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Protein Shift and Antigenic Variation in the S-Layer of *Campylobacter fetus* subsp. *venerealis* during Bovine Infection Accompanied by Genomic Rearrangement of *sapA* Homologs

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***Campylobacter fetus* subsp. *venerealis* isolated from a case of human vaginosis was inoculated into the uterus of a *C. fetus*-negative heifer. Isolates obtained weekly from the vaginal mucus exhibited variations in high-molecular-mass-protein profiles from that of the original inoculum, which had a dominant 110-kDa S-layer protein. Immunoblots of the weekly isolates with monoclonal antibody probes against the 110-kDa S-layer protein and other *C. fetus* S-layer proteins demonstrated antigenic shifts. Genomic digests of the isolates probed with a 75-mer oligonucleotide of the conserved *sapA* region also indicated that antigenic variation of the S-layer is accompanied by DNA rearrangement.**

Campylobacter fetus subsp. *venerealis* is a major cause of transient infertility and sporadic abortion in cattle (12, 27). In 1987, Holst and coworkers (13) described isolations of *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* from several women with vaginosis in southern Sweden. Three of these isolates have been confirmed as *C. fetus* subsp. *venerealis* by both conventional identification methods and pulsed-field gel electrophoresis (22). Thus far, the pathogenicity of the human *C. fetus* isolates possessing cultural and biochemical characteristics identical to those of bovine *C. fetus* subsp. *venerealis* has not been investigated.

Wild-type strains of *C. fetus* possess an S-layer consisting mainly of high-molecular-mass surface array proteins ranging from approximately 97 to 149 kDa. This paracrystalline surface structure has been the subject of a number of recent molecular studies (7, 8, 10, 18) since earlier reports suggested that it may play a major role in chronic venereal campylobacteriosis by the mechanism of antigenic variation (5, 23). Antigenic variation may be achieved by shifts in the expression of S-layer proteins which result in different immunodominant epitopes during in vivo persistence in the bovine genital tract (28). A gene (*sapA*) encoding the 97-kDa S-layer protein has been cloned (1), and it now is clear that both the wild type and spontaneous mutants lacking the S-layer proteins possess multiple *sapA* homologs (25). In a single strain, in vitro antigenic shift was associated with rearrangement of *sapA* homologs (26). In this paper, we provide data supporting a direct relationship between high-molecular-weight-protein shifts and antigenic variation of the *C. fetus* S-layer on the one hand and genomic rearrangement of the *sapA* homologs on the other during the course of infection in the bovine reproductive tract.

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MATERIALS AND METHODS

Bacterial strain, animal inoculation, and culture methods. *C. fetus* subsp. *venerealis* ADRI 1023 was one of five isolates from human cases of vaginosis in southern Sweden (13). The dominant S-layer protein of this strain migrates at 110 kDa and has remained stable despite repeated in vitro subculturing. For animal inoculation, strain ADRI 1023 was grown on Mueller Hinton agar (Unipath Inc., Nepean, Ontario, Canada) supplemented with 10% sheep blood for 48 h of incubation at 37°C in a microaerobic atmosphere consisting of 3.5% O_2, 10% CO_2, and 86.5% N_2. Cell growth was washed off with 0.1 M phosphate buffer (pH 7.2), and the concentration was adjusted to a McFarland no. 10 standard (ca. 3×10^9 cells per ml). A 9-month-old heifer which had been shown to be negative for *C. fetus* by three weekly cultures was inoculated with 1.0 ml of the cell suspension through an insemination pipette into the uterus. At weekly intervals, starting 3 weeks before inoculation, vaginal mucus samples were aspirated into plastic rods and inoculated directly onto cystine heart agar (Difco Laboratories, Detroit, Mich.) supplemented with 10% sheep blood, 0.2 U of polymyxin B sulfate per ml, 2 μg of novobiocin per ml, and 20 μg of cycloheximide per ml (all components from Sigma Chemical Co., St. Louis, Mo.). The plates were incubated at 37°C in a microaerobic atmosphere for 2 to 3 days.

SDS-PAGE, monoclonal antibody production, immunoblotting, and immunoelectron microscopy. Whole cells were washed and resuspended in 0.01 M Tris buffer, pH 7.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 0.75-mm-thick slab gels with Coomassie blue staining as previously described (3). Analysis of lipopolysaccharides (LPS) involved digestion of proteins with proteinase K and detection of electrophoresed LPS components by silver staining as previously described (11). For monoclonal antibody production, the 110-kDa protein from the *C. fetus* subsp. *venerealis* ADRI 555 S-layer was extracted with 0.2 M glycine-hydrochloride buffer (pH 2.2) as previously described (10). Purified 110-kDa S-layer protein mixed (1:1, vol/vol) with Titermax (CytRx, Norcross, Ga.) was injected subcutaneously into BALB/c mice at days 0 and 14, and 100 μl of the antigen was injected intravenously on day 49. Sp 2/0 murine myeloma cells were fused with mouse spleen cells at day 54, essentially as described by Kennett et al. (14). Hybridoma culture supernatants were screened by enzyme-linked immunosorbent assay against the 110-kDa S-layer protein antigen by using horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G diluted 1:5,000 in phosphate-buffered saline–Tween (Bethyl Laboratories, Montgomery, Tex.) and TMB (3,3′,5,5′-tetramethylbenzidine) Microwell Peroxidase Substrate System (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Md.). Positive cells were cloned and monoclonal antibody isotypes were determined by using commercial reagents (Idexx, Portland, Maine). One hybridoma, M442, was highly reactive to the purified 110-kDa S-layer protein and was selected for this study. Monoclonal antibodies 1D1, 6E4, and 2E11 recognize *C. fetus* S-layer proteins and have been used previously (28); 1D1 recognizes the 97-kDa proteins of either serotype A or B strains, 6E4 strongly recognizes the 96- to 98-kDa proteins of serotype A only, and 2E11 recognizes 80- to 149-kDa proteins of serotype A. Immunoblotting was performed as described elsewhere (24). Immunogold electron microscopy was done essentially by a previously described procedure (9). After reacting with

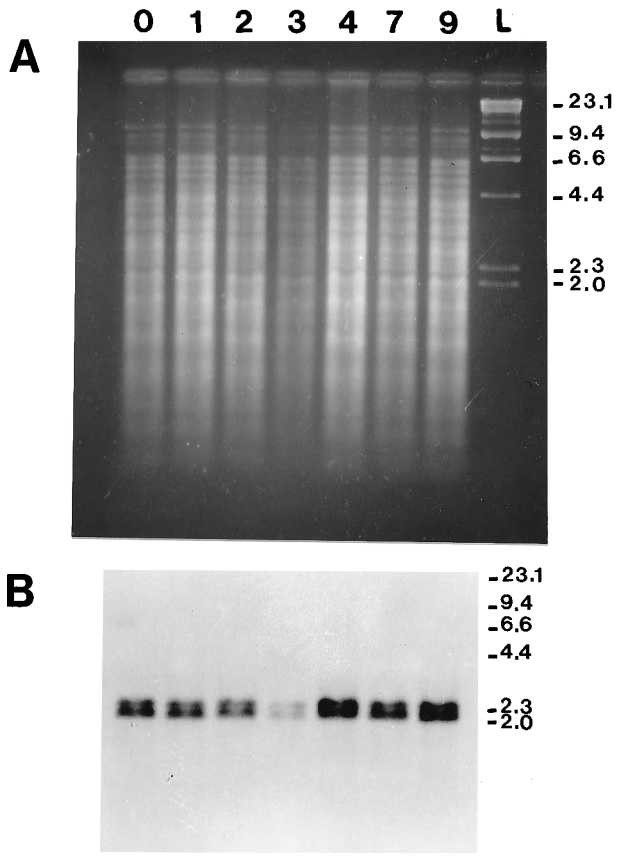

FIG. 1. (A) *Hin*dIII cleavage of genomic DNAs from weekly isolates from a heifer infected with strain ADRI 1023. Lane numbers represent weeks postinoculation. Lane L, lambda-*Hin*dIII DNA size marker (sizes shown in kilobases on the right). (B) Ribotyping of *Hin*dIII digest of genomic DNAs of the weekly isolates. A *C. fetus* subsp. *fetus* 16S rRNA probe was used for hybridization to the *Hin*dIII-digested genomic DNA.

M442, the cells were labeled with an anti-mouse immunoglobulin G conjugate with 30-nm-diameter gold particles (Cedarlane Laboratories, Ltd., Hornby, Ontario, Canada).

DNA techniques. Genomic DNA from the *C. fetus* isolates obtained weekly was extracted with guanidinium thiocyanate by a standard protocol (21) with minor modifications. Briefly, the precipitated DNA was resuspended in 10 mM Tris-HCl–1 mM EDTA buffer (pH 8.0) and purified further by hexadecyltrimethyl ammonium bromide (Sigma) treatment (17) to remove polysaccharides. Approximately 10 μg of purified DNA was digested to completion with *Hin*dIII (Boehringer Mannheim, Laval, Quebec, Canada) according to the manufacturer's suggestions and electrophoresed in 0.7% agarose. The gel was denatured, neutralized, and dried for in situ hybridization using a nick-translated cloned construct of 16S rRNA from *C. fetus* (6) and an end-labeled oligonucleotide spanning the first 75 bases of *sapA* (1). The latter represents a part of the conserved region of the *sapA* homologs (26) starting from the amino terminus. All hybridization and posthybridization steps were performed under stringent conditions (16), and the hybridizing genomic segments were visualized by autoradiography.

RESULTS AND DISCUSSION

Characterization of *C. fetus* isolates from the bovine reproductive tract. Weekly vaginal mucus samples yielded recoveries of strain ADRI 1023 at 1, 2, 3, 4, 7, and 9 weeks postinoculation. The organism was not recovered at 10 to 12 weeks postinoculation, at which time the experiment was terminated, since our interest concerned mainly the early stage of infection. In most cases, mixed cultures were observed on primary isolation plates despite the presence of selective agents in the medium. The number of suspect *C. fetus* discrete colonies on the

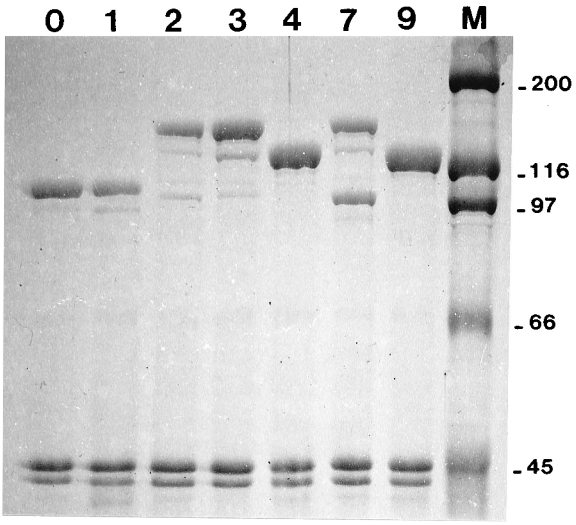

FIG. 2. SDS-PAGE of whole-cell proteins of weekly isolates from a heifer infected with strain ADRI 1023. Lane numbers represent weeks postinoculation. Lane M, molecular weight markers (indicated in thousands on the right). Protein molecular weight standards were myosin (200,000), β-galactosidase (116,250), phosphorylase *b* (97,400), bovine serum albumin (66,200), and ovalbumin (45,000) (Bio-Rad).

isolation plates ranged from 1 to 10; a representative single colony was selected for subsequent characterization. In a separate bovine infection study with *C. fetus* subsp. *venerealis* ADRI 555, we selected four or five colonies from each of four primary isolation plates, with each plate representing a sample

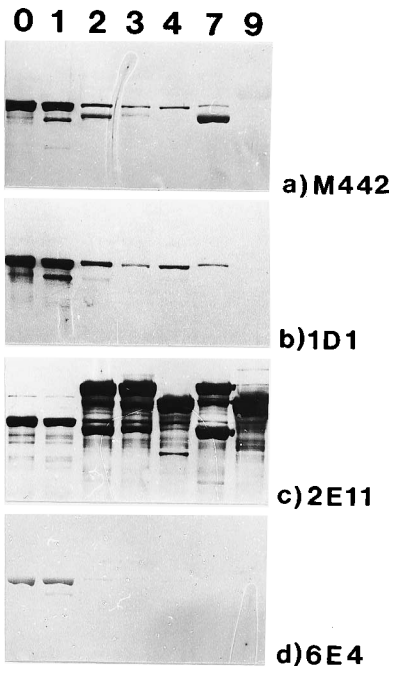

FIG. 3. Immunoblots of S-layer proteins of sequential isolates from a heifer infected with strain ADRI 1023. The following dilutions of monoclonal antibodies were used: M442, 1:50; 1D1, 1:80, 6E4, 1:50; and 2E11, 1:40. Immunoblots were visualized by employing an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G and 5-bromo-4-chloro-3-indolylphosphate toluidinium–nitroblue tetrazolium (BCIP-NBT) as a substrate. Lane numbers represent weeks postinoculation.

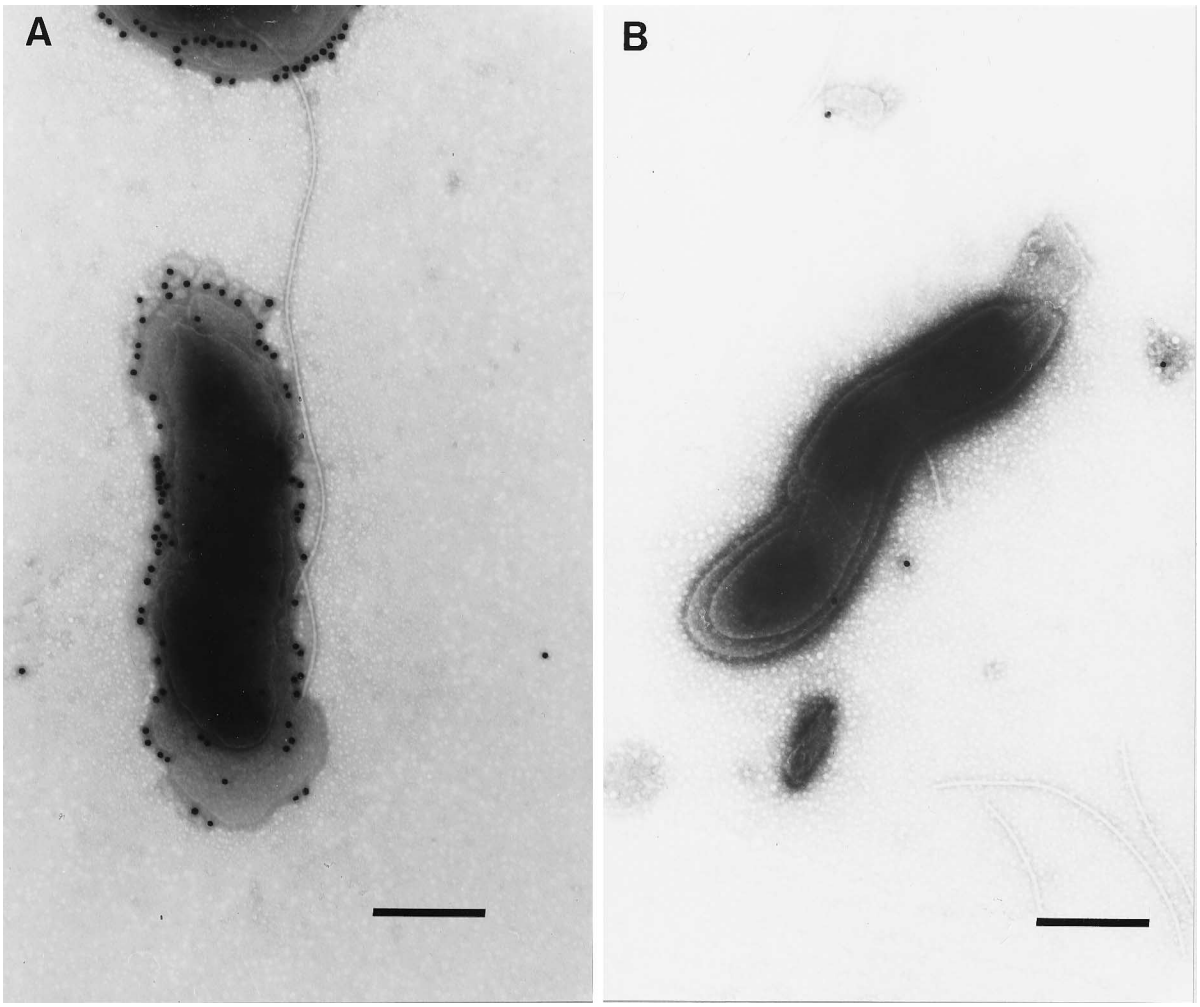

FIG. 4. Electron micrographs of immunogold-labeled S-layer proteins of week 0 (A) and week 9 (B) cells with monoclonal antibody M442. The numerous gold grains surrounding the week 0 (inoculant) cells confirm the surface location of the epitope present on the 110-kDa S-layer recognized by M442. Bars = 0.5 μm.

from a different week postinoculation. Colonies from each isolation plate showed identical protein profiles except in one plate in which one of the colonies showed a slightly different pattern. We believe that the single colonies of strain ADRI 1023 selected in the study described here were also representative. All subcultures of ADRI 1023 showed the morphological and biochemical characteristics typical of *C. fetus* subsp. *venerealis*, including sensitivity to 1.0% glycine. LPS profiles of the weekly isolates (not shown) were identical and exhibited the typical serotype A pattern (20). Similarly, restriction endonuclease analysis with *Hin*dIII of these isolates (Fig. 1A) showed no obvious variations and was further confirmed when the same digests exhibited a single type of riboprint (Fig. 1B). Combined with efforts to avoid cross-contamination through strict animal handling, these results indicated that the weekly isolates represented descendants of the original culture.

S-layer protein shift accompanied by genomic rearrangement during infection. The protein profiles of the weekly isolates revealed differences in the numbers and positions of bands only at molecular sizes greater than 90 kDa (Fig. 2). The predominant protein of the inoculant (strain ADRI 1023) had an approximate molecular mass of 110 kDa and a minor band of 97 kDa. No changes were noted for the isolate recovered 1 week after inoculation. However, protein shifts above 90 kDa were observed for subsequent isolates, starting from the second week postinoculation. The isolates from weeks 2, 3, and 7 contained a predominant protein band that had shifted to ca. 160 kDa, whereas those from weeks 4 and 9 showed a predominant band of ca. 127 kDa. The predominant 110-kDa band present in the week 0 and 1 isolates became less evident in the subsequent isolates and was not detected in the week 9 isolate. Two predominant bands with similar staining intensities corresponding to 100 and 160 kDa were observed at week 7 but were not evident in the week 9 isolate. Other dominant protein bands of approximately 45 kDa, which corresponded to the major outer membrane proteins (2, 15), were unchanged in the weekly isolates, as were bands with lower molecular masses (not shown). Immunoblots of similar gels indicated that except for the week 9 isolate, all isolates showed the presence of an epitope recognized by monoclonal antibody M442 (Fig. 3a). Immunoelectron microscopy also confirmed that the relevant 110-kDa S-layer epitope detected in immunoblots of the week 0 to 7 isolates, but not in the week 9 isolate, was surface exposed (Fig. 4). The absence of a reaction in the week 9 immunoblot was preceded by diminished intensity of the immunoblot reaction starting from week 2, suggesting that changes in the conformation (or presentation) of the epitope

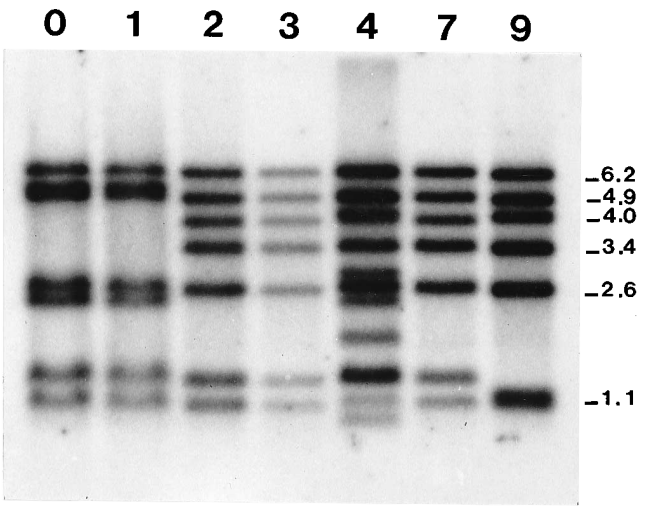

FIG. 5. *Hin*dIII digests of genomic DNAs from isolates of strain ADRI 1023 from the bovine vagina. Each was hybridized with a 75-mer 5′-conserved probe from *sapA*, encoding a 97-kDa S-layer protein gene. Lane numbers represent weeks postinoculation. Molecular sizes (in kilobases) are indicated on the right.

during the intervening weeks may have occurred. The reduced staining of the 110-kDa S-layer protein after week 1 also was observed in another infection study using bovine strain ADRI 555 (data not shown). Immunoblotting with monoclonal antibody 1D1 yielded results similar to those for M442 with respect to the intensity of staining of the 110-kDa band in the weekly isolates and the absence of a reaction in the S-layer protein of the week 9 isolate (Fig. 3b). The immunoblots with 2E11 showed multiple intensely reacting bands both below and above the 110-kDa band (Fig. 3c). However, most of the predominant antigens had masses of >90 kDa. By contrast, monoclonal antibody 6E4 recognized an epitope of the 110-kDa protein only for the week 0 and 1 isolates and a minor 97-kDa band for the week 1 isolate (Fig. 3d).

When probed with an oligonucleotide spanning the first 75 bases of *sapA*, the weekly isolates showed different hybridization patterns with respect to the number and size of bands (Fig. 5). The number of hybridizing bands ranged from at least six distinct bands for week 0, 1, and 9 isolates to 11 bands for the week 4 isolate, reflecting variation in the number of *sapA* homologs or the location of *Hin*dIII recognition sites. All isolates revealed common hybridizing bands at 6.2, 4.9, 2.6, 2.45, 1.35, and 1.1 kb. The disappearance of the 2.45-kb hybridizing segment from week 2, 3, 7, and 9 isolates was accompanied by the appearance of two other hybridizing bands at 4.0 and 3.4 kb, while three additional bands with molecular sizes of 2.9, 1.8, and 0.92 kb were apparent for the week 4 isolate. In a comparison with the corresponding protein profiles in Fig. 2, the hybridization patterns of the *Hin*dIII digests of genomic DNAs of these weekly isolates correlated directly with the shift in S-layer proteins. Interestingly, isolates from weeks 2 to 9 which showed major protein bands of more than 110 kDa also exhibited additional hybridizing bands of approximately 4.0 and 3.4 kb that were not observed for the week 0 and 1 isolates. The 1.35-kb hybridizing segment was no longer evident in the week 9 isolate, and thus far, repeated (five times) in vitro subcultures of this isolate have not shown any further changes in the DNA hybridization pattern (data not included).

The ability of the oligonucleotide probe based on the *sapA* sequence from *C. fetus* subsp. *fetus* to hybridize with multiple fragments of genomic DNA from *C. fetus* subsp. *venerealis* indicates that *sapA* is also conserved in the latter subspecies and that multiple *sapA* homologs are present in both subspecies. Previous studies indicated that an in vitro shift in S-layer protein expression by a single strain was associated with rearrangement of *sapA* homologs (26). We now show that this phenomenon occurs in vivo in the natural host. Furthermore, we provide evidence that each of the shifts in S-layer protein expression and antigenicity was associated with genomic rearrangement. This finding is consistent with the multiplicity of *sapA* homologs (25) and their organization, with both conserved and variable regions (26). Our combined observations indicate the dynamic changes in *C. fetus* S-layer protein antigen expression during vaginal colonization and show that this in vivo antigenic variation is accompanied by genomic rearrangement and possibly amplification of the *sapA* homologs, as indicated by the increase in copy numbers in week 2 to 9 isolates.

The results of this in vivo study not only reinforce previous work (28) by demonstrating that persistent colonization of the bovine vagina by *C. fetus* is associated with antigenic variation of the S-layer proteins but also indicate that this variation is a high-frequency event. It is interesting that the week 1 isolate appeared identical to the inoculant. If the development of a specific host antibody response selects for *C. fetus* clones with S-layer protein variants, then the 1-week time point may have been too early for a sufficient host response to develop. Indeed, Corbeil and coworkers (4) demonstrated that it took 14 days after intrauterine instillation of *C. fetus* in heifers before antibodies against the organism first appeared. The frequency of spontaneous clearance (or reappearance) in the bovine reproductive tract is not clearly understood, although host immune response may be a major factor. The presence of an S-layer, while apparently important for colonization of the bovine vagina, is not sufficient. *C. fetus* subsp. *fetus* 23D, which has an intact S-layer (10) and is virulent in mice (19), was not able to colonize the bovine reproductive tract despite repeated attempts (data not shown). The ability of ADRI 1023 to colonize the bovine vagina indicates that human isolates of *C. fetus* subsp. *venerealis* closely resemble their bovine counterparts in terms of antigenic structure, biochemical features, and now biological activity. Another human isolate of *C. fetus* subsp. *venerealis* (ADRI 1024) with identical biochemical and molecular properties (i.e., those determined by SDS-PAGE, restriction endonuclease analysis, and ribotyping) also colonized the bovine cervicovaginal tract. Since these two strains had been isolated from two women who had been involved with the same sexual partner, transmission (as in cattle) was most likely by a venereal route. Thus, it is probable that *C. fetus* subsp. *venerealis* infection can occur in humans as a sexually transmitted disease. The bovine model appears to be suitable for testing the virulence of human isolates of *C. fetus* subsp. *venerealis* and also will be useful for studying isogenic *C. fetus* mutants.

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