elsewhere (12). PVNT media were supplemented with 7 U of polymyxin B, 10 mg of vancomycin, 50 mg of naladixic acid, and 10 mg of trimethoprim lactate per ml and, for kanamycin- or chloramphenicol-resistant strains, with 30 mg of kanamycin or 15 mg of chloramphenicol, respectively, per ml. *E. coli* strains used in this study, including DH5a, HB101, and XL1-Blue (Stratagene, La Jolla, Calif.), were grown in L broth or on L plates (45).

Chemicals and enzymes. Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-galactoside (X-Gal) were purchased from Jersey Lab Supply (Livingston, N.J.) and used at 50 and 30 mg/ml, respectively. Restriction enzymes, T4 DNA ligase, *Taq* polymerase, and the *E. coli* DNA polymerase large (Klenow) fragment were from Promega and U.S. Biochemical Corp. (Cleveland, Ohio). Antibiotics were from Sigma Chemical Co. (St. Louis, Mo.). [α-^{32}P]dATP (650 mCi/mmol) was from ICN Radiochemicals (Irvine, Calif.).

Genetic techniques. Chromosomal DNA was prepared from 48-h plate cultures, as described previously (10). Plasmids were isolated by the procedure of Birnboim and Doly (2). All other standard molecular genetic techniques were done as described elsewhere (45). The nucleotide sequence for pRJ701 was determined for both strands by using the dideoxy chain termination reaction (47).

PCR amplification and cloning of *C. fetus recA*. Degenerate PCR primers were designed based on the conserved amino acid sequences of RecA (42). The forward degenerate primer (5′-GARATHTWYGGNCCNGA-3′) was based on the conserved amino acid sequence EI(Y/F)GPE; the reverse degenerate primer (5′-CCNGTNGTNGTYCNGG-3′) was based on the conserved amino acid sequence PETTTG. PCR, with 23D chromosomal DNA as the template, yielded a 444-bp fragment of the expected size (42), which was subcloned into pT7Blue (Novagene, Madison, Wis.) to create pJ15-3. A sequence analysis verified that this was part of an authentic *recA*. Using the cloned fragment as a probe, we screened a λ-Zap II bank of 2- to 8-kb fragments from *C. fetus* 82-40LP3 (12). Following identification of the desired clones by hybridization, plaques were purified and pBluescript plasmids harbored within the λ-ZapII phagemid were excised in vivo in accordance with the procedure described by the manufacturer (Stratagene). We identified the complete gene on pRJ701 (GenBank accession no., AF020677), a 5.8-kb pBluescript derivative.

Construction of the suicide and shuttle vectors. pRJ707KF, a suicide hybrid plasmid with the *recA* ORF disrupted with a kanamycin resistance gene (*km*), was constructed as follows. pRJ701 was digested with *Eco*RI, and the 2.9-kb fragment that included the entire *recA* ORF and the first 345 nucleotides of the *eno* ORF was ligated into the *Eco*RI site of suicide vector pILL570E (modified from pILL570 [30] by the introduction of *Eco*RI into the *Bam*HI polylinker site [37a]) to create pRJ707. A 1.4-kb *km* gene was purified from *Sma*I-digested pILL600 (31) and blunt end ligated into the *Eco*RV site located at bp 782 of the *recA* ORF to yield pRJ707KF. The orientation of the *km* insert within the *recA* ORF in pRJ707KF was verified by restriction enzyme mapping. A selectable tetracycline resistance marker for the shuttle vectors (see below) was constructed as follows. The 259-bp *sapA* promoter region was amplified by PCR with a forward (1F) primer upstream of the promoter (10) and oligonucleotide 2754 (5′-TTGAAACATCTGTTTTGTTTAACA-3′), the reverse complement of *sapA* ORF nucleotides 3 to 28 (57). A 1.8-kb promoterless *tetO* product from pUOA2 was amplified by PCR with a *tetO* forward primer that contained the ribosome binding site (RBS; 5′-TTGATAGGAAGAAAATCACATG-3′) and a *tetO* reverse primer (5′-CCCAATTCCCATTCATCATAA-3′) (33). The PCR products were gel purified as described previously (57), and following ligation the construct was reamplified by PCR with the 1F and *tetO* reverse primers to yield a 2.1-kb fusion product. This product was cloned into pT7Blue (Novagen) and further subcloned into pBluescript (Stratagene) following *Bam*HI and *Pst*I digestions to create pATJ100 and pATJ150, respectively. The 2.1-kb *Bam*HI fragment of pATJ150 was excised and ligated directly into shuttle vector pILL550 (32) to create pATJ200. To create *recA* shuttle vector pATJ271, the 2.9-kb pRJ701 *Eco*RI fragment was inserted directly into pATJ200.

Transfer of suicide and shuttle vectors from *E. coli* into *C. fetus*. pRJ707KF was transformed into HB101 harboring the pRK212.1 IncP helper plasmid (pJB3) (16) and mobilized into *C. fetus* 23D or 23D:AC100 by conjugal mating, as described elsewhere (7). Following conjugation of pRJ707KF *E. coli* donors with 23D and 23D:AC100 recipients, *C. fetus* transconjugants were selected on PVNT brucella plates with 30 mg of kanamycin per ml and screened as described previously (10). Southern hybridizations with pILL570, *km*, and conserved *recA* sequences as the probes were used to confirm the genotypes of newly created kanamycin-resistant strains 23D:RK100 and 23D:RKAC100. Mobilization of shuttle vectors pATJ200 and pATJ271 into 23D:RK100 and of pATJ271 into 23D:RKAC100 was essentially identical to the mobilization of pRJ707KF except that transconjugants were selected on PVNT brucella plates supplemented with 10 μg of tetracycline per ml. Southern hybridizations with the pILL550 sequence, PCR analyses, and demonstration of the continued presence of an appropriately sized plasmid were used to confirm that the shuttle vectors had not integrated into the chromosome.

MMS sensitivity assays. The determination of the susceptibilities of *C. fetus* strains to methylmethane sulfonate (MMS; Kodak Co., Rochester, N.Y.) was performed as described previously (56). Cells from 48-h cultures were harvested and diluted serially in brucella broth, and the suspensions were plated onto PVNT media containing 0 to 0.04% MMS and incubated for 5 days before

TABLE 2. Plasmids used in this study

Plasmid	Relevant genotype	Source or reference
pRK212.1	IncP helper plasmid	16
pILL550	Conjugative shuttle vector	32
pILL570	Conjugative suicide vector	30
pILL570E	Conjugative suicide vector (*Eco*RI in polylinker)	This study
pILL600	*km* in shuttle vector	31
pUOA2	*tetO* in pUC8	33
pYW70	*cm* in pUC9	61
pBSC103	*cm* in pBluescript	This study
pBG101	*sapA* (promoter and 5′ end) in pBluescript	57
pKO101	*sapA* (5′ end) in pILL570	This study
pKO500	*sapA* disrupted with *cm* in pILL570	This study
pJ15-3	*recA* (444-bp fragment cloned with degenerate primers)	This study
pRJ701	*recA* in pBluescript	This study
pRJ707	*recA* in pILL570	This study
pRJ707KF	*recA* disrupted with *km* in pILL570	This study
pATJ100	*sapA* promoter *tetO* in pT7Blue	This study
pATJ150	*sapA* promoter *tetO* in pBluescript	This study
pATJ200	*sapA* promoter *tetO* in pILL570	This study
pATJ271	*recA* in pATJ200	This study

colonies were counted. Survival rates were calculated as the ratios of the numbers of colonies grown in the presence of MMS to the numbers of colonies grown in the absence of MMS (56).

Bactericidal assays. The susceptibilities of the mutant strains to the bactericidal activity of normal human serum was assayed, and survival rates were determined as described previously (10), except that cultures were plated to PVNT media after serum incubation.

Production of antiserum to *C. fetus* SLPs and immunoblot analyses. Wild-type and transconjugant *C. fetus* strains were analyzed for SLP expression with antiserum to the 97-kDa SLPs of type A strain 82-40LP (39, 60) exactly as described previously (10).

Southern hybridizations and probes. *C. fetus* chromosomal DNA was digested with *Nde*I or *Pst*I and then electrophoresed, transferred to nylon membranes, and hybridized exactly as described previously (57). Probes included the gel-purified 0.5-kb *Cla*I-*Nde*I DNA fragment (*sapA* promoter region) from pBG101 (57), the 0.44-kb *Eco*RI-*Pst*I DNA fragment from pJ15-3, and PCR products specific for *km* and *cm* (11).

PCR. Chromosomal DNA from each *C. fetus* strain was prepared and used as a template for PCR (10). Samples were routinely processed through 30 cycles of amplification, denatured for 30 s at 95°C, annealed for 1 min at 5 to 10°C below primer melting temperature, and extended for 3 min at 72°C.

RESULTS

Cloning and characterization of *C. fetus recA*. To determine whether *C. fetus* possesses *recA*, we constructed degenerate oligonucleotides based on highly conserved *recA* sequences (42) to attempt to amplify a portion of the gene by PCR. This strategy yielded a fragment of 444 bp, which was the expected size based on the primer pair used, and sequence analysis followed by a FASTA search of the GenBank verified that this fragment was part of an authentic *recA*. Using the cloned fragment (in pJ15-3) as a probe and screening a *C. fetus* λ-Zap II bank, we identified the complete gene on pRJ701, a 5.8-kb pBluescript derivative. By double-stranded sequence analysis, we found an ORF of 1,035 bp encoding a deduced RecA protein of 37 kDa. The *recA* ORF is preceded by a putative (AGGA) RBS and consensus −35 and −10 promoter elements. A putative RBS is present just before the *recA* TAA translational stop codon, and a new ORF begins 8 bp later, overlapping the stop codon. The complete ORF of the second gene is not present in pRJ701; however, a Swiss Protein Bank search based on the amino acid sequence predicted from the first 620 bp of overlapping clones indicated that it has between 47.4 and 56.7% amino acid identity with enolase. A 10-bp inverted repeat resembling a rho-dependent transcriptional terminator ($\Delta G = 3.7$) was found within *eno*, 86 bp after the *recA* stop codon. Northern analysis and reverse transcription-PCR indicate that *recA* and *eno* are cotranscribed on the same 2.5-kb transcript (data not shown). An essentially identical arrangement of *eno* immediately downstream of *recA* also has been found for *Campylobacter jejuni* (20) and *Helicobacter pylori* (56). Southern blot analyses indicate that *recA* exists as a single copy within the genome (Fig. 1 and data not shown). The overall amino acid identity to 39 other RecA proteins was between 45.1 and 66.1%.

Construction and characterization of *recA* mutant strains. To assess whether the RecA function is important in *sapA* rearrangement, we sought to inactivate *recA*. Using insertional mutagenesis (7) by rescue of a marker from a suicide plasmid (pRJ707KF) we introduced a kanamycin resistance gene (*km*) into the *Eco*RV site located at bp 781 of the *recA* ORF in wild-type strain 23D and S^- mutant 23D:AC100, which had *cm* inserted into the *sapA* ORF (10), to create 23D:RK100 and 23D:RKAC100, respectively (Table 1). In Southern hybridizations of *Nde*I-digested chromosomal DNA, the probe to the promoter region hybridized to a 1.2-kb fragment in S^+ strains 23D and 23D:RK100, whereas hybridization of the promoter region and of the *cm* probes was to identical 2.3-kb fragments in S^- strains 23D:AC100 and 23D:RKAC100, consistent with the 1.1-kb size of the inserted *cm* cassette (Fig. 1, left two panels). For *Pst*I-digested chromosomal DNA the *recA* probe hybridized to a 2.0-kb fragment in $recA^+$ strains 23D and 23D:AC100, whereas hybridization of both this probe and the *km* cassette was to a 3.4-kb fragment in *recA* mutant strains 23D:RK100 and 23D:RKAC100, indicating that the 1.4-kb *km* cassette had been inserted into *recA* in the *recA* mutant strains (Fig. 1, right panel).

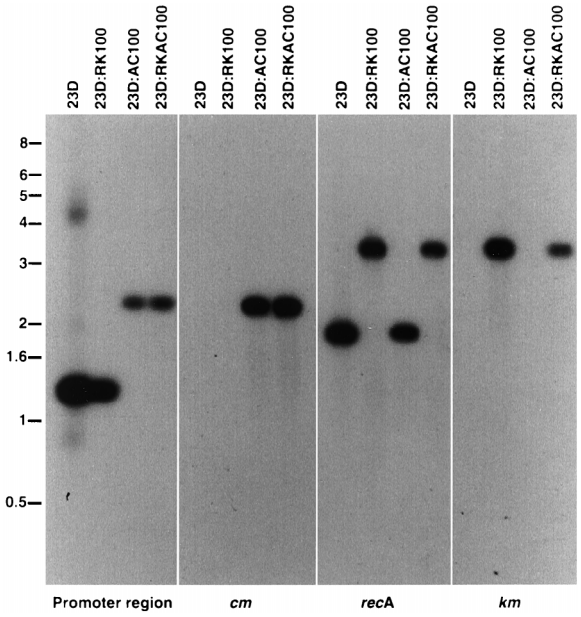

FIG. 1. Southern hybridization of *Nde*I (left two panels) and *Pst*I (right two panels) digestions of chromosomal DNA from *C. fetus* 23D, single mutants 23D:RK100 and 23D:AC100, and double mutant 23D:RKAC100 with probes to the *sapA* promoter region, the *cm* cassette, *recA*, and the *km* cassette. The hybridization patterns are consistent with *km* insertion into *recA* in strains 23D:RK100 and 23D:RKAC100 and *cm* insertion into *sapA* in strains 23D:AC100 and 23D:RKAC100.

Because *recA* and *eno* exist on the same transcript, insertion of the *km* cassette also may have a polar effect on the expression of *eno* or other 3′ genes. To account for this possibility and to determine the ability of *recA* to affect the phenotypes of the strains in which the chromosomal *recA* was disrupted, we also introduced shuttle plasmid pATJ200 or its derivative pATJ271 harboring *recA* into 23D:RK100 (Tables 1 and 2). *recA*-containing plasmid pATJ271 also was introduced into *recA* mutant strain 23D:RKAC100.

Susceptibilities of wild-type and *recA* mutant *C. fetus* strains to MMS. To assay for loss of activity of the *C. fetus* RecA protein, we examined the susceptibilities to the mutagenizing agent MMS of wild-type strains, *recA* mutant strains, and *recA* strains harboring the shuttle plasmid pATJ200 or pATJ271 (Fig. 2). *recA* mutants 23D:RK100 and 23D:RKAC100 were substantially more susceptible to MMS than was wild-type strain 23D, as expected (Fig. 2). Also as expected, introduction of shuttle plasmid pATJ200 into *recA* mutant 23D:RK100 had no effect on survival, whereas introduction of shuttle plasmid pATJ271 (containing *recA*) returned MMS resistance to wild-type levels, thus complementing RecA function in both *recA* mutant strains (Fig. 2). Southern and PCR analyses and demonstration of maintenance of the plasmid indicated that pATJ271 did not integrate into the chromosome (data not shown). Parallel experiments in which the wild type and *recA* mutants were exposed to UV light yielded similar results (data

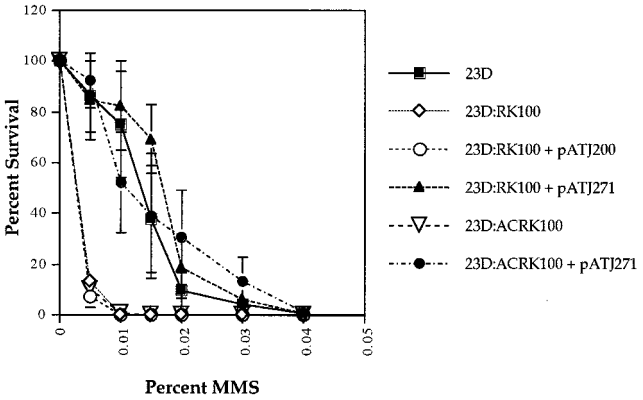

FIG. 2. Susceptibilities of wild-type and mutant *C. fetus* strains to mutagenizing agent MMS. Susceptibility is shown by plotting the percent survival of each strain versus the dose of MMS. Each point represents the mean ± standard deviation of two or more replicate determinations.

not shown). Since strains 23D, 23D:RK100 (pATJ271), and 23D:RKAC100 (pATJ271) were similar in their resistances to MMS, the presence of an S-layer and resistance to chloramphenicol, spectinomycin, tetracycline, or kanamycin had no effect on survival in the presence of MMS. Therefore, the observed phenotypes are consistent with the presence or absence of RecA function.

Immunoblot analysis of SLPs of mutant strains. Colonies of wild-type *C. fetus* strains represent mixed cultures because of the high ($\sim 10^{-4}$) rate of spontaneous *sapA* rearrangement (10). This often is discernible as the presence of both major and minor intensity SLP bands on immunoblots (7, 38, 60). Laboratory-passaged *C. fetus* cultures tend toward expression of 97-kDa SLPs (unpublished observations). However, *recA* mutant strains 23D:RK100 and 23D:RK100 (pATJ200) expressed a single SLP of 127 kDa; even after 6 months of serial passage, no minor SLPs were seen (Fig. 3, lanes 1 and 2). In contrast, 23D:RK100 complemented with pATJ271 (encoding RecA) demonstrated a shift from 127- to 97-kDa SLP expression during a single in vitro passage and predominately expressed a 97-kDa SLP after in vitro passage for 2 months (Fig. 3, lanes 3 and 4). Thus, in the absence of RecA function, variation in S-layer expression was not detected.

Serum susceptibility of the *C. fetus* mutants. Loss of the S-layer renders *C. fetus* susceptible to complement-mediated serum bactericidal activity (4–6, 38); conversely, serum incubation selects for SLP expression, which occurs at high frequency (7, 10, 11). S^+ *recA* mutant strain 23D:RK100 was serum resistant, as expected, because it expresses a full-length SLP. Since in strain 23D:AC100 insertion of *cm* into *sapA* disrupted SLP expression (11), serum incubation selected for those strains that switched to S^+ (7, 10). Thus, by determining survival frequencies following serum incubation of S^- mutant strains that are either wild type (23D:AC100) or mutant (23D:RKAC100) for *recA*, the role of *recA* function in SLP gene cassette rearrangement could be quantitated. In such experiments, we found that *recA* mutant strain 23D:RKAC100 was highly serum sensitive, with greater than a 3-log-unit decrease in serum survival compared with 23D:AC100 (Table 3); no S^+ serum survivors were ever recovered. Complementing strain 23D:RKAC100 with *recA* mutant-encoded functions on pATJ271 returned serum survival to the level observed for $recA^+$ 23D:AC100. These results confirm that RecA plays a major, if not essential, role in *sapA* rearrangement.

DISCUSSION

We have recently demonstrated that SLP diversity involves a single *sapA* promoter-containing element that may be inverted alone or together with one or more tandemly arrayed SLP gene cassettes by a process of nested DNA inversion (10, 11). By the creation of appropriate mutants and by using selection for phenotypic properties, our present experiments demonstrate that this DNA rearrangement is *recA* dependent. Although *recA* and *eno* are cotranscribed in *C. fetus* and *H. pylori* (56) and probably in *C. jejuni* (20), it is not apparent why such apparently unrelated genes are in the same operon. However, the use of the shuttle vectors permitted us to determine with certainty a role for *recA* in SLP gene cassette DNA rearrangement independent of potential polar effects on *eno* expression.

The present study represents the first reported use of a shuttle vector and expression of *tetO* in *C. fetus*. To facilitate expression, the *Campylobacter coli tetO* cassette (33) was positioned downstream of the highly active *sapA* promoter, which normally permits the expression of approximately 10^5 copies of SLPs per cell per generation (64). Attempts to express the tetracycline resistance gene in *C. jejuni* by using *tetO* with its native promoter were unsuccessful (19a). The expression of *C. coli cm* (61) also was dependent on the utilization of the *sapA* promoter, and the native *C. coli cm* promoter in *C. fetus* did not permit expression (reference 11 and unpublished data). Thus, the *sapA* promoter may enable the expression of genes that otherwise might be difficult to express in *C. fetus.*

Despite the presence of extensive homologies and the dependence on RecA, the rearrangement of SLP gene cassettes is anticipated to occur by a site-specific mechanism (10). The presence of IRs bracketing each of the inverting elements and the absence of random duplications of SLP gene cassettes argue against an obligate mechanism of generalized recombination. Although oppositely oriented homologous regions can undergo homologous (RecA-dependent) recombination resulting in inversion (15, 25, 40, 49), even-more-frequent duplications or deletions among the tandemly and oppositely oriented sequences (40, 48) would be expected through this

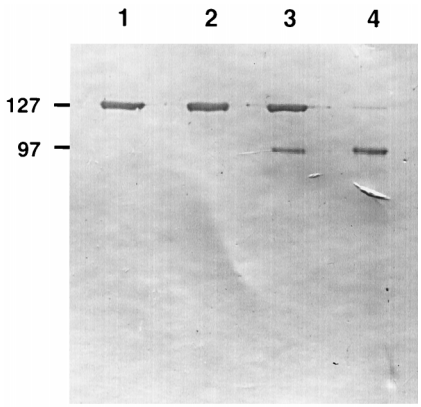

FIG. 3. Immunoblot of *recA* mutants produced by using high-titer antiserum to conserved *C. fetus* SLP determinants. $RecA^-$ strain 23D:RK100 expresses a single SLP of 127 kDa even after 30 serial passages (lane 1). The presence of pATJ200 in strain 23D:RK100, even after 30 serial passages, has no effect on SLP expression (lane 2), whereas following introduction of pATJ271 both 97- and 127-kDa SLPs could be detected even after a single passage (lane 3). After 30 serial passages, a 97-kDa SLP was predominantly expressed in 23D:RK100 harboring pATJ271 (lane 4). Note that *recA* mutant strains that have not been complemented (lanes 1 and 2) do not show even low-level expression of heterogeneous SLPs, indicating that *recA* is necessary for expression of SLP variants. Molecular mass markers are in kilodaltons.

TABLE 3. Serum susceptibility of *C. fetus* mutants

Strain	Genotype	Molecular mass (kDa) of SLP on immunoblot in initial culture	No. of expts	Survival frequency[b]	
				Mean	Range
23D	Wild type	97	5	1[c]	
23B	Undefined[a]	None	5	$<10^{-7}$	$<10^{-7}$
23D:AC100	*sapA* mutant	None	8	9.0×10^{-5}	1.7×10^{-5}–3.1×10^{-4}
23D:RK100	*recA* mutant	127	3	9.5×10^{-1}	0.9–1
23D:RKAC100	*sapA* and *recA* mutant	None	7	$<10^{-7}$	$<10^{-7}$
23D:RKAC100 (pATJ271)	*sapA* and *recA* mutant (*recA*$^+$)[d]	None	4	9.6×10^{-5}	8.5×10^{-5}–1.1×10^{-4}

[a] Spontaneous deletion mutant.
[b] After incubation with 10% normal human serum for 60 min; 10^{-7} represents the limit of assay sensitivity.
[c] Since incubation of wild-type strain 23D shows <0.05 $\log_{10}$ killing in this assay, strain survival was defined as 1. Values shown for all other strains are relative to that of 23D.
[d] pATJ271 harbored wild-type *recA*.

mechanism; neither are observed during *C. fetus* SLP gene cassette rearrangement (references 7, 10, 11, and 58 and unpublished results). Moreover, the presence of inverted sequence homology may not be sufficient for inversion (21, 24, 35). Inversion of the P1 phage C segment is dependent on site-specific invertase Cin, despite the presence of 600 bp of flanking homologies (24). Similarly, site-specific recombinase Sin is implicated in the inversion of DNA sequences located between 650-bp inverted repeats in penicillinase plasmids of *Staphylococcus aureus* (35–37). Thus, site-specific inversion and the presence of substantial homology are not mutually exclusive.

The known instances of site-specific inversion are RecA independent, as might be expected, and are catalyzed by recombinases that belong to one of two families, the Din invertase family (a subset of the Tn*3* resolvase family) and a family of recombinases related to the bacteriophage λ integrase (Int family). Both recombinases recognize specific IR sequences bracketing the invertible element (19, 59). The invertase and integrase families promote inversion by introducing either a single- or double-stranded break, respectively, at the IR (19, 22). Integrases are believed to promote single-strand exchanges through a Holliday junction intermediate, whereas invertases rotate the double-stranded breaks and religate the ends (9, 22, 27). In both instances, the break is repaired by the site-specific enzyme (9, 19). Since integrases are more promiscuous than invertases, i.e., they use both direct and inverted repeats as substrates (9, 43), catalyzing deletions as well as inversions, an integrase-like pathway is less likely to be utilized in *C. fetus* since the number of homologs does not change.

RecA requires at least three features to promote homologous recombination: (i) extensive nucleotide sequence homology, (ii) a region of single-stranded DNA on at least one of the substrates, and (iii) in RecBCD-dependent pathways, a free DNA end on at least one of the substrates (51). In contrast to invertases, RecA cannot introduce DNA cleavage; therefore, DNA cleavage and single-stranded DNA must be provided by another protein(s), and in *C. fetus* DNA cleavage presumably precedes RecA function. We hypothesize that the RecA protein is necessary for rearrangement because it repairs DNA breaks introduced by a separate site-specific endonuclease that cleaves within the 5′ regions of identity among SLP gene cassettes. Precedent for such a system is found in yeast mating type switching, where site-specific endonuclease HO creates a double-stranded break that initiates homologous recombination leading to gene conversion at the MAT locus (28, 53, 62; see references 23, 52, and 55 for reviews). A similar mechanism may account for the creation of a hot spot for homologous recombination following Tn*7* excision along with the introduction of double-stranded breaks within *lacZ* heteroalleles (21). The movement of type I introns also occurs by a double-stranded DNA break repair process following the activity of an intron-encoded endonuclease (44).

We speculate that, for *C. fetus*, introduction of a double-stranded break may be mediated by an endonuclease that specifically recognizes the IR located in the 5′ conserved region of the SLP gene cassettes, immediately upstream of the ORF. The DNA ends provide a site for homologous double-stranded-break repair (51, 54) for which the RecBCD enzyme plays a principal role, acting both as an endonuclease and helicase (29). Chi sites act by arresting the ExoV activity embodied in RecBCD as it approaches from a 3′ direction starting at the double-stranded DNA end, but leave the RecBCD helicase activity intact, permitting continued 5′ unwinding of the DNA (29). In *C. fetus*, a potential Chi site (10) located 15 bp 5′ to the IR is consistent with this model. The extent of *sapA* homology extending 5′ from the site at which the nuclease activity is expected to halt is at least 50 nucleotides, sufficient for homologous-strand exchange (51). The resulting frayed end may provide the necessary substrate for RecA-mediated homologous repair and, in *C. fetus*, presumably DNA inversion.

The mechanism utilized by *C. fetus* to rearrange the SLP gene cassettes indicates that it differs fundamentally from previously described RecA-independent programmed DNA inversion mechanisms, perhaps in the manner we propose. Regardless of the exact mechanism, the inversion of *C. fetus* SLP gene cassettes mimics in appearance a wholly site-specific phenomenon yet clearly is RecA dependent and thus may be a hybrid of these phenomena.

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