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A Lipopolysaccharide-Binding Domain of the *Campylobacter fetus* S-Layer Protein Resides within the Conserved N Terminus of a Family of Silent and Divergent Homologs

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Campylobacter fetus **cells can produce multiple S-layer proteins ranging from 97 to 149 kDa, with a single form predominating in cultured cells. We have cloned, sequenced, and expressed in *Escherichia coli* a *sapA* homolog, *sapA2*, which encodes a full-length 1,109-amino-acid (112-kDa) S-layer protein. Comparison with the two previously cloned *sapA* homologs has demonstrated two regions of identity, approximately 70 bp before the open reading frame (ORF) and proceeding 550 bp into the ORF and immediately downstream of the ORF. The entire genome contains eight copies of each of these conserved regions. Southern analyses has demonstrated that *sapA2* existed as a complete copy within the genome in all strains examined, although Northern (RNA) analysis has demonstrated that *sapA2* was not expressed in the *C. fetus* strain from which it was cloned. Further Southern analyses revealed increasing *sapA* diversity as probes increasingly 3′ within the ORF were used. Pulsed-field gel electrophoresis and then Southern blotting with the conserved N-terminal region of the *sapA* homologs as a probe showed that these genes were tightly clustered on the chromosome. Deletion mutagenesis revealed that the S-layer protein bound serospecifically to the *C. fetus* lipopolysaccharide via its conserved N-terminal region. These data indicated that the S-layer proteins shared functional activity in the conserved N terminus but diverged in a semiconservative manner for the remainder of the molecule. Variation in S-layer protein expression may involve rearrangement of complete gene copies from a single large locus containing multiple *sapA* homologs.**

Campylobacter fetus is a spiral, microaerophilic gram-negative bacterium that causes infertility and infectious abortion in sheep and cattle and extraintestinal infections in humans (16, 23, 43, 45). There are two serotypes of *C. fetus* (A and B), a property based on their lipopolysaccharide (LPS) composition (34). As with many other bacterial species (2), *C. fetus* possesses an outermost crystalline surface layer of regular closely packed protein subunits (S-layer proteins). The S-layer proteins of *C. fetus* have been shown to be critical in resistance to host immune defenses (5–7, 32, 51). *C. fetus* strains possessing S-layer proteins (S^+) but not spontaneous mutants or chemically treated strains lacking S-layers (S^-) are resistant to C3b binding and phagocytosis by polymorphonuclear leukocytes (5, 7).

The *C. fetus* S-layer proteins must share relatively conserved features, as dictated by requirements for crystalline structure and binding to divalent cations (19, 22, 53) and as demonstrated by amino-terminal sequencing (33) and cross-reactivity with polyclonal antisera (6, 18, 49). In addition, S-layer proteins bind exclusively to the LPS of homologous (type A or B) cells, which is a property that is localized to the N-terminal half of the molecule (53). Cloning of two genes (*sapA* and *sapA1*) encoding 97-kDa forms of S-layer proteins has demonstrated that the S-layer proteins share identical amino termini extending for 219 amino acid residues but diverge for the remainder of the molecule (4, 47).

A characteristic of the *C. fetus* surface layer is that a single bacterial strain may produce one of three different S-layer proteins ranging from 97 to 149 kDa, with one form predominating in mixed cultures (8, 18, 22, 33). *C. fetus* strains may shift the size of the S-layer protein being produced, resulting in a shift in antigenicity and possible evasion of host immune defenses (8, 15, 18, 19, 47, 49, 50). However, the mechanisms by which such changes occur are unknown. Southern blot analysis using the conserved amino-terminal coding region as a probe demonstrates that multiple (8) *sapA* homologs exist within the *C. fetus* genome (46); however, it is unknown whether size variation involves reassortment of complete (silent) gene copies or assemblage of partial coding gene segments (47).

To address this question, we cloned a gene from strain 82-40 LP3, which expresses an S-layer protein with an apparent molecular mass of 127 kDa, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Analysis of this gene provided information as to whether larger S-layer proteins are due to longer open reading frames (ORFs) or reflect posttranslational modification. Further analyses pertaining to the organization of the *sapA* homologs, the conservation of *sapA* homolog sequences, and the domains involved in specific LPS binding by the gene product also were performed.

MATERIALS AND METHODS

Bacterial strains and culture condition. Six *C. fetus* subspecies *fetus* strains, of either S^+ or S^- phenotype and LPS serotype A or B, were used in this study (Table 1). *C. fetus* 23D was isolated from the vagina of a cow in New York before 1970; *C. fetus* 82-40 LP was isolated from the blood of a human in Pittsburgh, Pa., in 1981. Stock cultures were kept at −70°C in brucella broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 15% glycerol. For study, cultures were grown in a microaerobic atmosphere (5% oxygen, 10% carbon dioxide, 85%

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TABLE 1. *C. fetus* subspecies *fetus* strains used in this study

Strain	Origin	Predominate S-layer protein[a] (kDa)	LPS type[b]
84-32 (23D)	Wild type	97	A
84-54 (23B)	Spontaneous mutant	—[c]	A
82-40 LP	Wild type	97	A
82-40 HP	Spontaneous mutant	—	A
82-40 LP3	Spontaneous mutant	97, 127	A
83-88	Wild type	—	B

[a] Major surface array protein expressed by each strain.
[b] LPS types as previously described (34).
[c] —, no S-layer protein expressed.

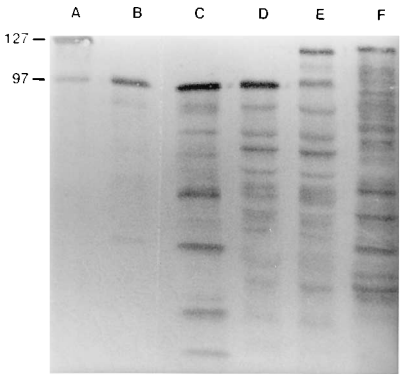


FIG. 1. Immunoblot analysis of native and recombinant *C. fetus* S-layer proteins with polyclonal antiserum to the 97-kDa S-layer protein from strain 82-40 LP. Lanes: A, whole-cell lysate of *C. fetus* 82-40 LP3; B, whole-cell lysate of *C. fetus* 23D; C to F, whole-cell lysates from *E. coli* recombinant clones pMJ110, pMJ100, pMJ2, and pMJ1, respectively. Molecular masses (in kilodaltons) refer to the major S-layer proteins of *C. fetus.*

nitrogen) at 37°C on brucella agar plates as described previously (33). The *Escherichia coli* strains that were used in this study, including DH5α and XL1-blue (Stratagene, La Jolla, Calif.), were grown in LB medium or on L plates (39).

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

Genetic techniques and DNA sequencing analysis. Chromosomal DNA was prepared as described previously (41). Plasmids were isolated by the procedure described by Birnboim and Doly (3). All other standard molecular genetic techniques were used, as described elsewhere (39). To sequence plasmid pMJ1, which harbors the complete ORF for *sapA2*, a series of 200-bp exonuclease III nested deletions were created from either direction of the insert using the Erase-a-base system (Promega, Madison, Wis.). The nucleotide sequence was determined unambiguously on both strands by using these nested deletions as double-stranded DNA templates and the dideoxy chain termination procedure as described previously (40). Primers common to the vector sequence flanking the insert and additional primers required to complete the sequencing were synthesized at the Vanderbilt University DNA Core Facility with a Milligen 7500 DNA synthesizer. Nucleotide sequences were compiled and analyzed with the aid of the DNA Strider program (CEA, Gif-Sur-Yvette, France).

Amino acid sequence analysis. Predicted primary amino acid sequences were compared by using the Genetics Computer Group sequence analysis programs (University of Wisconsin, Madison, Wis.) with the Gap algorithm (31), with a gap weight of 3 and a length weight of 1.

Construction of a *C. fetus* genomic library. Partial *Alu*I digests of chromosomal DNA from *C. fetus* 82-40 LP3 containing fragments of 3 to 10 kb were ligated to *Eco*RI linkers, digested with *Eco*RI, and ligated into pBluescript harbored within the vector λZapII (Stratagene). Packaged material was plated onto XL1-blue and yielded 9×10^5 PFU; 99% of the plaques were white on plates containing X-Gal and IPTG, indicating insertional inactivation of the vector β-galactosidase by foreign DNA fragments. The library was amplified on XL1-blue to obtain a higher titer stock (10^9 PFU/ml).

Detection and purification of recombinant clones. The amplified λZapII bank was screened for S-layer protein expression with an absorbed high-titer rabbit antiserum to the purified 97-kDa S-layer protein from *C. fetus* 82-40 LP (33). More than 10 signals per 1,000 plaques were detected. After 20 plaques were repicked and reprobed, 15 recombinants were selected, and single isolated plaques from each were picked and amplified for further study. The plasmids harbored within these phagemids were excised in vivo as detailed by the manufacturer (Stratagene).

Characterization of the recombinant proteins. To characterize further these clones, XL1-blue cells infected with the recombinant phagemid were induced with IPTG, and the lysates were recovered. The lysates were analyzed by SDS-PAGE, as described elsewhere (25). Immunoblotting was performed as detailed elsewhere (32) by using a 1:5,000 dilution of antiserum to the 97-kDa S-layer protein from *C. fetus* 82-40 LP (33) and goat anti-rabbit immunoglobulin alkaline phosphatase conjugate as the second antibody. The plasmids harbored within the λZapII phagemid that encoded S-layer proteins of 97 kDa or larger were excised in vivo, as described by the manufacturer, and were used for further study.

Southern hybridizations. *C. fetus* chromosomal DNA was digested with *Hae*III, electrophoresed, transfered to nylon membranes, and hybridized exactly as described elsewhere (46). The probes were gel-purified DNA fragments from pMJ1 or PCR products and were radiolabeled by primer extension with random hexameric oligonucleotides (21). The filters were washed successively three times for 30 min each in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× SSC, and 0.5× SSC at 65°C and were exposed to XAR-2 X-ray film.

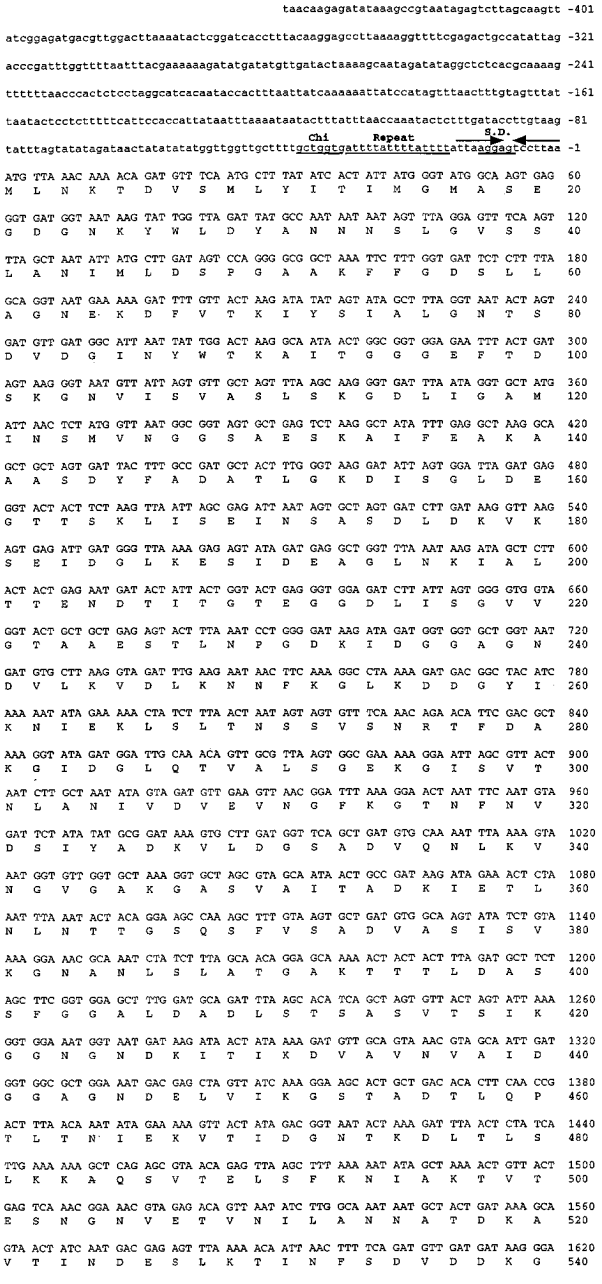
RNA isolation and Northern (RNA) hybridization analysis. For RNA isolation, *C. fetus* strains were grown overnight on brucella plates, cells were harvested, and RNA was recovered by one round of hot phenol extraction as described previously (1). The aqueous phase was reextracted once with phenol-chloroform that had been saturated with 0.02 M sodium acetate (pH 5.2) and twice with chloroform, and then the RNA was precipitated in 3 volumes of absolute ethanol at −70°C for 30 min. For Northern hybridization, RNA samples (10 μg) were subjected to electrophoresis on formaldehyde-agarose gels and then transferred to nylon membranes (39). Northern blots then were probed in the presence of 50% formamide (39).

PFGE. Overnight cultures of *C. fetus* 23D, 23B, 82-40 LP, 82-40 HP, and 82-40 LP3 were embedded in agar, lysed, and stored in 0.05 M EDTA at 4°C as described elsewhere (38). DNA was digested with *Sal*I, *Sac*II, *Mlu*I, and *Bgl*I also as described elsewhere (38). Bacteriophage λ (48.5-kb) concatemers were used as size standards. The contour-clamped homogeneous electric field (CHEF) system (Bio-Rad, Melville, N.Y.) of pulsed-field gel electrophoresis (PFGE) was used to separate DNA fragments in 1% agarose gels. The gels were subjected to electrophoresis for 24 h at 8°C at 175 V with pulse times of 10 to 25 s and for a further 3 h at 175 V with a pulse time of 0.5 to 1.0 s or for 20 h with a pulse time of 1 to 12 s in 0.05 M Tris-borate-EDTA buffer. After electrophoresis, the gels were stained with ethidium bromide, and the DNA fragments were visualized with a UV transilluminator to verify proper digestion. The gels then were transferred to nylon membranes and Southern hybridization was performed as described above.

In vitro reattachment of recombinant S-layer protein to the *C. fetus* cell surface. The *C. fetus* S$^-$ strains, 83-88 (serotype B) and 23B (serotype A), were used as templates for reattachment of recombinant proteins. Cells were harvested from plate culture and washed twice with C buffer (1 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.5], 20 mM $CaCl_2$), pelleted, and stored at −20°C until later use. Recombinant *C. fetus* protein products were recovered from sonicated recombinant *E. coli* cultures suspended in C buffer and centrifuged to remove cellular membranes. Protein quantities were determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.). Approximately 10 μg of lysate from recombinant *E. coli* strains was then mixed with 100 μg of *C. fetus* cells (~2 μl per cell pellet) in 30 μl of C buffer, and the mixture was incubated for 1 h at 37°C. Following incubation, cells were washed free of unbound recombinant S-layer protein by washing and pelleting successively in 1 ml of C buffer. Reattachment then was assessed by immunoblot analysis with anti-97-kDa protein following SDS-PAGE, as previously described (53).

RESULTS

Cloning of *C. fetus sapA2* homolog. Our initial goal was to determine whether the genes encoding S-layer proteins larger than 97 kDa exist as complete copies within the genome or whether they are constructed by assembly of partial gene cassettes similar to that seen in other pathogenic organisms (9, 17, 29, 42, 52). For this purpose, we constructed a λZapII library containing *Alu*I-digested chromosomal fragments from strain 82-40 LP3, a mixed culture which predominantly expresses a 127-kDa S-layer protein (Table 1). The library was screened with immune serum to the 97-kDa S-layer protein (33), and



immunoreactive plaques were purified and screened for the size of the expressed protein by immunoblotting. Four clones expressed immunoreactive proteins of ≥97 kDa, and a plasmid excised from one of these, pMJ1, expressed a 127-kDa form in *E. coli*, which is referred to as the *sapA2* product (Fig. 1).

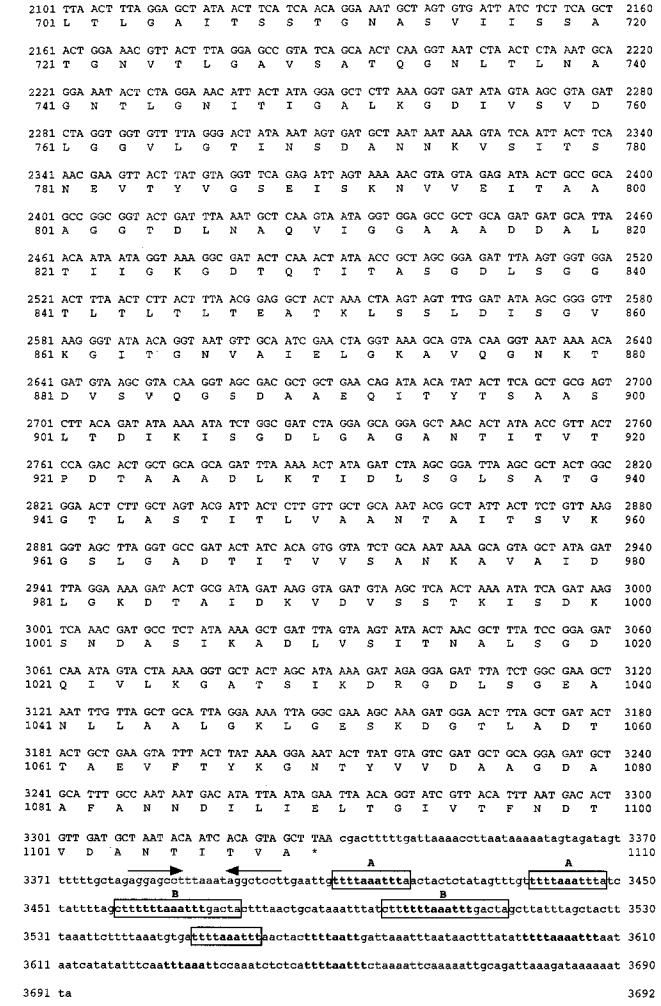


FIG. 2. The nucleotide sequence of pMJ1, including the deduced amino acid sequence of *sapA2*. The DNA sequence was determined in both directions as described in Materials and Methods. Nucleotides are numbered from the first letter of the initiation codon for the *sapA2* gene. The asterisk indicates the stop codon. Putative Shine-Delgarno (SD) and chi sequences (−13 to −9 and −38 to −31, respectively) upstream of the initiator methionine, as well as the pentameric repeat (ATTTT; −31 to −17), are indicated. Converging arrows indicate inverted repeat sequences ending at the ATG initiation codon and 3′ to the ORF. The DNA sequence beginning at base 3336 may form a dual stem structure ($\Delta G = -19.4$). A 10-bp TTTTAAATTT motif is repeated five times within the downstream noncoding region (boldface in boxes), as are nearly identical sequences (boldface only). Repeat pairs (A and B) show additional flanking sequence similarity or identity.

Nucleotide sequence of *sapA2* and comparison of *sapA* homologs. Nested deletions using exonuclease III were made in both directions of pMJ1 relative to the insert, and the insert was sequenced on both strands using common vector primers. Figure 2 shows the 4,132-nucleotide and deduced amino acid sequence of *sapA2* and its flanking regions. Only a single ORF greater than 500 bp was found in any of the six possible frames. This 3,327-bp ORF encoded a polypeptide of 1,109 amino acids with a predicted molecular mass of 111.8 kDa and an apparent molecular mass of 127 kDa, as estimated by SDS-PAGE (Fig. 1). Searches of the protein and gene banks failed to reveal any similarity to other known proteins except other S-layer proteins of *C. fetus* (4, 47). Sequence data (Fig. 2) revealed that the region upstream of the *sapA2* ORF which was

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2943  TTTAGACCAAA-CCTTAATAAA--TAGTAGATAGTTTTTT--GCTAAGAGGAGC
      :::::::::: :::::::::  ::::::::::::::   ::::: ::::::
2846  TTTAGACCAAA-CCTTAATAAA--TAGTAGATAGTTTTTTTTGCTAAAAGGAGC
      ::: ::  ::: ::::::::::  ::::::::::::::  ::: : ::::::
3336  TTTTGATTAAAACCTTAATAAAAATAGTAGATAGTTTTTT--GCT-AGAGGAGC

      TAGGAGAACTA----GCTCCTTG----------AATA--TTTTTAAATTTATT      sapA
      : : . :       :::::::            ::::  ::::::::::::::
      TTTGCATATAGCATGGCTCCTTAATTTTCCAACCAATACCTTTTTAAATTTAAT      sapA1
       ::  : :::    ::::::::            :::   ::::::::::::
      CTTTAA-ATA----GGCTCCTTG----------AATTG--TTTTAAATTTAAC      sapA2
```

FIG. 3. Comparison of *sapA*, *sapA1*, and *sapA2* downstream nucleotide sequences. Converging arrows indicate inverted repeat sequences within a region of strong homology. Colons indicate identical nucleotides. Numbers represent nucleotide positions in relation to the translation initiation codon (ATG) for *sapA* homologs. The boldface indicates a 10-bp repeat as in Fig. 2.

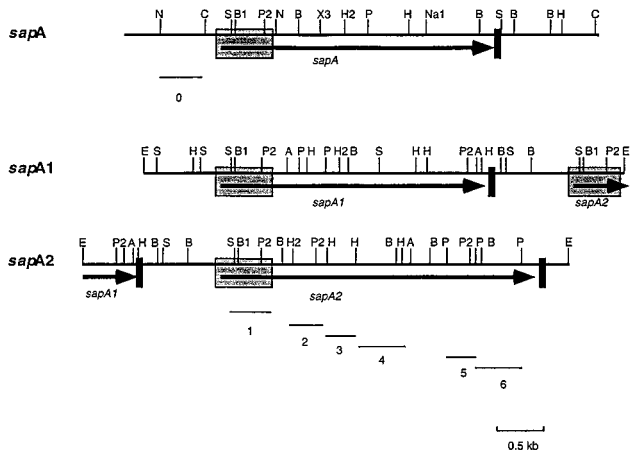

FIG. 4. Physical map comparisons of regions surrounding *sapA* homologs. Mapping was performed in pBG1 and pBG101 for *sapA*, pMT101 for *sapA1*, and pMJ1 for *sapA2*. Arrows represent the locations of genes and directions of transcription. Hatched boxes indicate the upstream and N terminus-encoding conserved regions. The smaller black boxes indicate homologous regions downstream of each ORF as indicated in Fig. 3. The locations of DNA probes used for Southern and Northern hybridizations are indicated by the numbers 0 to 6. 0, 0.5-kb *Cla*I-*Nde*I fragment of the *sapA* promoter region; 1, 0.42-kb PCR fragment of conserved origin; 2, 0.36-kb PCR fragment proximal to the N terminus; 3, 0.35-kb *Hin*dIII fragment of the middle region; 4, 0.46 *Hin*dIII fragment of the middle region; 5, 0.33-kb *Pst*I fragment of the C-terminal region; 6, 0.46-kb *Pst*I fragment of the C-terminal region. Restriction endonuclease cleavage sites: A, *Acc*I; B, *Bgl*II; B1, *Bst*nI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; H2, *Hin*cII; N, *Nde*I; Na1, *Nal*I; P, *Pst*I; P2, *Pvu*II; S, *Ssp*I; X3, *Xma*III.

cloned from *C. fetus* 82-40 LP3 was identical to the partial sequence located downstream of *sapA1* which was cloned from the unrelated *C. fetus* strain 23B (47); this is consistent with the plasmid restriction maps (see Fig. 4). These findings suggested that *sapA1* and *sapA2* exist in tandem on the chromosome and that this property is highly conserved among *C. fetus* strains. Comparison with the two previously cloned *sapA* homologs demonstrated two regions of identity. The first began 74 bp before the ORF, proceeding 552 bp into the ORF. Within this shared region, a sequence (GCTGGTGA) sharing seven of eight bases with the *E. coli* RecBCD (Chi) recognition site (GCTGGTGG) (12, 13) was found upstream of the ATG start codon and was followed by three pentameric (ATTTT) repeats and a potential ribosome-binding site (AGGAG; Fig. 2). A perfect 7-bp palindrome with a 1-bp spacer ending exactly at the ATG initiation codon and encompassing the ribosome-binding site also was present (Fig. 2). The second region of identity (Fig. 3) began 6 nucleotides from the *sapA2* translation stop codon and extended for 51 bases, with subsequent brief areas of homology over the next 50 bases. A putative transcriptional termination signal with a ΔG of -19.4 also was observed. Within the downstream noncoding region, a 10-bp motif (TTTTAAATTT) was repeated five times, and nearly identical motifs also were present (Fig. 2). The first repeat commenced at the 3′ end of the 50- to 60-bp conserved region shared among all *sapA* homologs (Fig. 3). From there it was repeated a variable number of times downstream of each *sapA* homolog (not shown). Sequence flanking the repeat also may have been duplicated (Fig. 2). Within the downstream conserved region, an 8-bp palindromic sequence similar to that found immediately upstream of the initiation codon was observed (Fig. 2 and 3). The presence of these homologous sequences at both the 5′ ends of these *sapA* homologs and 3′ to them suggested that these regions might play a role in homologous recombination, as previously reported (47).

Sequence analysis does not demonstrate the presence of a typical σ^{70}-like promoter (utilized by at least one S-layer protein expressed in *C. fetus* [46]) or any other known promoter upstream of *sapA2* (Fig. 2). Northern blot analysis using a unique *sapA2*-specific 0.46-kb *Pst*I fragment as probe (probe 6 [Fig. 4]) indicated that *sapA2* was not expressed in strain 82-40 LP3, from which it was cloned (data not shown). However, for the laboratory-passed strain 23D, which represents a mixed culture expressing both 97- and 127-kDa S-layer proteins, and a laboratory variant created in a manner similar to that described elsewhere (47), the *sapA2*-specific probe hybridized to 3.3-kb messages in both (data not shown). Thus, *sapA2* can in fact be expressed by *C. fetus*. These results indicated that, consistent with the lack of an upstream promoter-like sequence, *sapA2* represents a silent gene in the strain (82-40 LP3) from which it was cloned but is actively expressed in other strains producing 127-kDa S-layer proteins.

Comparison of predicted primary amino acid sequences. Comparison of *sapA2* with *sapA* or *sapA1* demonstrated an identical N-terminal 184-amino-acid sequence (Fig. 4). For the remainder of the ORF, *sapA2* shared 28% identity (47.7% similarity) and 26% identity (48.8% similarity) with *sapA* and *sapA1*, respectively. *sapA2* and *sapA* shared an identical span of eight residues (starting at position 234); no other significant regions of identity were found between *sapA2* and *sapA* or *sapA1*. In contrast, comparison between *sapA* and *sapA1* beyond residue 184 demonstrated a 45.4% identity (64.8% similarity) with extension of the N-terminal region of identity to residue 219 and five identical clusters of identity of six or more amino acids in the remainder of the molecules. Thus, *sapA* and *sapA1* were more closely related to each other than to *sapA2.*

PFGE. Given the tandem nature of the two *sapA* homologs, we sought to determine whether the family of *sapA* homologs (4, 46, 47) are clustered on the chromosome. When chromosomal DNAs from five *C. fetus* strains were digested with the infrequent cutter *Sal*I and resolved by PFGE (Fig. 5, left panel), the fragmentation pattern was nearly identical for all. Subsequent Southern blotting (Fig. 5, right panel) with the conserved 5′ region of the *sapA* homologs as a probe (probe 1 [Fig. 4]) demonstrated a limited hybridization pattern. Strain 23D showed a hybridizing band of 125 kb, whereas its spontaneous S^- mutant 23B showed a hybridizing fragment at 116 kb. Strain 82-40 LP and its two spontaneous variants, 82-40 HP and 82-40 LP3, all showed two hybridizing signals; a 54- and 81-kb band were seen for strains 82-40 LP and 82-40 LP3, whereas 82-40 HP possessed two hybridizing signals at 54 and 73 kb. Consistent findings also were found for *Sac*II, *Bgl*I, and *Mlu*I digests of *C. fetus* 23D; *Sac*II digestion demonstrated a unique hybridizing band of 93 kDa, *Bgl*I digestion demonstrated three hybridizing bands totalling less than 130 kb, and *Mlu*I digestion

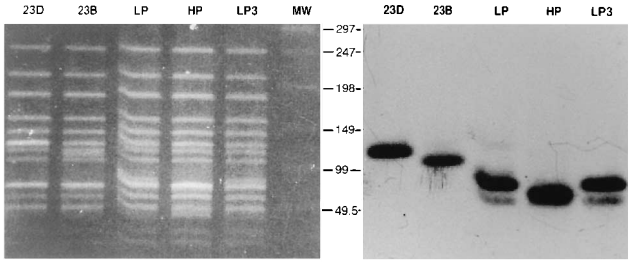

FIG. 5. PFGE and Southern hybridization using probe 1 of genomic DNA from *C. fetus* 23D, 23B, 82-40 LP, 82-40 HP, and 82-40 LP3. Genomic DNAs were digested overnight with 60 U of *Sal*I while embedded in an agar plug. Digested material was electrophoresed with a CHEF system for 24 h at 185 V and 8°C, with a ramped pulse time of 10 to 25 s in a 1% agarose gel. The gel was photographed (left panel) and transferred to nylon membrane for Southern hybridization with probe 1 (right panel). Molecular size (MW) markers are in kilobases.

demonstrated two hybridizing bands totalling less than 95 kb (data not shown). Because the hybridization patterns were limited to one or two restriction fragments totalling as little as 93 kb, these results indicated that the *sapA* homologs were clustered on fragments representing less than 8% of the chromosome (37). The spontaneous mutants 23B and 82-40 HP appeared to differ from their wild-type parents by deletion of approximately 8 to 9 kb within this region. Reblotting using the *sapA* promoter region (probe 0 [Fig. 4]) demonstrated a single *Sal*I-hybridizing band in S^+ strains at 125 kb in strain 23D and 81 kb in strains 82-40 LP and 82-40 LP3 but no band for 82-40 HP or 23B, as expected (46) (not shown).

Southern analysis with *sapA2* probes. To investigate the genomic organization of *sapA2* within the strain from which it was cloned (82-40 LP3) and within other *C. fetus* strains that produce smaller S-layer proteins, a series of Southern hybridizations were performed with probes 1 to 6 from pMJ1 (Fig. 4). As shown in Fig. 6, probes increasingly 3′ within the ORF hybridized to progressively fewer *Hae*III fragments. The N-terminally conserved probe (probe 1) hybrized to multiple (six) bands, some of which appeared as doublets, in all strains (Fig. 6A, left panel), although fewer hybridizing bands were resolved in the spontaneous S^- mutant strains 23B and 82-40 HP. Probe 2, which flanks the conserved N-terminal region at its 3′ end, hybridized strongly to three bands and weakly to one (23D) or two (LP, HP, and LP3) bands (Fig. 6A, middle band). A strongly hybridizing band of approximately 2.0 kb in the other four strains was absent in strain 23B (Fig. 6A and B). A highly conserved 4.3-kb *Hae*III fragment containing *sapA2* sequences was present, since probes 2 to 6 recognized it in all strains (Fig. 6A and B). Probe 3 from the middle region of *sapA2* hybridized to the *sapA2* 4.3-kb fragment as well as two homologs of 2.0 and 1.0 kb, with the exception of 23B (Fig. 6A, right panel). Only two hybridization signals at 4.3 and 2.0 kb were observed with probes 4 and 5 (Fig. 6B, middle and left panels). In all strains examined, probe 6 from the extreme C-terminus-encoding region of the *sapA2* ORF hybridized only to the 4.3-kb *sapA2* fragment (Fig. 6A and B). Consistent results were also observed for *Acc*I-, *Bgl*II-, and *Hin*dIII-digested genomic DNAs, since *sapA2* probes increasingly 3′ hybridized to progressively fewer fragments (data not shown). Taken together, these results demonstrated that *sapA2* existed as a complete copy within the genome of each of the *C. fetus* strains examined and that homology between *sapA2* and other *sapA* homologs diminished toward the 3′ end of the gene. These data also clearly demonstrated that the deletions observed in strains 23B and 82-40 LP were different.

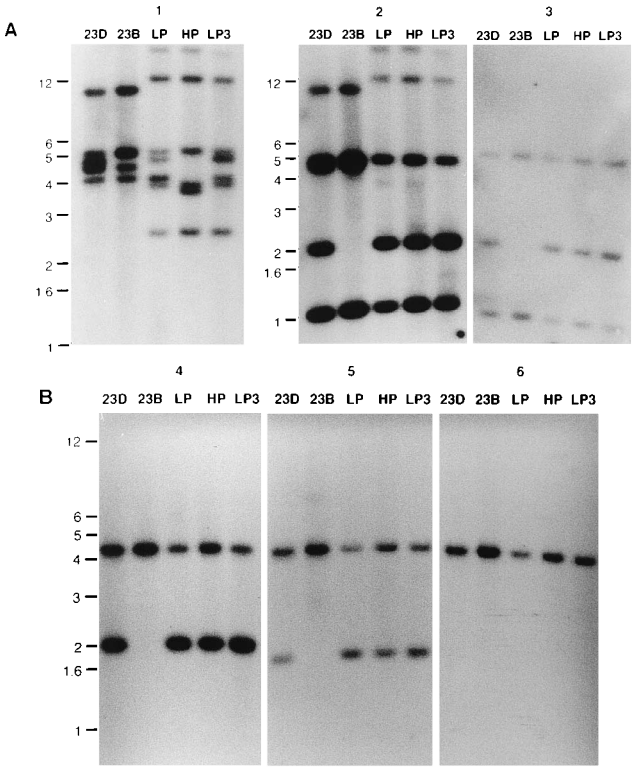

FIG. 6. Southern hybridization with six *sapA2* probes to genomic DNAs from *C. fetus* 23D, 23B, 82-40 LP, 82-40 HP, and 82-40 LP3. Chromosomal DNAs from these strains were digested with *Hae*III; the restriction fragments were separated an a 0.7% agarose gel, transferred to a nylon membrane, and hybridized with the ^{32}P-labeled *sapA2* probes indicated in Fig. 4 (probes 1 through 3 [A] and probes 4 through 6 [B]). The positions of molecular size markers (in kilobases) are indicated to the left of each panel.

Reattachment assays with truncated products. S-layer proteins of *C. fetus* exclusively adhere to the LPS layer in a serotype-specific manner (53). On the basis of recombinant proteins reattaching to the surface of S^- *C. fetus* mutants, the LPS-binding domain of the 97-kDa S-layer protein has been shown to be present within the N-terminal half of the molecule (53). Because the three type A S-layer proteins that have been examined have been deduced to share the same 184 N-terminal amino acids and then diverge, it was anticipated that this 5′ region encoded the type A binding domain. To verify this hypothesis, we performed reattachment assays using *E. coli*-expressed cloned full-length and truncated *sapA2* recombinant products, derived from exonuclease III-generated nested deletions. The full-length *sapA2* recombinant S-layer protein reattached to type A cells but not to type B cells (Fig. 7), as expected (53), demonstrating that the product of *sapA2*, hitherto unexpressed in *C. fetus*, possessed functional binding capacity that was specific for type A LPS. Both the 40 (C16-4)- and 23 (C17)-kDa deletion mutants also reattached to type A but not to type B S^- cells (Fig. 7). As determined by dideoxy DNA sequencing, the mutant C17 was truncated at amino acid residue 189. Thus, the conserved 189-amino-acid N-terminal peptide contained at least one of the LPS type-specific binding sites. More extensively truncated recombinant proteins were not sufficiently immunoreactive to be detectable by immunoblotting so as to narrow further the LPS-binding domain.

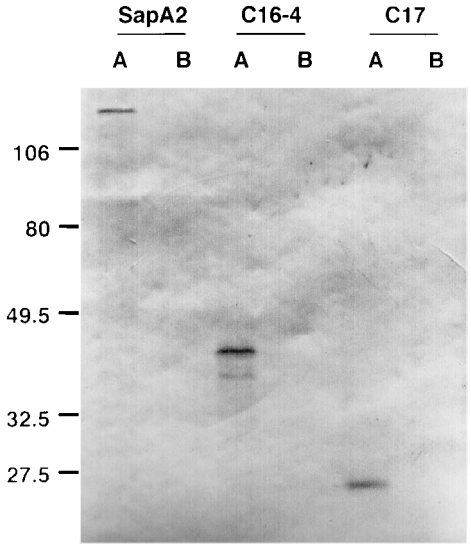

FIG. 7. Attachment of recombinant type A S-layer protein and truncated S-layer protein products from *E. coli* lysates to S^- *C. fetus* cells as shown by immunoblotting with rabbit anti-97-kDa S-layer protein from strain 82-40 LP. The following lysates are shown: lysates of *E. coli* pMJ1 producing full-size type A (127-kDa) S-layer protein (encoding SapA2) incubated with type A cells of S^- strain 23B (SapA2 lane A) or type B cells of strain S^- 83-88 (SapA2 lane B), lysates of *sapA2* truncated mutant C16-4 (40-kDa truncated S-layer protein) incubated with type A (23B) (C16-4 lane A) or type B (83-88) (C16-4 lane B) cells, and lysates of *sapA2* truncated mutant C17 (encoding only the first 189 amino acids) incubated with cells of type A strain 23B (C17 lane A) or type B strain 83-88 (C17 lane B). As shown, the recombinant type A S-layer proteins and fragments specifically reattach to the type A (lanes A) but not to the type B cells (lanes B).

DISCUSSION

These studies have broadened our understanding of the organization and expression of the genes encoding *C. fetus* S-layer proteins and have further characterized the functional domains of the proteins. First, there is the observation from our Southern hybridization studies that the cloned *sapA2* existed as a complete copy within the genome of all strains examined, including spontaneous mutants that lack S-layer protein expression. Heretofore, only the genes encoding 97-kDa forms of S-layer proteins had been cloned from *C. fetus*. The present observation supports the hypothesis that genes encoding S-layer proteins larger than 97 kDa include complete ORFs within the *C. fetus* genome and are not assemblages of partial gene cassettes as seen in other bacteria. Moreover, the conserved nature of *sapA2* and its homologs in the *C. fetus* genome, even among unrelated strains (Fig. 4 and 6), also indicated that these coding regions were stable. Southern hybridization of *Hae*III digests (Fig. 6) and *Hin*dIII and *Acc*I digests (data not shown) also indicated that for unrelated strains the flanking regions were at least partially conserved as well. Together, these data indicated that if rearrangements occur within the ORFs (47), a mechanism must be present to preserve the antecedent coding sequences and their relationship with their flanking regions.

Second, the Southern hybridization analyses have further detailed the extent of homology that the *sapA2* ORF shared with other *sapA* homologs in the *C. fetus* genome. Previous examination of two cloned *sapA* homologs showed that each shares a deduced N-terminal 219-amino-acid sequence (4, 47), which is consistent with experimentally derived sequence data (33) and serological studies (6, 18, 49). Beyond residues 219 and 184, the coding regions of *sapA* and *sapA1* and of *sapA2*, respectively, appeared to diverge completely from one another. However, detailed Southern analyses (Fig. 6) indicated that the divergence in the 3′ region is not as complete as previously assumed, since probes increasingly 5′ within the *sapA2* ORF (probes 2 to 6 [Fig. 4]) hybridized to progressively more fragments (Fig. 6). Similar results were also observed when *sapA*-specific probes were used (data not shown). From the present study, we have learned that all three cloned *sapA* homologs encoded the identical N-terminal 184 amino acids, that there was partial conservation beyond the conserved N terminus between *sapA* and *sapA1*, and that diversity increased toward the 3′ end of the gene for all of the homologs.

During infection of its native host, *C. fetus* establishes a transient bacteremia from which it seeds the biliary tract, resulting in long-term carriage (10, 11, 14). Variation in S-layer protein expression may allow the organism to evade both systemic and mucosal humoral immunity (49). Conservation of N-terminal residues and variation in surface-exposed C-terminal epitopes is a mechanism by which organisms can maintain functional protein domains while evading the host immune response. The Southern hybridization data for the *C. fetus sapA* homologs were consistent with this hypothesis, since diversity increased toward the 3′ end of the ORF, and the reattachment studies with recombinant S-layer proteins clearly demonstrated that the specificity of binding to LPS (53) was determined by the conserved N terminus. Our results do not rule out the possibility of further LPS-binding domains beyond this region. Although divalent cations are necessary for binding (53), the N-terminal sequence does not possess a consensus divalent cation-binding site (24). These results suggest that divalent cations may function to neutralize the charge of the acidic S-layer proteins, help with steric organization of the molecules, or serve as an electrostatic bridge between the molecule and the cell surface, as reported for *Caulobacter* S-layers (24, 48).

Northern hybridization results indicated that *sapA2* was not expressed in the strain from which it was cloned and are consistent with the absence of a typical σ^{70} promoter necessary for expression of at least two other related genes, *sapA* (46) and *sapB* (unpublished data). However, *sapA2* was expressed in other strains, and PCRs using a forward promoter-specific primer and a reverse *sapA2*-specific primer demonstrated that *sapA2* is in continuity with the *sapA* promoter in these later strains (data not shown). No upstream consensus promoter was found for *sapA1* (47), which was also unexpressed (unpublished results). Our previous studies suggest that the *sapA* homologs rearrange via reciprocal recombination (47). Together with the present results, these data suggest that variation of *C. fetus* S-layer proteins involves rearrangement of silent gene cassettes into a unique (*sapA*) expression locus. The promoter upstream of *sapA* (46) is the only such locus that has been identified in type A strains, and loss of this promoter in spontaneous mutants is associated with loss of transcription and S-layer protein expression (46). A unique promoter responsible for expression of multiple silent gene homologs has been identified in other microbes undergoing antigenic variation (9, 29, 35, 36, 42). Experiments are currently under way to determine whether *C. fetus* possesses more than one promoter permitting S-layer protein expression.

Gene rearrangement may be facilitated by the proximity of the *sapA* homologs to one another on the chromosome, as indicated by both PFGE (Fig. 5) and sequence analysis (Fig. 2 and 4). In addition, the spatial relationships of the *sapA* homologs were at least partially conserved among unrelated strains. On the basis of both restriction digestion and sequencing data for pMT101 and pJM1, *sapA1* and *sapA2* existed in

tandem (Fig. 4) in two unrelated *C. fetus* strains, 23B and 82-40 LP3. Moreover, the Southern hybridization patterns of the *sapA2* homologs among unrelated strains were highly conserved when *Hae*III (Fig. 6), *Hin*dIII, and *Acc*I (data not shown), which digest at sites both within and flanking the *sapA2* ORF, were used. These findings suggest that each *sapA2* homolog may have been located in the same genomic position even among unrelated strains. Despite reciprocal recombinatory events, such conservation may be explained if recombination proceeds in a vectorial fashion similar to that seen in trypanosomes (20, 36). During a vectorial process, recombination proceeds in a sequential manner, in that events occur either with respect to spatial constraints, e.g., proximal to distal genetic elements, or are constrained by the extent of homology, e.g., most to least homologous. In such a model, the organism may preserve the spatial relationship of the homologs during multiple rounds of reciprocal exchange. Whether this accounts for our observations remains to be determined. The function of the 10-bp repeat motif seen exclusively 3′ to the ORF of the *sapA* homologs is uncertain. It may represent a targeting signal for recombination, since it was present within the 3′ conserved box shared among all *sapA* homologs, or it may simply represent a region of duplication from past recombinatorial events. The palindromic sequences found within both conserved upstream and downstream sequences may also be involved in recombination events.

Although they are continuously undergoing recombinatorial events on the order of 10^{-2} to 10^{-3} per generation (8), the S-layer-encoding genes of *C. fetus* are remarkably stable. Several lines of evidence support this view. In all strains examined, *sapA*, *sapA1*, and *sapA2* are conserved in the genome (Fig. 6 and data not shown), as are the homologs that hybridized with the *sapA2*-specific probes (Fig. 6); in the unrelated strains 23B and 82-40 LP3, both *sapA1* and *sapA2* exist in the same spatial relationship to one another (Fig. 4), with identical noncoding sequence between them. A stable genotype in spite of frequent recombinatorial events could be due to a mechanism involving duplicative transfer of genetic information from a silent locus to the expression site, thereby preserving the original silent genetic elements. Although previous experiments (47) involving in vitro antigenic variation demonstrate a mechanism of reciprocal recombination involving exchanges near the N terminus, these mechanisms need not be exclusive.

The stability of the coding region also suggests conservation of protein function, which has been clearly demonstrated for the N-terminal region. However, our Southern hybridization data also demonstrated that the remaining coding sequences of the *sapA* homologs were present among unrelated strains. This conservation further suggested that each homolog may possess some feature(s) necessary for function that was not universal among the homologs. Variation in S-layer expression may subserve functions other than altering surface epitopes, such as altering adherence properties similar to those seen for pili (26, 27, 30). Electron microscopy studies indicate that the tetragonal or hexagonal crystalline pattern of the *C. fetus* S-layer is dependent on the size of the expressed S-layer protein (22). Such crystalline pattern changes may alter the layer's physical properties in a manner which alters, for example, its potential molecular sieve functions (28, 44). Alternatively, each of the homologs may be conserved because this constellation of silent genes allows the most efficient intragenic recombination within the stringent constraints of protein export, LPS binding, and crystal formation under which *C. fetus* must operate.

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