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Rearrangement of *sapA* homologs with conserved and variable regions in *Campylobacter fetus*

(bacterial pathogenesis/*Campylobacter fetus* surface-layer proteins/antigenic variation)

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ABSTRACT **The *Campylobacter fetus* surface-layer (S-layer) proteins mediate both complement resistance and antigenic variation in mammalian hosts. Wild-type strain 23D possesses the *sapA* gene, which encodes a 97-kDa S-layer protein, and several *sapA* homologs are present in both wild-type and mutant strains. Here we report that a cloned silent gene (*sapA1*) in *C. fetus* can express a functional full-length S-layer protein in *Escherichia coli*. Analysis of *sapA* and *sapA1* and partial analysis of *sapA2* indicate that a block of ≈600 bp beginning upstream and continuing into the open reading frames is completely conserved, and then the sequences diverge completely, but immediately downstream of each gene is another conserved 50-bp sequence. Conservation of *sapA1* among strains, the presence of a putative Chi (RecBCD recognition) site upstream of *sapA*, *sapA1*, and *sapA2*, and the sequence identities of the *sapA* genes suggest a system for homologous recombination. Comparison of the wild-type strain (23D) with a phenotypic variant (23D-11) indicates that variation is associated with removal of the divergent region of *sapA* from the expression locus and exchange with a corresponding region from a *sapA* homolog. We propose that site-specific reciprocal recombination between *sapA* homologs leads to expression of divergent S-layer proteins as one of the mechanisms that *C. fetus* uses for antigenic variation.**

Structural variation of surface components is an effective means of immune evasion used by many pathogenic microorganisms (1–4). *Campylobacter fetus*, a Gram-negative spiral bacterium that causes infectious abortion in sheep and cattle (5) and extraintestinal infections in humans (6), expresses several variable high-molecular-size (95–149 kDa) proteins that form paracrystalline surface layers (S-layers) (7–9). *C. fetus* strains possessing S-layer proteins (S^+), but not spontaneous mutants lacking S-layers (S^-), are resistant to C3b binding and to phagocytosis by polymorphonuclear leukocytes (10, 11).

The *C. fetus* S-layer proteins must share relatively conserved features, as dictated by requirements for crystalline structure and binding to divalent cations and specific lipopolysaccharide (LPS) molecules (12) and as demonstrated by amino-terminal sequencing (7, 8) and cross-reactivity with polyclonal antisera (7). However, *C. fetus* S-layer protein expression is subject to high-frequency antigenic variation (8, 9, 13).

Recently, Blaser and Gotschlich (14) reported the isolation of the *sapA* gene encoding a 97-kDa S-layer protein from *C. fetus* strain 23D. Southern hybridization revealed that both wild-type strains and spontaneous S^- mutants possess several *sapA* homologs, but S^- mutants do not contain expression sequences (15). We now report the presence of a full-length *sapA* homolog in S^- strain 23B§ that, when cloned in *Escherichia coli*, can express its product. This gene (*sapA1*) is conserved in all *C. fetus* strains studied, and all three *sapA* homologs that we have studied contain both conserved and variable domains. Finally, we show that phenotypic variation of S-layer proteins is associated with site-specific reciprocal recombination of *sapA* homologs.

MATERIALS AND METHODS

Bacterial Strains, Vectors, and Growth Conditions. *C. fetus* strains 84-32 (23D), 84-54 (23B), 82-40LP (LP), 82-40HP (HP), and 83–88 were from the culture collection of the Vanderbilt University *Campylobacter* Laboratory, have been extensively characterized (10, 11, 16), and were grown and maintained as described (10). Strain 23D-11 was a spontaneous variant of strain 23D, observed after passage on blood agar plates when immunoblotting of resulting colonies indicated that it expressed a 127-kDa S-layer protein. *E. coli* strain DH5α (Stratagene) was used for transformation. Cosmid ScosI (Stratagene) was used as a vector for construction of the genomic library, and pGEM3Z (Promega) was used as a cloning vector. Plasmid pBG1 contains the *sapA* gene on a 4.0-kb insert in pUC9 (14).

Genetic Techniques and DNA Sequencing Analysis. Chromosomal DNA was prepared as described (17). Plasmids were isolated by the procedure of Birnboim and Doly (18). All other standard molecular genetic techniques were done as described (19). Nucleotide sequence was determined unambiguously on both strands by using double-stranded DNA templates and the dideoxynucleotide chain-termination procedure, as described (20). Oligonucleotide primers were synthesized by using a Milligen model 7500 DNA synthesizer. Nucleotide sequences were compiled and analyzed with the aid of the DNA-STAR program (DNAstar, Madison, WI).

Construction of a Genomic Library. Partial *Sau*3A digests of chromosomal DNA from strain 23B containing fragments of 20–40 kb were ligated to the *Xba* I-digested, dephosphorylated, and *Bam*HI-digested ScosI vector, packaged, and transduced into *E. coli* strain DH5α. Colonies of recombinants were hybridized at 67°C for 16 hr under high-stringency conditions (15) to a radiolabeled *C. fetus sapA* probe [4.0-kb *Eco*RI fragment from pBG1 (14)], as described (19). Cosmid DNA was isolated from purified positive clones, restriction enzyme-cleavage maps were generated, and a 12.5-kb *Eco*RI fragment carrying the *sapA* homolog was subcloned into pGEM3Z to create pMT100. The *sapA* gene was further localized to a 5.0-kb *Eco*RI–*Eco*RV fragment and subcloned in pGEM3Z to generate pMT101 (Fig. 1).

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Abbreviations: S-layer, *Campylobacter fetus* surface-layer; ORF, open reading frame; LPS, lipopolysaccharide.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. L15800).

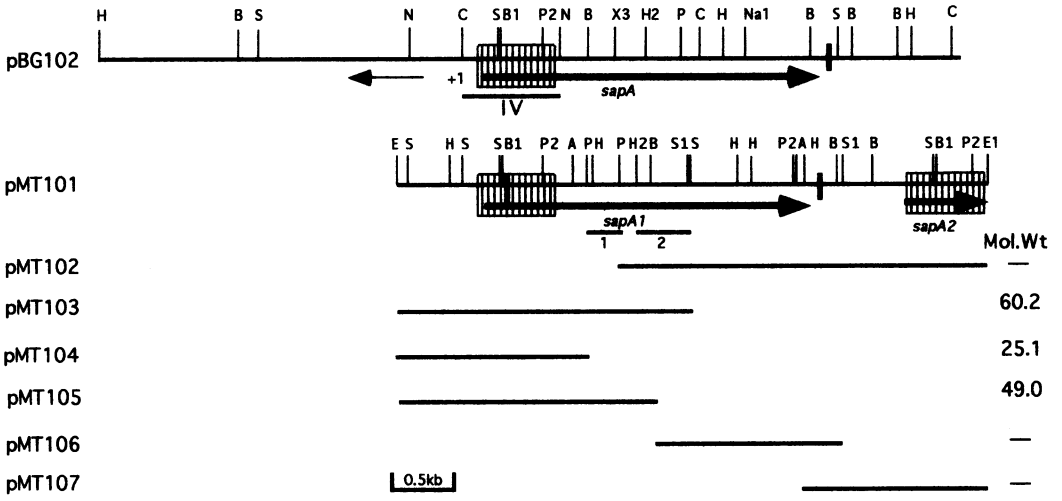

FIG. 1. Structure of *sapA* and *sapA1*-containing recombinant plasmids. Plasmid pBG102 contains the *sapA* gene, and pMT101 contains a 5.0-kb *Eco*RI–*Eco*RV fragment from *C. fetus* strain 23B, which hybridized to a *sapA* probe (14). Plasmids pMT102-107 represent different deletion clones derived from pMT101. Numbers at right refer to molecular weight (Mol.Wt) of truncated *sapA1* products. Hatched boxes indicate the conserved regions of *sapA*, *sapA1*, and *sapA2*; small solid bars indicate the homologous region downstream of each open reading frame (ORF). Numbers IV and 1 and 2 indicate the regions of *sapA* or *sapA1* used as DNA probes. Large arrows beneath pMT101 and pBG102 represent the location of the genes and direction of transcription. The small arrow beneath pBG102 represents a cryptic ORF. Restriction endonuclease cleavage sites are as follows: A, *Acc* I; B, *Bgl* II; B1, *Bst*NI; C, *Cla* I; E, *Eco*RV; E1, *Eco*RI; H, *Hin*dIII; H2, *Hin*cII; N, *Nde* I; Na1, *Nae* I; P, *Pst* I; P2, *Pvu* II; S, *Ssp* I; S1, *Sac* I; X3, *Xma* III.

Gel Electrophoresis and Immunoblot Analysis. Lysates of strain DH5α carrying pMT100 or pMT101 were prepared and analyzed by SDS/PAGE, as described (21). Immunoblotting was done as detailed (22) by using a 1:3000 dilution of antiserum to the 97-kDa S-layer protein from *C. fetus* strain 82-40LP (7) and goat anti-rabbit immunoglobulin alkaline phosphatase conjugate as the second antibody. Alternatively, monoclonal antibody 2H11 raised against the 97-kDa S-layer protein from strain 82-40LP was used (34) with an anti-mouse conjugate.

Attachment of the Recombinant S-Layer Protein to the *C. fetus* Cell Surface. DH5α cells carrying pMT101 were grown overnight, lysed, and centrifuged; the supernatant was tested for reattachment to cells of strains 23B (LPS type A) or 83-88 (LPS type B), as described (12). As a positive control, water-extracted proteins from *C. fetus* 23D were used. Binding of S-layer proteins was analyzed by immunoblot of the washed, pelleted cells, as described (12).

Southern Hybridizations. *Hin*dIII-digested *C. fetus* chromosomal DNA was electrophoresed and transferred to nylon membranes (19) and hybridized at 67°C under high-stringency conditions with radiolabeled (23) fragments derived from pMT101 (*sapA1*) or pBG1 or pBG101 (*sapA*), exactly as described (15). Probes I–VII are restriction fragments from the *sapA* locus used for hybridizations with 23D and 23D-11 genomic DNA.

RESULTS

Cloning of *C. fetus sapA* Homolog. Because S^- *C. fetus* strain 23B possesses several homologs to *sapA* (15) but does not express S-layer proteins, we sought to determine whether the homologs are full-length or partial. For this purpose we constructed a cosmid library containing partial *Sau*3A-digested chromosomal fragments from strain 23B and screened transformed colonies by hybridization using a *sapA* probe. Recombinant cosmid DNA from one of the hybridizing isolates was purified and digested by restriction endonucleases *Eco*RI, *Pst* I, and *Eco*RV. A 12.5-kb *Eco*RI fragment hybridizing with the *sapA* probe was subcloned into pGEM3Z to generate pMT100, and then a 5.0-kb *Eco*RI–*Eco*RV hybridizing fragment was subcloned into pGEM3Z to create pMT101 (Fig. 1).

Analysis of *sapA1* Gene Product. We next sought to determine whether the product of the cloned *sapA* homolog can be expressed in *E. coli*. With antiserum specific for *C. fetus* S-layer proteins, immunoblots of lysates of *E. coli* carrying pMT100 or pMT101 revealed an antigenic protein of ≈97 kDa (Fig. 2), whereas cells carrying vector alone showed no reactivity (data not shown). The *sapA* homolog expressed in *E. coli* was reattached to the surface of S^- *C. fetus* 23B (type A LPS) but was not reattached to S^- 83-88 (type B LPS) cells, exactly as did the *sapA* gene product expressed by pBG1 (Fig. 3). These studies indicate that strain 23B possesses a gene that can express a functional S-layer protein in a suitable genetic background (such as *E. coli*). However, despite common size and shared antigens of this protein with the *sapA* gene product, the restriction map (Fig. 1) clearly differentiated *sapA* from the homolog, which we now named *sapA1*. To determine the location of the coding region in pMT101, we created a series of deletion mutants by enzyme digestion (Fig. 1). The *Bgl* II- and *Sac* I-deleted plasmids (pMT105 and pMT103, respectively) encoded truncated immunoreactive proteins having apparent molecular sizes of ≈49 and 60 kDa, respectively, allowing us to localize *sapA1*.

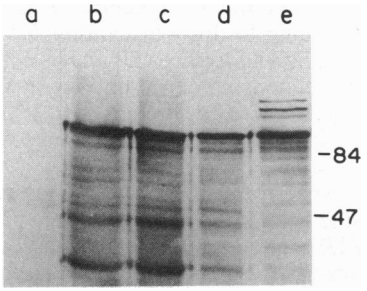

FIG. 2. Immunoblot analysis of recombinant *sapA1* gene product. *E. coli* DH5α cells containing pMT100 (lane b), pMT101 (lane c), or pBG1 (14) (lane d) were grown overnight, and the lysates were used for immunoblot analysis with antiserum to the 97-kDa S-layer protein of *C. fetus* strain 82-40LP. Controls are lysates of S^- *C. fetus* strain 23B (lane a) and S^+ *C. fetus* strain 23D (lane e). Numbers at right indicate positions of the molecular-weight ($\times 10^{-3}$) markers.

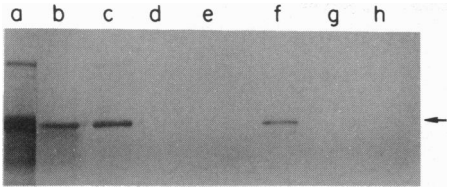

FIG. 3. Reattachment of recombinant *sapA1* gene product to *C. fetus* cell surface. *C. fetus* cells [23B (type A LPS) or 83-88 (type B LPS)] in Hepes buffer were incubated with $CaCl_2$, and the *sapA1* gene product was expressed from pMT101. Nonspecific binding of the *sapA1* gene product was removed by washing with excess C buffer, and attachment was analyzed by immunoblot with antiserum to the 97-kDa S-layer protein of *C. fetus* strain 82-40LP. The arrow at right represents the migration position of *sapA* and *sapA1* gene products. Lanes: a, *C. fetus* 23D whole cells; b, water extract from *C. fetus* 23D; c, 23B cells plus water extract; d, 83-88 cells plus water extract; e, 23B cells alone; f, 23B cells plus *sapA1* gene product; g, 83-88 cells plus *sapA1* gene product; h, 83-88 cells alone.

DNA Sequence Analysis of the *sapA1* Locus. To more precisely characterize the structure of *sapA1* and to understand its lack of expression in strain 23B, we determined its nucleotide sequence from pMT101, using pMT101 deletion derivatives (Fig. 1). Translation of the nucleotide sequence of a 5043-bp pMT101 insert in all possible reading frames on both strands revealed an ORF of 2760 nt. The sequence encodes a protein of 920-amino acid residues, with a calculated molecular size of 95,010 Da, which is in general agreement with the migration of the product on SDS/PAGE (Fig. 2). A potential ribosomal-binding site AGGAG is located 6 bases upstream of the methionine start codon. The downstream flanking region of the gene shows a palindromic sequence with complementarity for 23 of 26 bp, which could form a strong stem-loop structure (ΔG,20.4) expected for a rho-independent prokaryotic transcription terminator. Unexpectedly, we located a second ORF in this insert, which we call *sapA2* (Fig. 1). The sequence for the complete ORF has not been determined because no further downstream sequence is available.

Comparison of the Nucleotide Sequences of the *sapA* Homologs. At the nucleotide level, the *sapA*, *sapA1*, and partial *sapA2* sequences are highly conserved in the 5′ region. From nt 1–654, the *sapA1* ORF is 99% identical to that of *sapA*. Of the first 552 bp of the *sapA2* ORF for which sequence information is available, there is near identity with *sapA* and *sapA1* until the final 27 bp, which diverge. The first 74 nt upstream of the *sapA*, *sapA1*, and *sapA2* ORFs are identical (Fig. 4*A*). The untranslated leader RNA for *sapA* (15) contains three pentameric (ATTTT) repeats, which is conserved in both *sapA1* and *sapA2*. Sequences upstream of the *sapA1* and *sapA2* ORFs continue identity for another 39 bases (to −113), but unlike *sapA* (15), neither has a putative promoter sequence upstream. No homology was seen for the next 700 bp upstream of these three genes. A sequence (GCTGGTGA) sharing 7 of 8 bases with the consensus RecBCD (Chi) recognition site GCTGGTGG (24) is conserved in each of the three *sapA* homologs (Fig. 4*A*). Downstream of the *sapA1* and *sapA* ORFs, homology begins 80 bases from the translational stop codon of *sapA1* and extends 51 bases, with subsequent brief areas of homology over the next 65 bases (Fig. 4*B*). The presence of homologous sequences both 5′ and 3′ to these *sapA* homologs suggests that these regions might play a role in site-specific recombination.

Analysis of the Products of the *sapA* Homologs. As expected, the first 218 and 175 residues of the deduced amino termini of the sapA1 and sapA2 products, respectively, are nearly identical to those of the sapA product. Subsequently, the sequences are mostly divergent, but short stretches of homology are maintained. The predicted isoelectric points for

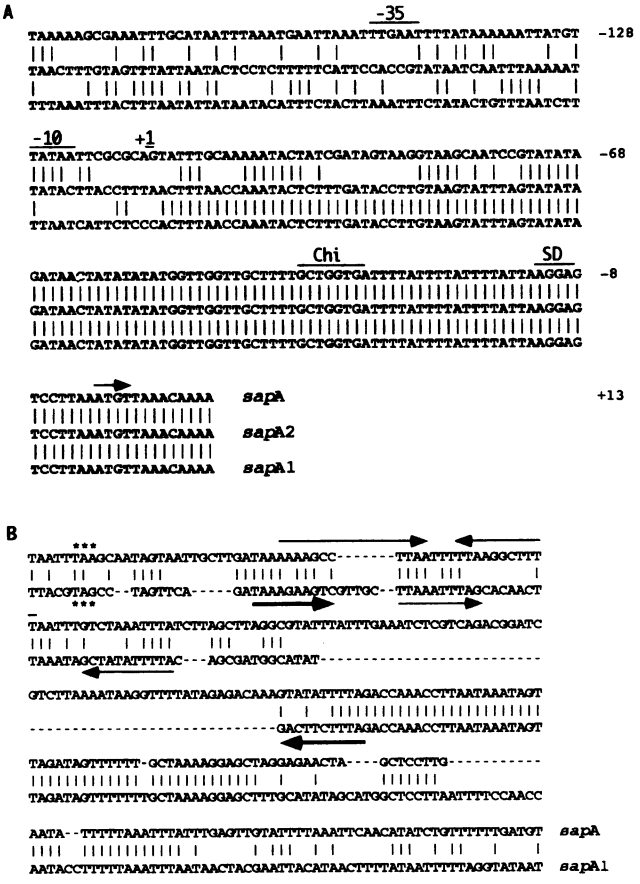

FIG. 4. Nucleotide sequence comparisons of *sapA* genes. (*A*) Upstream region. DNA sequences of the upstream regions of *sapA* (15), *sapA1*, and *sapA2* are compared. The consensus −10 and −35 promoter determinants, the transcription start site (+1), and the ATG codon and direction of transcription (arrow) for *sapA* are indicated. The ribosomal-binding site is identified by SD. (*B*) Comparison of downstream regions of *sapA* and *sapA1*. Stars indicate translational stop codons. Converging arrows beneath *sapA* and *sapA1* sequences indicate putative transcription terminators. Vertical dashes between nucleotides indicate sequence identity.

the *sapA1* and *sapA* gene products are 4.3 and 4.5, respectively. Although both proteins are predominantly hydrophobic, their secondary-structure profiles differ significantly beyond the conserved amino-terminal regions (data not shown).

Conservation of the *sapA1* Gene. To determine whether the *sapA1* gene is present in other *C. fetus* strains, we used probes from the conserved and divergent regions in Southern hybridizations under high-stringency conditions. The conserved amino-terminal probe hybridized to several *sapA* homologs in both S^+ and S^- strains, as expected (Fig. 5*A*). Probes 1 and 2 hybridized to fragments of the same size in both S^+ wild-type (23D, LP) and S^- spontaneous mutant (23B, HP) strains (Fig. 5*B*). These results indicate that a single copy of full-length *sapA1* is present on conserved restriction fragments in both wild-type and spontaneous mutant *C. fetus* strains.

Rearrangement of Chromosomal DNA Accompanies Phenotypic Change. Strain 23D-11, a derivative of strain 23D from laboratory passage, expresses a major S-layer protein of 127 kDa (encoded by a gene provisionally called *sapA3*) rather than of 97 kDa (Fig. 6*A*). To determine whether this phenotypic shift was associated with rearrangement in the *sapA* locus, the chromosomal DNA of strains 23D-11 and 23D was examined by Southern hybridization. A series of probes (I–VII) that surround the *sapA* locus was used to determine

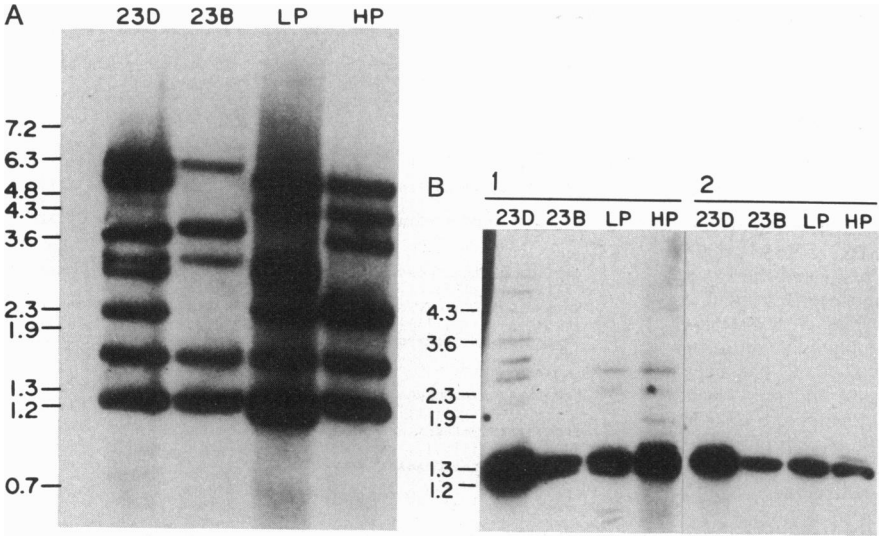

FIG. 5. Southern hybridization of conserved and variable regions of *sapA1* to genomic DNA from *C. fetus* strains. DNA from *C. fetus* strains 23D, 23B, LP, and HP was digested with *Hin*dIII, electrophoresed in 0.7% agarose, transferred to a nylon membrane, and hybridized with ^{32}P-labeled *sapA1* probes. (*A*) The 0.7-kb *Cla* I–*Nde* I fragment designated as probe IV in Figs. 1 and 6 (conserved amino-terminal region). (*B*) Variable regions of *sapA1* (designated as probes 1 and 2 in Fig. 1).

whether rearrangement had occurred. Analysis of results using these probes on *Hin*dIII, *Ssp* I, *Hin*cII, *Cla* I, and *Bgl* II digestions indicates that in strain 23D-11 the divergent region of *sapA* has become continuous with the 5′ end of the gene that we call *sapA3*, whereas the divergent region of *sapA3* is now continuous with the 5′ end of *sapA* (Fig. 6*B*). A schematic representation of the results of the hybridizations (Fig. 6*C*) indicates that reciprocal recombination had occurred. The locus of the cross-over occurs within the conserved region, but the exact site is not known. The conserved region of *sapA* (probe IV) hybridized to multiple bands present in both the *Hin*dIII and *Ssp* I digestions of chromosomal DNA as expected. The differences in the intensity of *Hin*dIII fragments that hybridize with probe IV indicate that multiple copies are present in similar size fragments. Hybridization to a smaller *Ssp* I fragment(s) is due to the existence of an *Ssp* I site within the conserved region and another *Ssp* I site located upstream to the *sapA* homolog.

DISCUSSION

S-layer proteins are critical for *C. fetus* virulence (25, 26) and undergo antigenic variation (8, 9, 13), and the presence of multiple *sapA* homologs (15) provides a potential mechanism for the variation. We now show that S$^-$ *C. fetus* strain 23B contains a conserved *sapA* homolog (*sapA1*) that can encode a full-length S-layer protein with specific cell-attachment functions. That *sapA1* can be expressed in *E. coli* but is not expressed in strain 23B and does not appear to be preceded by an active promoter indicates that it represents a silent

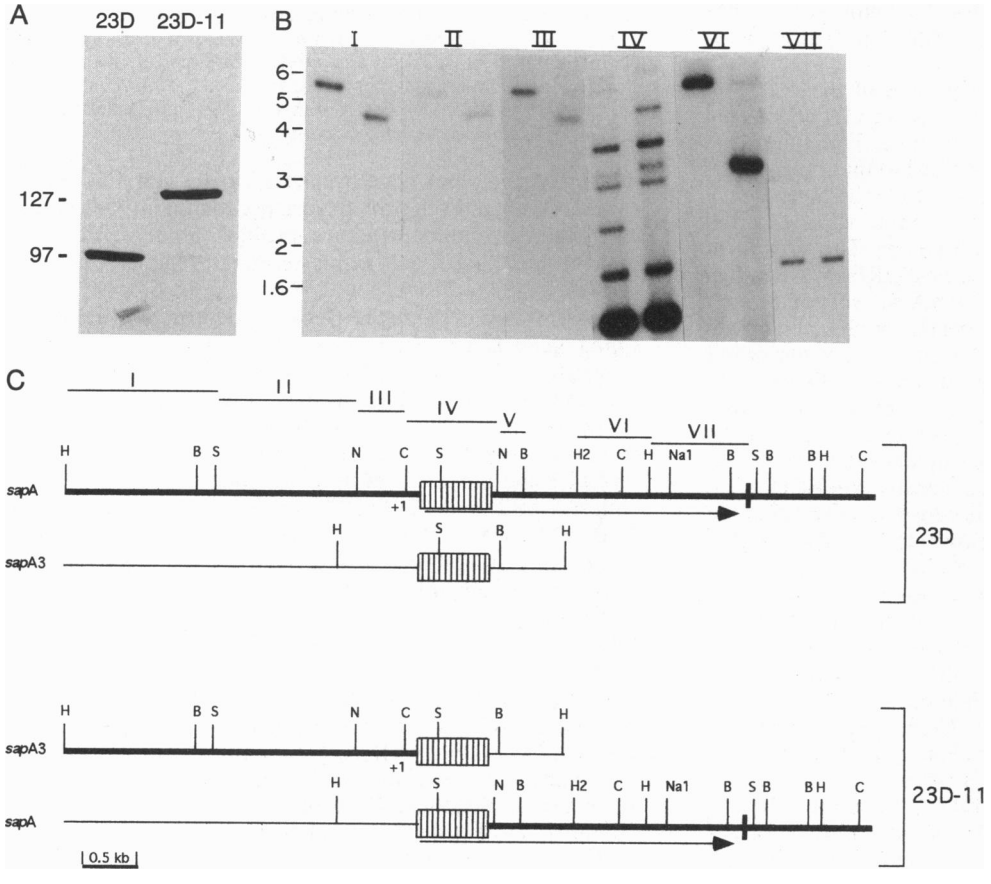

FIG. 6. Comparison of *C. fetus* strains 23D and 23D-11. (*A*) Immunoblot using monoclonal antibody 2H11. (*B*) Southern hybridizations of chromosomal DNA from the two strains (left lanes are strain 23D, and right lanes are strain 23D-11) digested with *Hin*dIII and hybridized with probes I–VII derived from the *sapA* locus from strain 23D. (*C*) Physical map of *sapA* and putative *sapA3* loci in strains 23D and 23D-11, as revealed from Southern hybridizations of chromosomal DNA digested with *Hin*dIII (H), *Ssp* I (S), *Hin*cII (H2), *Cla* I (C), *Bgl* II (B), and *Nae* I (Na1) (see legend for Fig. 1).

gene; in *E. coli*, vector promoter sequences likely permit expression.

Nucleotide analysis of the pMT101 insert identified both the *sapA1* gene and the 5′ region of another gene (*sapA2*), each of which contains a highly conserved sequence of >600 bp beginning 74 bp upstream of the translational start site (Fig. 1). This observation is not altogether unexpected because several S-layer proteins from *C. fetus* strains with type A LPS contain identical amino-terminal amino acid sequences (7). However, although subsequent regions of the *sapA1* and *sapA* ORFs are quite dissimilar, another identical region is located immediately downstream of potential transcriptional terminators. These observations and the identification of a putative RecBCD recognition (Chi) site (24) support the hypothesis that site-specific recombination may be a mechanism that *C. fetus* uses for S-layer protein antigenic variation. The 5′ homology does not include the *sapA* promoter site described previously (15), suggesting that the recombinational events observed occur immediately downstream of an active promoter; this is an efficient arrangement for expression of variant proteins.

DNA rearrangements in prokaryotes may be responsible for alterations in gene expression (1, 4, 27–32). The mechanism of gene activation by recombination of silent genes into an expression site is known in eukaryotes (1). The Chi site is known to terminate DNA unwinding by RecBCD (33), suggesting that for *sapA* the promoter region may be unchanged in the expression locus; only the actual gene copies might be involved in exchange.

The hybridization data we present indicate that the phenotypic change in S-layer protein expression from strain 23D to 23D-11 was accompanied by chromosomal rearrangement with site-specific reciprocal recombination originating within the conserved region of two *sapA* homologs. As shown by the Southern analysis, the other silent homologs were unaffected by this switch. In *E. coli*, RecBCD-mediated recombination occurs just downstream of the Chi site (33). The location of the putative Chi site near the 5′ end of the conserved *sapA* region is consistent with this mechanism. Strain 23D-11 shows a small amount of the 97-kDa protein, similar to observations of other *C. fetus* strains (7, 12). This result may reflect high-frequency rearrangement, creating mixed populations or low-level expression from alternative loci.

The downstream homology, 3′ to each ORF and its putative transcriptional terminator, suggests that recombination could involve the entire variable region and may not affect unrelated downstream genes. This hypothesis is consistent with RNA analysis of sapA mRNA, indicating that the transcript is of sufficient length for only a single *sapA* gene (15). However, Southern analysis (Fig. 6*B*) indicates that the downstream region is continuous with the divergent region of the *sapA* homologs even after rearrangement. Thus, we have not yet defined a functional role for this downstream homologous sequence.

The *sapA1* and *sapA2* gene products do not contain cleaved signal sequences, indicating that the absence of a leader sequence described previously for sapA (14) reflects a common theme. The identities observed at the amino termini of the sapA, sapA1, and sapA2 products may reflect involvement of this region in important conserved functions such as specific LPS binding, binding of divalent cations, or complement resistance. The sapA1 product expressed in *E. coli* was recognized by polyclonal antisera directed against *C. fetus* S-layer proteins but was recognized by only one of four monoclonal antibodies, whereas the sapA product was recognized by all four monoclonal antibodies (data not shown). This antigenic difference is consistent with the differences in both primary amino acid and deduced secondary structures of the *sapA* and *sapA1* gene products and supports the hypothesis that the function of the diverged region is the expression of antigenic variability.

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