# *Campylobacter fetus* Surface Layer Proteins Are Transported by a Type I Secretion System

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The virulence of *Campylobacter fetus*, a bacterial pathogen of ungulates and humans, is mediated in part by the presence of a paracrystalline surface layer (S-layer) that confers serum resistance. The subunits of the S-layer are S-layer proteins (SLPs) that are secreted in the absence of an N-terminal signal sequence and attach to either type A or B *C. fetus* lipopolysaccharide in a serospecific manner. Antigenic variation of multiple SLPs (encoded by *sapA* homologs) of type A strain 23D occurs by inversion of a promoter-containing DNA element flanked by two *sapA* homologs. Cloning and sequencing of the entire 6.2-kb invertible region from *C. fetus* 23D revealed a probable 5.6-kb operon of four overlapping genes (*sapCDEF*, with sizes of 1,035, 1,752, 1,284, and 1,302 bp, respectively) transcribed in the opposite direction from *sapA*. The four genes also were present in the invertible region of type B strain 84-107 and were virtually identical to their counterparts in the type A strain. Although SapC had no database homologies, SapD, SapE, and SapF had predicted amino acid homologies with type I protein secretion systems (typified by *Escherichia coli* HlyBD/TolC or *Erwinia chrysanthemi* PrtDEF) that utilize C-terminal secretion signals to mediate the secretion of hemolysins, leukotoxins, or proteases from other bacterial species. Analysis of the C termini of four *C. fetus* SLPs revealed conserved structures that are potential secretion signals. A *C. fetus sapD* mutant neither produced nor secreted SLPs. *E. coli* expressing *C. fetus sapA* and *sapCDEF* secreted SapA, indicating that the *sapCDEF* genes are sufficient for SLP secretion. *C. fetus* SLPs therefore are transported to the cell surface by a type I secretion system.

*Campylobacter fetus* is a gram-negative pathogen that causes infertility and infectious abortion in sheep and cattle and extraintestinal infections in immunocompromised humans (35, 55). Similar to many bacteria (54), wild-type *C. fetus* has a paracrystalline surface layer (S-layer) composed of S-layer proteins (SLPs) (23, 25). SLPs are the most abundant proteins in *C. fetus*, constituting as much as 10 to 15% of the total cell protein (19, 50). The *C. fetus* S-layer inhibits binding of complement factor C3b and therefore results in resistance to phagocytosis and to complement-mediated killing by normal or immune serum (13). Mutants lacking the S-layer are significantly less virulent in animal models than are those expressing the S-layer (11, 49).

Two types of SLPs exist (A and B), based on their specific binding to serotype A or B lipopolysaccharide. However, within each of the types are a number of SLP variants that range in size from 97 to 149 kDa. In *C. fetus* 23D, SLPs are encoded by a family of eight *sapA* homologs (26). A single *C. fetus* cell has the ability to change the type of SLP that it expresses by the *recA*-dependent inversion of a DNA segment containing a unique outward-facing *sapA* promoter (22). The minimum invertible DNA segment is 6.2 kb in size and is flanked by *sapA* homologs, although larger and more complex inversions allow expression of alternate *sapA* homologs (24, 31).

The majority of bacterial SLPs have N-terminal signal sequences and are secreted via the type II (*sec*-dependent) secretion pathway (15). The SLPs encoded by *C. fetus* (SapA

homologs) and *Caulobacter crescentus* (RsaA) lack N-terminal signal sequences and therefore are probably secreted by a different mechanism (15). C terminally truncated versions of *C. fetus* and *C. crescentus* SLPs are not secreted, suggesting that the secretion signal lies in the C terminus of the protein (6, 8, 14). Furthermore, the C terminus of *C. crescentus* RsaA is sufficient to allow secretion of heterologous proteins from *C. crescentus*, and recent mutational studies on its C terminus have begun to delineate sequences important for RsaA secretion (7). SLPs lacking N-terminal signal sequences recently were identified in *Serratia marcescens* (38) and *Campylobacter rectus* (62).

The type I pathway uses C-terminal secretion signals on the targeted protein for secretion from gram-negative bacteria. Proteins secreted by this pathway include *Escherichia coli* α-hemolysin and other bacterial RTX toxins and proteases from *Erwinia chrysanthemi*, *S. marcescens*, and *Pseudomonas aeruginosa* (51, 61). The secretion apparatus is composed of three proteins homologous to HlyB, HlyD, and TolC of *E. coli* or PrtDEF of *E. chrysanthemi*. These three proteins form a transmembrane complex by which a C-terminal signal in the cognate protein is recognized, initiating its secretion from the cytoplasm directly into the extracellular medium. The HlyB homologs are cytoplasmic membrane proteins that are members of the ABC transporter family and are responsible for recognizing the C-terminal secretion signal on the protein to be transported (46, 61). HlyD-like proteins also localize to the cytoplasmic membrane but belong to the MFP (membrane fusion protein) family (18) and probably facilitate the direct interaction of the cytoplasmic and outer membranes during the secretion process. TolC homologs reside in the outer membrane and appear to form a pore as the outer component of the transport machinery (42). Recently, SLP-transporting type I

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TABLE 1. Strains and plasmids used in this study

| Strain or plasmid | Relevant genotype | Reference or source |
|---|---|---|
| *C. fetus* | | |
| 23D | *sapA*⁺ *sapCDEF*⁺ | 48 |
| 23B | Δ*sapAp* | 48 |
| 97-205 | 23D *sapD*::*aphA* | This study |
| 84-107 | *sapB*⁺ *sapCDEF* | 27 |
| *E. coli* | | |
| C600 | | 3 |
| DH5αMCR | | 37 |
| S17-1 | | 53 |
| Plasmids | | |
| pIR100 | *sapCDEF* in pAMP1 | This study |
| pBGYC1 | *sapA* in pACYC184 | This study |
| pIR13 | *sapD* in pBluescript | This study |
| pIR131 | *sapD*::*aphA* in pBluescript | This study |
| pILL570 | ReppBR322 *mob* Spc^r | 43 |
| pILL131 | pIR131 insert in pILL570 | This study |
| pILL600 | ReppBR322 *aphA* | 44 |

systems have been characterized in *C. crescentus* and *S. marcescens* (2, 38). In *C. crescentus*, the ABC (RsaD) and MFP (RsaE) components are encoded by DNA immediately downstream of the SLP structural gene (*rsaA*), while the outer membrane component has not been identified (2). The *S. marcescens* SLP (SlaA) is secreted by the LipBCD type I transporter and thus shares this pathway with the *S. marcescens* extracellular lipase, LipA (38).

To investigate whether the invertible region contains genes involved in the expression, antigenic variation, or secretion of *C. fetus* SLPs, we cloned and sequenced the invertible regions from type A strain 23D and type B strain 84-107. Since each DNA sequence predicted four genes (*sapCDEF*), three of which were homologous to genes encoding type I secretion proteins, we created a mutation in *sapD* and showed that this mutant did not produce or secrete SLPs. Coexpression of the *sapA* and *sapCDEF* genes in *E. coli* showed that the *sapCDEF* genes are sufficient to allow secretion of SapA from the bacterial cell.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *C. fetus* strains were grown at 37°C under microaerobic conditions in a GasPak jar using a CampyPak Plus gas generator (BBL Microbiology Systems, Cockeysville, Md.) on brucella agar (Difco Laboratories, Detroit, Mich.) containing antibiotics at the following concentrations: 7-U/ml polymyxin B, 10-μg/ml vancomycin, 10-μg/ml trimethoprim lactate, 15-μg/ml nalidixic acid (designated PVNT), and 40-μg/ml kanamycin (PVNTK) for kanamycin-resistant strains. Strains were also grown in brucella broth containing the above concentrations of PVNT under microaerobic conditions at 37°C. *E. coli* strains were grown on LB plates or broth (52) supplemented with trimethoprim lactate (10 μg/ml), kanamycin (40 μg/ml), tetracycline (15 μg/ml), or ampicillin (50 μg/ml) when appropriate.

**DNA and protein techniques.** Restriction enzymes, the Klenow fragment of *E. coli* DNA polymerase I, and T4 DNA ligase were used as suggested by the manufacturer, either New England Biolabs (Beverly, Mass.), or Promega (Madison, Wis.). The sequences of the invertible regions from strains 23D and 84-107 were obtained by primer walking or direct sequencing of PCR products by using an ABI 377 (PE Applied Biosystems, Foster City, Calif.) automated sequencer by the Vanderbilt University Cancer Center Core Laboratory, and oligonucleotides were synthesized by the Vanderbilt University Molecular Biology Core Laboratory. DNA sequence analysis was done by using the GCG sequence analysis programs (17). Database similarity searches were performed by using the BLAST algorithms maintained by the National Center for Biotechnology Information (Bethesda, Md.). Searches of the PROSITE and MotifDic libraries for protein motifs were done by using the MotifFinder e-mail server (motiffinder @genome.ad.jp). Parsimony analysis of protein sequences was performed by

using PAUP 3.1 (Smithsonian Institution, Washington, D.C.) with 1,000 bootstrap replicates.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting.** Whole-cell lysates and water extracts of strains 23D, 23B, and 97-205 were prepared by previously described methods (50), and protein concentrations were assayed by using the Pierce BCA Protein Reagent Assay (Pierce, Rockford, Ill.). Protein samples were analyzed by sodium dodecyl sulfate–10%-polyacrylamide gel electrophoresis, and SLPs were detected by immunoblotting (1:10,000 dilution) by using polyclonal rabbit serum against *C. fetus* SLPs, generated by Cocalico Biologicals (Reamstown, Pa.). The secondary antibody (1:2,000 dilution) was goat anti-rabbit immunoglobulin G-2 alkaline phosphatase (Boehringer Mannheim, Indianapolis, Ind.).

**Cloning of the invertible region.** Clones representing the invertible region from type A strain 23D were isolated as follows. First, the entire invertible region was amplified by PCR by using primer 2754 (59), which binds in the conserved sequences at the 5′ end of each flanking *sapA* homolog (see Fig. 1). This 6.2-kb product was subcloned into pAMP1 (Gibco-BRL, Gaithersburg, Md.) to yield pIR100, and the pIR100 insert was used as a probe to identify genomic clones for DNA sequence analysis. Clones were isolated from a genomic library (26) constructed in λZAPII (Stratagene, La Jolla, Calif.) from random fragments resulting from partial *Alu*I digestion of chromosomal DNA from wild-type *C. fetus* 23D (48). Three λ clones (IR12, IR13, and IR15) hybridizing to the pIR100 insert were selected and characterized by restriction digestion, PCR, and DNA sequence analysis. A λ clone (IR20) covering a gap at the right end of the invertible region was identified by using as a probe a fragment released from pIR100 by *Nde*I digestion, corresponding to nucleotides 4715 to 5345 of the invertible region sequence. Plasmids (pIR12, pIR13, pIR15, and pIR20) derived from these purified λ clones were recovered by following the in vivo excision protocol recommended by the manufacturer (Stratagene). No clones were isolated for a region that represented a small gap between pIR13 and pIR12. We therefore amplified this region from *C. fetus* 23D chromosomal DNA by using primers A5598 (5′ TGTATCGTTTATGCTGCG; nucleotide positions 1677 to 1694) and C2968 (5′ CCGTCCGGAAGTCTTAGTATC; positions 2963 to 2983). The resulting 1,305-bp PCR product was cloned into pT7Blue (Novagen, Madison, Wis.), and the DNA sequences of three independent subclones were determined.

The sequence of the invertible region from type B strain 84-107 was compiled from a series of PCR products amplified by using primers derived from the sequence of the 23D invertible region. Automated direct DNA sequencing of each PCR product was performed on both strands as described above.

**Construction of a *C. fetus sapD* mutant.** Clone pIR13 was digested with *Bgl*II and ligated with the *aphA* (Km^r) cassette excised from pILL600 by *Bam*HI digestion, yielding pIR131. To create a mobilizable plasmid for conjugation, the pIR131 insert was released by *Bam*HI and *Acc*I digestion and ligated with *Cla*I/*Bam*HI-digested pILL570. The resulting plasmid (pILL131) was transformed into *E. coli* S17-1, which was mated with *C. fetus* 23D by previously described methods (14). Transconjugants were recovered after 3 days of growth on plates containing PVNTK.

**Southern hybridization.** Chromosomal DNAs from *C. fetus* 23D and 97-205 were prepared by using the cetyltrimethylammonium bromide method (1). The DNA was digested with *Nde*I, and 1 μg was electrophoresed on a 0.7% agarose gel and then transferred to a nylon membrane (MSI, Westborough, Mass.). The membrane was hybridized with DNA probes labeled by using the Renaissance nonradioactive chemiluminescence kit supplied by NEN Research Products (Boston, Mass.). The membrane was probed with either *Bam*HI-digested pIR13, *Bam*HI-digested pILL570, or the *aphA* cassette from *Sma*I-digested pILL600 and subsequently exposed on Biomax MR scientific imaging film from Eastman Kodak (Rochester, N.Y.) for 1 h.

**Primer extension analysis.** The levels of *sapA* mRNA in wild-type and *sapD* mutant *C. fetus* were determined by primer extension analysis as described previously (30), with the following modifications. Total cellular RNA was prepared from *C. fetus* 23D (wild type) or 97-205 (*sapD* mutant) grown for 48 h on brucella agar plates by the hot-phenol method (63). Reverse transcription was initiated by using primer 2754 (59), which binds 117 nucleotides downstream of the *sapA* transcriptional initiation site.

**Serum susceptibility assay.** *C. fetus* 23D (SapA⁺), 23B (SapA⁻), and 97-205 (*sapD*::*aphA*) were harvested from 48-h plate cultures and assayed for killing by normal human serum (NHS) by previously described methods (12), with the following modifications. Dilutions of *C. fetus* cells (10⁻⁴ to 10⁻⁷) were incubated in a 37°C 5% CO₂ environment for 60 min in the presence of 40% pooled NHS or heat-inactivated NHS (HINHS). Samples then were plated on tryptic soy agar with 5% sheep blood (BBL Microbiology Systems), and CFU were counted after 72 h. Net killing represented the difference between counts of cells incubated in HINHS and NHS as previously described (12).

**_E. coli_ SapA secretion assay.** To construct *E. coli* strains for assaying the secretion of SapA, we created an *E. coli* strain containing *C. fetus sapA* and *sapCDEF* on compatible plasmids. First, the *sapA*-containing *Eco*RI fragment of pBG1 (10) was subcloned into the *Eco*RI site of pACYC184 to create pBGYC1. Next, we transformed *E. coli* C600 with pBGYC1 and either pIR100 (*sapCDEF*) or pSPORT1 (vector control; Gibco-BRL). This pair of C600 strains (pSPORT1 plus pBGYC1 or pIR100 plus pBGYC1) was grown to mid-log phase, and the cells were harvested by centrifugation. The supernatants were passaged through 0.2-μm-pore-size filters (Micro Filtration Systems, Dublin, Calif.) to rid the
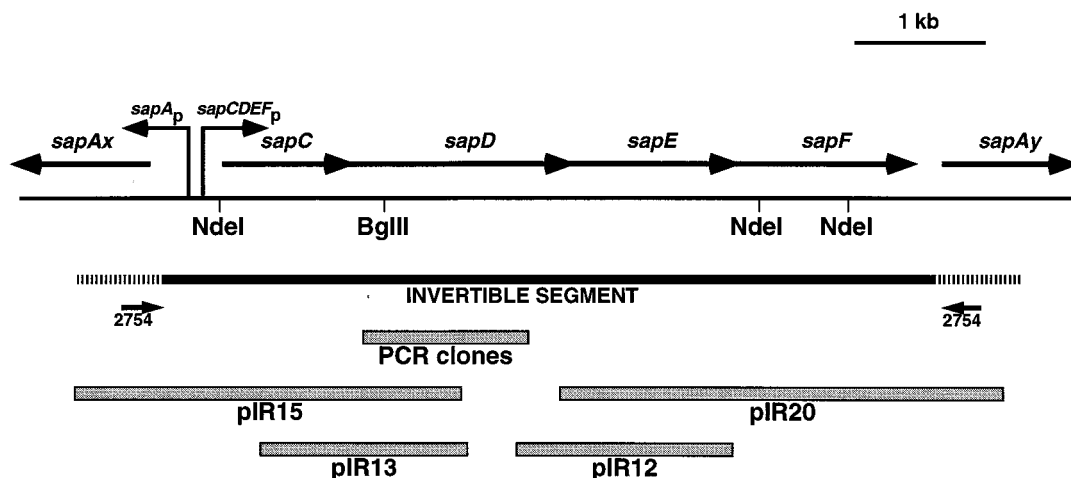
FIG. 1. Schematic representation of the *sapA* invertible region showing the *sapCDEF* genes, the locations of the divergent *sapA* promoter and the putative *sapCDEF* promoter(s), and the clones (pIR15, pIR13, pIR12, and pIR20) from which the invertible region sequence was determined. The hatched areas represent the ca. 600-bp conserved regions at the 5′ ends of *sapA* homologs flanking the invertible region (here designated *sapAx* and *sapAy*), at which recombination occurs as the basis of *sapA* homolog rearrangements during SLP antigenic variation (22, 24).

supernatants of residual bacterial cells. Trichloroacetic acid (TCA) precipitation of proteins was performed as previously described (28), and the proteins were resolved by Western blot analysis with rabbit anti-SLP serum as described above.

**Nucleotide sequence accession numbers.** The DNA sequence of the type A invertible region has been deposited in the GenBank database under accession no. AF027405. The DNA sequence of the 84-107 invertible region has been deposited in the GenBank database under accession no. AF071883.

## RESULTS

**Cloning of the type A invertible region.** Since bacterial genes involved in similar functions are often clustered, we sought to characterize the 6.2-kb invertible region between two *sapA* homologs. We first amplified the 6.2-kb fragment by PCR and subcloned the product into pAMP1 to yield pIR100. Next, we used this subcloned fragment as a probe to isolate a series of overlapping plasmid clones derived from a *C. fetus* genomic library constructed in λZAPII. Four of these, designated pIR15, pIR13, pIR12, and pIR20, represented the majority of the invertible region (Fig. 1) and were subjected to DNA sequence analysis. We were unable to isolate a clone that bridged a small gap between pIR13 and pIR12; this segment was amplified from *C. fetus* 23D genomic DNA by PCR using appropriate primers and subcloned into pT7Blue. To avoid the potential problem of PCR-induced errors, we sequenced three independent subclones, and in each case, the sequence was the same.

**Analysis of type A invertible region features.** The DNA sequence of the 6,229-bp invertible region from strain 23D predicted four open reading frames (ORFs), which we designated *sapCDEF$_A$*. The *sapC* gene began 596 bp from the initiation codon of the oppositely oriented upstream *sapA* homolog (Fig. 1). The *sapC* ORF was 1,035 bp in length and was immediately followed by the *sapD*, *sapE*, and *sapF* ORFs, which were 1,752, 1,284, and 1,302 bp long, respectively. Each gene in this cluster had a typical ribosome binding site, and they overlapped the preceding genes by 14 bp (*sapC/D*), 1 bp (*sapD/E*), and 11 bp (*sapE/F*). The *sapF* gene ended 287 bp upstream of the *sapA* homolog located downstream. The 74-bp sequences preceding the ATG codons initiating translation of the *sapA* homologs flanking *sapCDEF* were identical to each other (Fig. 1). These conserved segments have previously been noted upstream of the three characterized *sapA* homologs and

may play a role in the inversion of this DNA segment (22, 24, 60). As a potential component of this mechanism, sequences resembling χ (RecBCD recognition) sites were present at positions 31 to 38 and 6192 to 6199. Several potential $\sigma^{70}$-like promoters were noted 44 to 243 bp upstream of *sapC*. These putative *sapCDEF* promoters were oriented in the opposite direction from the *sapA* promoter, with the two −35 regions separated by 200 to 380 bp. Due to the overlapping nature of the *sapCDEF* genes and the lack of other putative promoters, it is likely that they are cotranscribed. No putative transcriptional terminators were evident within the invertible region.

**Similarities of SapCDEF to other proteins.** The *sapC* ORF predicted a protein product of 344 amino acids (aa) (39.7 kDa) that had no significant similarities in the nonredundant database maintained by the National Center for Biotechnology Information. A search by the MotifFinder server also did not reveal any recognizable protein motifs in SapC, and it did not have an N-terminal sequence suggestive of extracytoplasmic localization. In contrast, the products of the *sapDEF* genes had high similarities to proteins encoding type I secretion systems. SapD (584 aa, 64.0 kDa) was found to be related to the ABC family of transporters, especially those that are involved in translocation by the type I secretion systems of proteases, lipases, hemolysins, and leukotoxins across the envelopes of gram-negative bacterial pathogens. The degree of similarity found between SapD and these proteins was between 68% (with *P. aeruginosa* AprD) and 50% (*Actinobacillus actinomycetemcomitans* LktB; Fig. 2). In addition, SapD contained two motifs found in such proteins, an ATP/GTP binding site (GPS AAGKS; Walker box A) and a peptide (LSGGQRQRVALA) that is a signature sequence for ABC transporters. The SapE protein (428 aa, 47.9 kDa) was similar to the MFP proteins of type I transporters, typified by *P. aeruginosa* AprE (52% similar) and *E. coli* HlyD (49% similar). SapF (434 aa, 49.4 kDa) was related to the outer membrane component of type I systems such as *P. aeruginosa* AprF (45% similar), *E. chrysanthemi* PrtF (41% similar), and *E. coli* TolC (47% similar). The high similarity of each of the SapD, SapE, and SapF proteins to each of the proteins of type I secretion systems suggested that these proteins are involved in the secretion of *C. fetus* SLPs. Since the ABC component of type I transporters is responsible

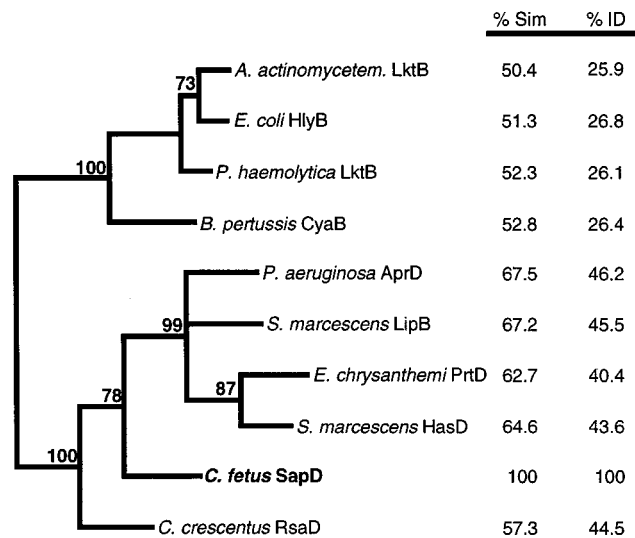|  | % Sim | % ID |
|---|---|---|
| *A. actinomycetem.* LktB | 50.4 | 25.9 |
| *E. coli* HlyB | 51.3 | 26.8 |
| *P. haemolytica* LktB | 52.3 | 26.1 |
| *B. pertussis* CyaB | 52.8 | 26.4 |
| *P. aeruginosa* AprD | 67.5 | 46.2 |
| *S. marcescens* LipB | 67.2 | 45.5 |
| *E. chrysanthemi* PrtD | 62.7 | 40.4 |
| *S. marcescens* HasD | 64.6 | 43.6 |
| *C. fetus* **SapD** | 100 | 100 |
| *C. crescentus* RsaD | 57.3 | 44.5 |

FIG. 2. Phylogram, generated by parsimony analysis, demonstrating the relatedness of the cytoplasmic membrane ABC proteins of type I secretion systems. Percentages of amino acid similarity (% Sim) and identity (% ID) with the *C. fetus* protein are shown at the right. The bold numbers adjacent to the phylogram branches indicate the percentages of 1,000 bootstrap replicates supporting the clustering of those branches. Branches without bootstrap values were clustered in less than 70% of the bootstrap replicates.

for substrate recognition (46), phylogenetic analysis of these proteins (SapD and homologs) is most likely to be informative with respect to the relationships between families of type I transporters. Parsimony and bootstrap analyses supported the classification of the *C. fetus* and *C. crescentus* SLP transporters on phylogenetic branches adjacent to the protease transporters but distinct from these and other type I secretion apparatuses (Fig. 2). Parsimony analysis of the MFPs (SapE and homologs) resulted in a similar tree, supporting this conclusion (data not shown). Phylogeny based on the type I outer membrane proteins showed few significant distinctions between subfamilies of transporters (data not shown).

**Invertible region features from type B strain 84-107.** To determine whether the features found in the invertible region of type A strain 23D also were conserved in a type B strain, we sequenced the invertible region from strain 84-107. The invertible region from this strain was 6,217 bp in length and was 99.1% identical to the invertible region from strain 23D. Similar to the conserved regions at each end of the type A invertible region, the first 67 nucleotides of the type B sequence were an exact inverse repeat of the last 67 nucleotides. However, these 67-bp segments at the ends of the type B invertible region were only 68.7% identical to the analogous sequences of the type A invertible region. Similar to the type A sequence, the type B invertible region sequence predicted a probable *sapCDEF* operon of 5.6 kb (designated *sapCDEF*$_B$). The putative *sapCDEF*$_B$ promoter sequences and ribosome binding sites were identical to their counterparts in the 23D invertible region (data not shown). Most of the differences between the invertible regions of 23D and 84-107 were found in the noncoding regions upstream and downstream of *sapCDEF*, with the coding regions sharing 99.8% nucleotide identity. Due to the high sequence similarity between the two invertible regions, the SapC, SapD, SapE, and SapF proteins from the type A and B strains were nearly identical, sharing 99.7, 100, 99.5, and 100% amino acid identity, respectively.

**Construction of a *sapD* mutant.** To test the hypothesis that genes in the *C. fetus* invertible region encode proteins responsible for SLP secretion, we constructed a derivative of type A strain 23D containing an insertional mutation in *sapD*. Since homologs of SapD (ABC transporters) are essential components of type I secretion systems (5, 41, 46), *C. fetus* mutants lacking SapD would be expected to be secretion deficient. To generate a *sapD* mutation, we chose a unique *Bgl*II site 224 bp from the upstream end of the gene (Fig. 1). The *aphA* cassette from pILL600 was inserted into this site, and transformants were selected on plates containing kanamycin. Plasmid DNA from kanamycin-resistant *E. coli* colonies was analyzed by *Eco*RI and *Hin*dIII digestion, and the *aphA* cassette was found to have been incorporated such that *sapCDEF* and the antibiotic resistance gene were transcribed in the same direction. This new clone was designated pIR131. The *sapD* insert containing the resistance fragment was subcloned into suicide vector pILL570 to yield pILL131, which was transformed into *E. coli* S17-1. Transformants were selected from trimethoprim-kanamycin plates, and verification of pILL131 was made by digestion with *Hin*dIII. *E. coli* S17-1 containing IncP DNA transfer functions and pILL131 was used as the conjugation donor, and wild-type *C. fetus* 23D was the recipient. Approximately 5,000 transconjugants were recovered (frequency of 4 × 10$^{-6}$ transconjugants per recipient), 6 of which were picked for further screening. Chromosomal DNAs extracted from these six strains were digested with *Nde*I for Southern analysis by using hybridization probes for *sapD*, *aphA*, and pILL570. The hybridization results indicated that one of these strains (97-205) was derived from a double-crossover event in which only the mutagenized *sapD* allele was incorporated into the chromosome (data not shown). The remaining five strains produced results consistent with single crossovers in which the pILL570 vector also was introduced into the chromosome (data not shown). PCR experiments using *sapD*- and *aphA*-specific primers confirmed the Southern hybridization data (not shown). These results indicate that a strain containing an insertional mutation in *sapD* was successfully constructed.

**Properties of a *C. fetus* *sapD* mutant.** We next sought to determine whether the *C. fetus* *sapD* mutant 97-205 was able to export SLPs and to assemble a functional S-layer on its surface. First, we tested the abilities of the wild-type and *sapD* mutant strains to resist complement-mediated killing, a phenotype consistent with the presence of the S-layer. Suspensions of cells were exposed either to NHS or to HINHS, and the extent of complement-mediated killing was determined. As expected, S$^+$ strain 23D was completely resistant to killing (Fig. 3A). In contrast, as expected, there was approximately 3 log$_{10}$ killing of strain 23B, which is unable to express an S-layer (48, 59) (Fig. 3A). Results for strain 97-205, the *sapD* mutant, were nearly identical to those for 23B and are consistent with the absence of a functional S-layer on the surface of *sapD* mutant strain 97-205.

To understand the basis for the serum susceptibility of the *sapD* mutant, we performed immunoblot analysis to detect the presence of SLPs on the cell surface. SLPs can readily be removed from the surfaces of S$^+$ cells by washes with distilled water (50). Therefore, water washes and whole-cell proteins of wild-type and *sapD* mutant cells were analyzed by immunoblotting with polyclonal antiserum against SLPs. SLPs were detected in both whole-cell samples and water washes of the wild-type strain, as expected for cells expressing a functional S-layer (Fig. 3B, lane 1), whereas no SLPs were detected in S$^-$ strain 23B (Fig. 3B, lane 2). However, SLPs were detected neither extracellularly nor in whole-cell samples of the *sapD* mutant, (Fig. 3B, lane 3). Thus, disruption of *sapD* by the
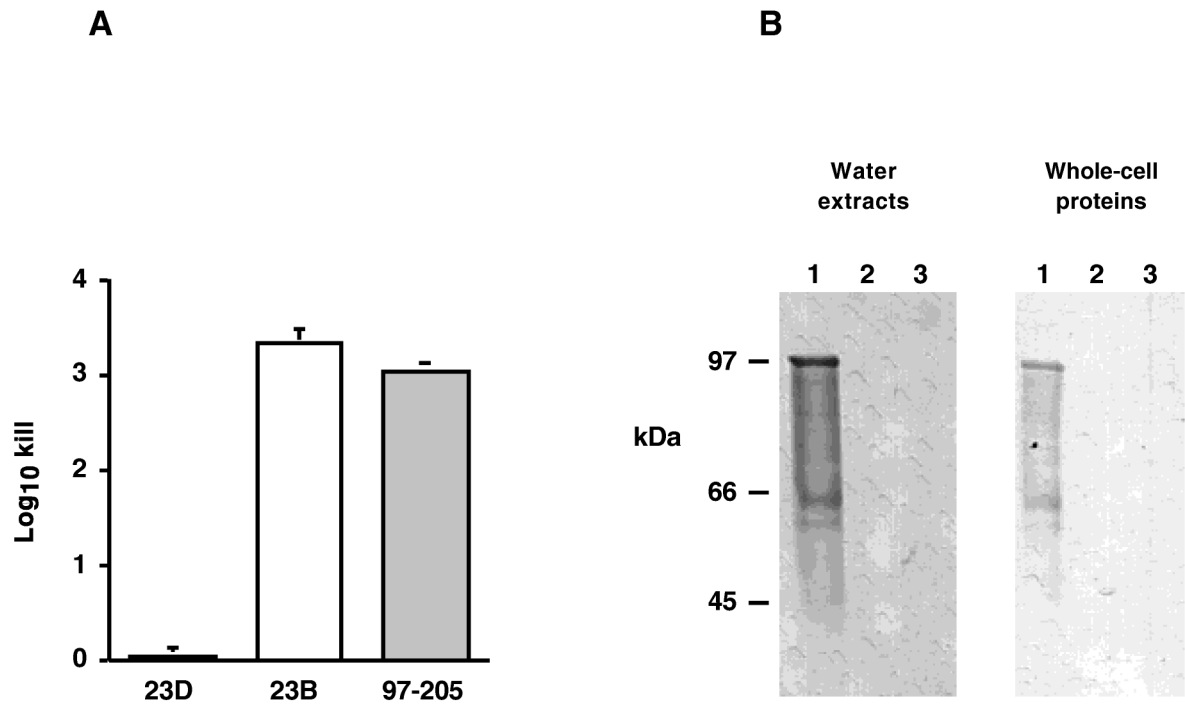
**A**

**B**



FIG. 3. Phenotypes of *C. fetus* wild-type (23D), spontaneous S$^-$ mutant (23B), and *sapD* mutant (97-205) cells. (A) Serum susceptibilities of *C. fetus* strains. Serial dilutions of $10^3$ to $10^5$ CFU/ml were suspended in either 40% NHS or HINHS (56°C, 30 min) for 60 min. The amount of killing relative to that of the time zero inoculum was determined and is presented as mean $\log_{10}$ killing $\pm$ the standard error of the mean. (B) Western blot of *C. fetus* strains. Cell surface water extracts and whole-cell lysates were probed with polyclonal serum against *C. fetus* SLPs. Lanes: 1, strain 23D (S$^+$); 2, strain 23B (S$^-$); 3, strain 97-205 (*sapD* mutant). The positions of molecular mass markers are shown at the left.

insertion of an antibiotic resistance cassette had the effect of eliminating SLP expression altogether, as well as SLP secretion. It could not be ascertained from these experiments whether the effect of the *sapD* mutation on the inability to detect SLPs in the cytoplasm was due to a regulatory effect on SLP gene expression or whether full-length SLPs were simply degraded in the absence of a functional secretion apparatus. Primer extension experiments indicated that wild-type levels of *sapA* mRNA were present in strain 97-205 (Fig. 4), showing that potential regulatory effects of the *sapD* mutation on SapA expression did not occur through diminished *sapA* transcription.
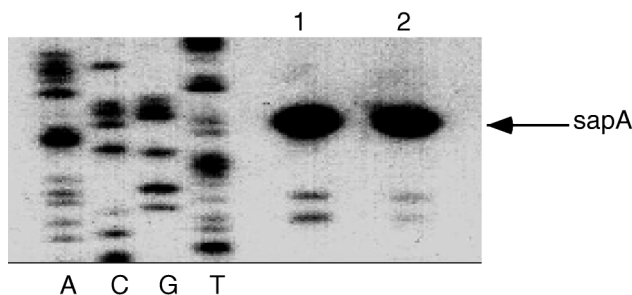


FIG. 4. Primer extension analysis of *sapA*-specific mRNA in wild-type and *sapD* mutant *C. fetus* strains. The levels of *sapA* transcription were determined by reverse transcription of standardized 40-μg RNA samples by using primer 2754 (59). The sequencing ladder was generated by using primer 2754 and plasmid pIR15, which contains the *sapA* promoter and 5′ region (Fig. 1). Lanes: 1, strain 23D (wild type); 2, strain 97-205 (*sapD* mutant). The +1 site for strain 23D is identical to that determined previously (59).

**Secretion of SapA by *E. coli* expressing *sapCDEF*.** We had cloned the PCR-amplified *sapCDEF*-containing invertible region (pIR100) for use as a hybridization probe with which to identify genomic clones as described above. To determine whether *sapCDEF* permits the secretion of SapA, we tested the ability of *E. coli* strains carrying pIR100 to mediate the specific secretion of *C. fetus* SapA (Fig. 5). *E. coli* C600 strains were isolated that contained pBGYC1, a pACYC184-derived plasmid containing *C. fetus* *sapA*, and either pIR100 (*sapCDEF*) or pSPORT1 (vector control). SapA secretion was assayed by using immunoblots to indicate the appearance of SapA in filtered, TCA-precipitated culture supernatants prepared from these strains. As expected (10), both *E. coli* strains produced SapA (Fig. 5, whole-cell proteins). Secretion of SapA was detected only in supernatants from *E. coli* cells expressing *sapCDEF* (Fig. 5, lane 4). These results indicate that the *C. fetus sapCDEF* genes are sufficient to allow *E. coli* to secrete SapA.

**Conserved features of SLP C termini.** Proteins that are transported by type I secretion systems do not have the N-terminal signal peptides that are conserved in proteins exported by type II systems, instead relying on signals that are located at their extreme C termini (51, 61). However, these signals tend to have little primary sequence homology, and their exact structures have been difficult to elucidate. In an attempt to define candidate sequences or structures for C-terminal secretion signals in bacterial SLPs, we aligned the C-terminal 70 aa of four *C. fetus* SLPs for which the sequences are known (SapA, SapA1, SapA2, and SapB), as well as RsaA, the SLP from *C. crescentus* (9). Several conserved peptides were evident (Fig. 6). The sequences GDGS(T/G), GxTYV V(V/I)D, and DxxIKLxG were each present in at least four of
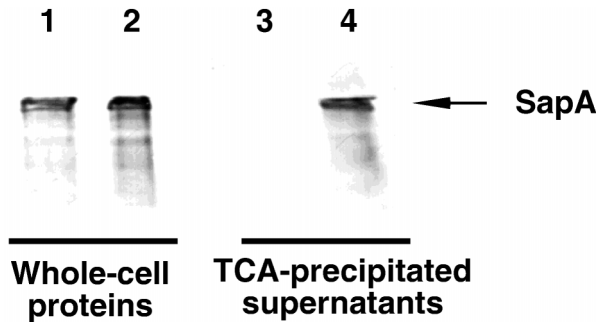
FIG. 5. Secretion of SapA from *E. coli* expressing *C. fetus sapCDEF_A*, detected by immunoblotting with antiserum to *C. fetus* SLPs. One microgram of whole-cell proteins (lanes 1 and 2) or the amount of TCA-precipitated protein present in 250 μl of culture supernatant (lanes 3 and 4) was analyzed. Lanes: 1 and 3, C600(pSPORT1)(pBGYC1); 2 and 4, C600(pIR100)(pBGYC1).

the five SLPs (Fig. 6). The DVIV motifs implicated in protease secretion (33) were not present per se at the extreme C terminus of any of the SLPs, although a similar sequence (DGSVI) was found at the C termini of the SapA and SapB proteins (Fig. 6). Similar to type I secreted toxins and related exoproteins (56, 58), the SLPs had one to four hydroxylated residues (S or T) within the C-terminal 10 amino acids.

To investigate whether the C-terminal domains of these SLPs also could form similar secondary structures, we analyzed each of these peptides by using the programs of Garnier et al. (32). The predicted C-terminal secondary structures (α-helix, β-sheet, amphipathic peptides, and turn-forming residues) were then superimposed on the alignment of the five C-terminal peptides. Four of these peptides (SapA, SapA1, SapB, and RsaA) consisted of segments predicted to form a sheet-sheet-helix-sheet structure, with turn-forming residues separating the domains (Fig. 6). The C-terminal domain of SapA2 was predicted to form a helix-sheet-helix-sheet structure. Furthermore, the sheet-forming regions of all five proteins, as well as the most N-terminal helix-forming region of SapA2, were predicted to be amphipathic. Taken together, these results suggest that the C termini of four *C. fetus* SLPs and one *C. crescentus* SLP contain conserved sequences and secondary structures that are candidates for secretion signals.

Since SapC did not have any database homologies, yet *sapC* was situated immediately upstream of *sapDEF*, we also compared the C terminus of SapC to the conserved SLP C termini. The C terminus of SapC did not at all resemble the C termini of the other proteins (data not shown); therefore, it is likely that SapC is not secreted by the *C. fetus* type I system.

## DISCUSSION

Three genes in the *sapA* invertible region (*sapDEF_A*) and in the homologous *sapB* invertible region (*sapDEF_B*) encode proteins that are homologous to type I transporters, typified by the *E. coli* α-hemolysin and *S. marcescens* LipB lipase secretion systems. The *sapDEF* genes are adjacent to the *sapA* homologs that encode the proteins that are substrates for the *C. fetus* type I secretion system. Genes encoding type I secretion systems of other bacteria often show such a clustered arrangement (51, 61), although the gene encoding the outer membrane component is not necessarily adjacent (2, 4, 51). The first gene, *sapC*, predicts a protein that has no database homologies, no amino acid motifs, and no other recognizable features such as signal sequences or helix-turn-helix structures. Furthermore, since the C-terminal 100 aa bore little resemblance to those of the SLPs and proteases, SapC does not appear to be another substrate for the SapDEF secretion apparatus. Therefore, the function of SapC is unclear.

Some aspects of type I secretion systems have been well characterized, and studies by a number of researchers have yielded the following model of type I secretion. Three proteins form the secretion apparatus. ABC transporters homologous to SapD (e.g., HlyB and PrtD) localize to the cytoplasmic membrane, probably as homodimers (41, 61), and serve a number of functions in the initial stages of the transport process. ATP hydrolysis by the ABC protein plays a role in energizing the secretion process (40). Recognition of the C terminus of the secreted protein by the ABC protein sequesters, in turn, the MFP and the outer membrane proteins, completing the assembled apparatus (46). The MFP is located mostly within the cytoplasmic membrane but is thought also to associate with the outer membrane and mediate its juxtaposition with the cytoplasmic membrane (18). The outer membrane protein (TolC homolog) may serve as a pore through which the secreted protein is extruded (42). The secreted proteins are devoid of multiple cysteine residues and therefore lack disulfide bridges during their transit through both membranes. The high degree of conservation between each of the *C. fetus* transport proteins and each constituent of other type I transporters strongly suggests that the secretion mechanism of SLPs is similar to that shown for other proteins. However, the phylogram generated from comparisons of the ABC components of the secretion apparatus (Fig. 2) shows that the *C. fetus* and *C. crescentus* systems represent a third family of type I transporter, divergent from the toxin or protease transporters.

Analysis of the sequence of the C terminus of *E. coli* α-hemolysin revealed a potential signal consisting of a charged region, followed by an amphipathic α-helix, and a number of hydroxylated amino acid residues at the extreme C terminus of the protein (56). The importance of these structures, as well as



FIG. 6. Conserved primary and secondary structures of the C termini of four *C. fetus* SLPs (SapA, SapA1, SapA2, and SapB) and RsaA, the SLP of *C. crescentus*, predicted by GCG PILEUP and by the algorithm of Garnier et al. (32). The consensus line indicates amino acid residues that were conserved in at least three of the five SLPs. Secondary-structure features shown are regions of predicted α-helix (boxed), β-sheet (light shading), random coil (no shading), and strong or weak amphipathicity (A or a, respectively) and strong or weak turn-forming residues (T or t, respectively).

of individual amino acid residues was supported by mutational analyses (16, 39, 56). Similar features are found in the C termini of other exoproteins that can be secreted by the HlyBD secretion apparatus (58). In contrast, these features are not universally found in other proteins secreted by type I systems. For example, in proteases, a C-terminal amino acid motif of DVIV is involved in secretion (33, 64). This exact motif is found in neither type I-secreted toxins nor SLPs. The remainder of the C termini among type I-secreted proteases, lipases, and the *S. marcescens* heme-binding protein HasA share few structural similarities, although amphipathic α-helices have been proposed as components of secretion signals of some of these (21, 56, 58, 64). Nevertheless, many of these proteins can be secreted by heterologous type I transporters (5, 21, 45, 47, 57), suggesting the conservation of secretion signals within divergent C termini.

It is likely that the secretion signals of *C. fetus* SLPs are located at the C termini of the proteins, for the following reasons. None of the four characterized SLPs has an N-terminal signal sequence (10, 26, 27, 60). A 50-kDa C terminally truncated form of SapA was not secreted by *C. fetus*, suggesting that the signal directing its transport was located at the C terminus of the protein (14). Furthermore, *E. coli* K-12 strains are unable to secrete SapA (10; this work), suggesting that proteins different than the *E. coli* type II (*sec*) apparatus are necessary for its transport. Now we have shown that type I secretion proteins are responsible for transporting *C. fetus* SLPs to the cell surface, and with the exception of colicin V (34), the signals recognized by type I transporters are found at the extreme C terminus of the secreted protein (61). The SLP of *C. crescentus*, which also is secreted by a type I system, contains a secretion signal within its C-terminal 242 aa (7). Alignment of the C-terminal 70 aa of four *C. fetus* and one *C. crescentus* SLPs revealed a surprisingly large number of conserved amino acid residues (Fig. 6). In fact, the C termini of *C. fetus* SapA and SapB appeared to be more closely related to that of *C. crescentus* RsaA than to the C termini of the *C. fetus* SapA1 and SapA2 proteins. These conserved residues, as well as predicted β-sheet and α-helical regions, provide attractive targets for mutational analyses in determining the important components of SLP C-terminal secretion signals. The availability of several related *C. fetus* SapA homologs with somewhat different C termini allows the testing of a variety of hypotheses regarding the secretion signals and offers advantages to comparing the structures of type I-secreted proteins from heterologous bacterial species.

The mutation of *sapD* had an inhibitory effect on SapA synthesis by *C. fetus* 97-205, although this is most likely related to the inability of the *sapD* mutant to export SLPs. Since SLPs are the most abundant protein produced by *C. fetus*, their accumulation in the cytoplasm would probably be deleterious to growth or viability. The detection of wild-type levels of *sapA* mRNA in the *sapD* mutant precludes the possibility that a mutation occurred (in either *sapD* or another locus) that ablated transcription from the *sapA* promoter. We therefore consider two main possibilities for the lack of detection of SLPs in the *C. fetus sapD* mutant. First, nonsecreted SLPs may simply be degraded. Smit and colleagues were unable to detect derivatives of *C. crescentus* RsaA resulting from disruption of the C-terminal secretion signal, presumably due to the degradation of the secretion-deficient protein (7). A similar phenomenon previously had been noted for nonsecreted *E. coli* α-hemolysin (16), *P. aeruginosa* AprA, or hybrids of AprA with *Pseudomonas fluorescens* lipase (20, 21, 36). Therefore, the disruption of the secretion process of these proteins may necessitate their degradation. Second, the accumulation of SLPs within the cytoplasm may have an inhibitory effect on their own synthesis at a posttranscriptional level. It seems desirable for the expression of such an abundant protein to be tightly regulated, and in fact, this occurs with the SLPs of *Thermus thermophilus* (29). Although the *T. thermophilus* SLPs are secreted by the general secretory pathway (type II secretion), recent studies have shown that the C terminus of the *T. thermophilus* SLP is able to bind to the 127-bp 5′ untranslated region (UTR) of its own message and inhibit SLP synthesis (29). The *C. fetus sapA* mRNA has a highly structured 114-bp 5′ UTR, and it is possible that a similar repression of SLP synthesis occurs in *C. fetus*. If *C. fetus* SLPs can inhibit their own synthesis in such a manner, then it is probably also dependent on the C-terminal half of the protein, since the expression of a 50-kDa N-terminal (secretion-deficient) fragment of SapA is tolerated (14). We cannot rule out either of these hypotheses, and further studies, such as pulse-chase labeling of SLPs to examine protein turnover and genetic studies with SLPs truncated at the C terminus, will address these possibilities.

That *sapCDEF* are sufficient for the secretion of *C. fetus* SLPs was demonstrated by the ability of these genes to direct the transport of SapA from *E. coli*. Therefore, it appears that, similar to other type I systems, three proteins (SapDEF) are probably necessary and sufficient for the secretion of *C. fetus* SLPs. In contrast to the inability to detect nonsecreted SLPs in *C. fetus*, large quantities of SapA are present in *E. coli* lacking *sapCDEF*. This difference may have been due to the 5′ UTR of *sapA* in the clone used in this secretion experiment. The *sapA* gene encoded by pBGYC1 is not expressed from its native promoter and has only 23 bp of the 114-bp 5′ UTR found in *C. fetus sapA* homologs (10). The ability to reconstruct the *C. fetus* SLP secretion system in *E. coli* now facilitates genetic studies intended to define the interactions between SapA and the SapDEF transport apparatus and to investigate the possible autoregulation of *sapA*.

We have characterized the SapDEF system that is responsible for the secretion of *C. fetus* SLPs and shown that it is a related but somewhat divergent member of the type I secretion family. Future studies intended to define the C-terminal secretion signals of *C. fetus* SLPs and their recognition by the SapDEF apparatus, as well as by heterologous type I transporters, may provide new insight into transporter-substrate interactions within these systems.

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