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Correlation between Molecular Size of the Surface Array Protein and Morphology and Antigenicity of the *Campylobacter fetus* S Layer

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The correlation between the molecular size of the surface layer protein (S protein) and both structure and antigenicity of the *Campylobacter fetus* surface layer (S layer) was investigated in several clinical strains and their spontaneous variants which produce S proteins of molecular weights (MW) different from those of the parents. Only three molecular sizes of the S proteins were observed (98, 127, and 149 kDa) in the parental and variant strains. Immunologically, the 98-kDa protein and the 149-kDa protein but not the 127-kDa protein were cross-reactive. Freeze-etching analysis showed that the 98-kDa S protein formed a hexagonal arrangement with a 24-nm center-to-center space and that the S proteins with larger MW (127 or 149 kDa) formed tetragonal ones with an 8-nm center-to-center space. Thus, the MW changes of the S proteins seen in the variant strains were associated with both morphological and antigenic changes in S layer. These observations support the hypothesis that the pattern and antigenicity of the *C. fetus* S layer is determined by the particular type of S protein. Furthermore, the presence of the two different S layer patterns on a single bacterial cell indicates that multiple S proteins can be produced and expressed in a single cell.

Campylobacter fetus subspecies *fetus* (*C. fetus*), a veterinary pathogen, is now recognized as a human pathogen causing systemic infections such as sepsis or meningitis in compromised hosts (5, 21). In *C. fetus* infection a surface layer (S layer) plays an important role in invasion and survival within the host (2, 9, 19).

S layers cover cell surfaces of numerous bacterial species. They are composed of single subunits of a glycoprotein or a protein called a surface array protein (S protein). S layers also are called regular surface arrays or crystalline surface layers because they have a highly periodic hexagonal, tetragonal, or oblique pattern (for reviews, see references 13, 23, and 24).

Since the S layer of *C. fetus* was described as an antiphagocytic antigen (15, 18), information about its value to the organism has been accumulating. Strains with the S layer usually resist phagocytosis by polymorphonuclear leukocytes, but this anti-phagocytic ability is lost in the presence of specific opsonizing antibodies (3). The S layer inhibits binding of several lectins to the *C. fetus* cell surface (8), and the presence of the S layer is associated with increased virulence in experimentally infected mice (19).

The S proteins of *C. fetus* represent a family of high-molecular-weight (MW) proteins that share biochemical and antigenic characteristics (20); proteins of 98 to 100, 127, and 149 kDa have been demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20). Morphologically, hexagonal, tetragonal (9), and oblique (7) S layers all have been reported. Although this diversity of size and structure of the S layer of *C. fetus* has been reported, it was considered a strain-specific taxonomical feature. However, recently the presence of *C. fetus* variants obtained during in vitro passage which produce S proteins with MW different from that of the parent have been observed (6, 26, this paper).

Our work with several *C. fetus* strains and their variants, isolated under different conditions in widely separated places, demonstrates a regularity of the MW change in the S protein. We now report that the variants which differ from their parents by MW of their S proteins possess different S layers both in morphology and antigenicity. These observations suggest a correlation between the size of an S protein and both the structure and antigenicity of the *C. fetus* S layer.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *C. fetus* strains TK and M were clinical isolates in Japan and were kindly donated by T. Morooka (Fukuoka University Hospital, Fukuoka, Japan) and by S. Miake (Hakujuji Hospital, Fukuoka, Japan), respectively. Strains 84-112 and 82-40LP were clinically isolated in the United States. *C. fetus* 82-40LP3, a spontaneous variant of 82-40LP, was obtained during in vitro passage on trypticase soy agar with 5% sheep erythrocytes (BBL Microbiology Systems, Cockeysville, Md.). Strain 84-112AP3 was isolated from a mouse liver inoculated with strain 84-112 (9).

These strains were kept at −70°C in brucella broth (Difco Laboratories, Detroit, Mich.) immediately after isolation. They were cultured on brucella agar plates (Difco Laboratories) at 37°C for 24 or 48 h in a GasPak jar without catalyst (BBL Microbiology Systems) for use in experiments.

Animal passage in suckling mice and isolation of variants. The method of oral inoculation was essentially the same as described previously (9, 17). One-tenth mililiter of bacterial suspension was inoculated into a stomach of a suckling mouse (ddY, 5 to 6 days old), through a fine polyethylene tube attached to a small injection syringe with a 23-gauge

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needle. Three days after inoculation, bacteria were cultured from the mouse liver by homogenizing the organ with a Potter type glass homogenizer. Brucella agar plates containing antimicrobial agents (polymyxin B, 2,500 IU/liter; vancomycin, 10 mg/liter; trimethoprim, 5 mg/liter) were used for the isolation of *C. fetus*.

Extraction of S protein. An S protein was extracted from each strain by the method of McCoy et al. (16). In short, bacterial cells cultured for 48 h were suspended in 100 ml of 0.2 M glycine-hydrochloride buffer (pH 2.2) at a concentration of 5×10^9 CFU/ml. After gently rocking on a shaker for 20 min at room temperature, cells were removed by centrifugation at 12,000 × *g* for 30 min. The pH of the supernatant was then adjusted to 7.5 with NaOH, and proteins were precipitated by adding $(NH_4)_2SO_4$ (50 g/100 ml). The precipitate was dissolved in 5 ml of 0.05 M Tris-HCl buffer (pH 7.5) and dialyzed overnight at 4°C against the same buffer. The dialysate was concentrated by using a PM5 membrane filter (Amicon Corp., Lexington, Mass).

Anti-S-protein antisera. Six ddY mice (male, 4 weeks) were immunized intraperitoneally with 0.2 ml of S protein mixed with an equal volume of Freund's complete adjuvant (Difco Laboratories). After two booster injections at 1-week intervals, the mice were bled and sera were separated.

Freeze-etching method. Freeze-etching was carried out either with a Balzers' device (BAF301, Balzers Union, Lichtenstein) or with a JEOL's device (JFD9000, JEOL Co. Ltd, Tokyo) as described previously (9). A specimen was frozen in Freon 25 cooled by liquid nitrogen. After fracturing at −110°C at a pressure of less than 10^{-6} torr (10^{-6} mmHg), the specimen temperature was raised to −100°C for 10 min to etch the fractured surface. A replica of the freeze-etched surface was made by shadowing with platinum-carbon at an angle of 45° followed by evaporating the carbon in the vertical position. The replica was cleaned with sodium hypochlorite and finally washed with distilled water. The replica then was placed on a copper grid and was examined with a JEM 2000EX electron microscope (JEOL) at 100 kV.

Image processing. Micrographs were scanned with Luzex 3 (Nikon, Tokyo, Japan), an image processor, and selected for subsequent computer image analysis. Digitized data (1,024 by 1,024 pixels) were preprocessed and Fourier transformed.

Electrophoresis. SDS-PAGE was performed by the method of Laemmli (14) with modifications as described (9).

Immunoblotting. Electrophoresed whole-cell lysates were electrotransferred to a nitrocellulose sheet (membrane filters, 0.45-μm pore size; Schleicher & Schuell, Dassel, Germany) by the method of Towbin et al. (25). After transfer, the sheet was blocked by soaking in 0.5% (wt/vol) bovine serum albumin-containing PBS-Tween (phosphate-buffered saline, pH 7.8, supplemented with 0.05% Tween 20) and then washed three times with PBS-Tween. The sheet was incubated with an anti-S-protein antiserum at room temperature for 90 min and washed three times with PBS-Tween for 15 min each. The reactivity of antibody probe to the S protein was detected by incubating the sheet with peroxidase-conjugated goat anti-mouse immunoglobulin G (Zymed Laboratories, Inc., San Francisco, Calif.) at room temperature for 60 min. After being washed three times with PBS-Tween, the sheet was soaked in a solution of 4-choloro-1-naphthol (40 mg of substrate in 100 ml of 50 mM Tris-HCl, pH 7.5), to which 35 μl of 30% (wt/vol) hydrogen peroxide had been added.

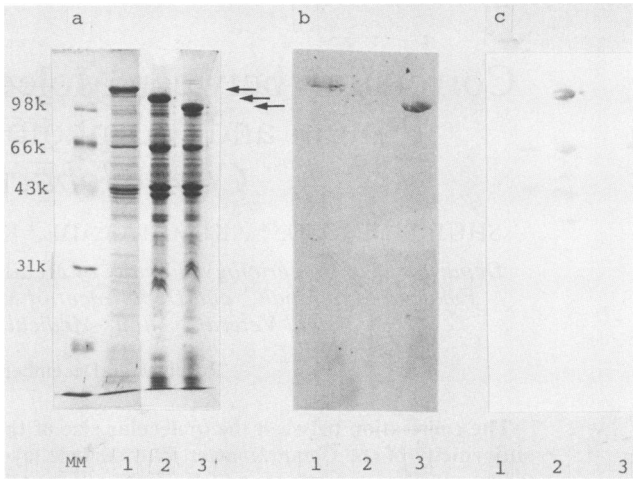

FIG. 1. SDS-PAGE (12.5% acrylamide) and immunoblots of whole-cell preparations from different *C. fetus* strains. Lanes 1, 2, and 3, preparations from *C. fetus* strains 84-112, M, and TK, respectively. (a) Coomassie blue-stained preparation. The three strains showed different major bands migrating at 98 kDa (TK), 127 kDa (M), and 149 kDa (84-112) (arrows). (b and c) Immunoblots with mouse antiserum TK-SP (b) and M-SP (c). The antiserum TK-SP, raised against the 98-kDa S protein of strain TK, reacted with both 98- and 149-kDa S-protein bands (b, lanes 1 and 3) but not with the 127-kDa band (b, lane 2). In contrast, antiserum M-SP, raised against the 127-kDa S protein of strain M, reacted only with the 127-kDa S-protein bands of strains M (c, lane 2) and TK (c, lane 3). Sera were diluted 1:100.

RESULTS

Antigenic and molecular-size variations among *C. fetus* S proteins. SDS-PAGE of three clinical isolates, 84-112, M, and TK, showed that the MW of each major S protein were 149,000, 127,000, and 98,000, respectively (Fig. 1a, arrows). Although the major S proteins of these strains differ in molecular size, other S proteins also were present as minor bands which stained faintly by Coomassie blue (Fig. 1a). Western blot (immunoblot) analysis with antisera to the S proteins showed antigenic variation among these S proteins. As shown in Fig. 1b and Fig. 1c, the S protein of strain 84-112 (149 kDa) and that of strain TK (98 kDa) were antigenically cross-reactive but the S protein of strain M (127 kDa) did not react with either the anti-84-112 or the anti-TK serum.

Two morphological types in *C. fetus* S layer. We examined the morphology of the S layers of the above-mentioned three strains by the freeze-etching technique. The results are shown in Fig. 2. The 98-kDa S protein of strain TK formed a hexagonal arrangement with a 24-nm center-to-center space (Fig. 2a). In contrast, strain M and 84-112, which possessed 127- and 149-kDa S proteins, respectively, had tetragonal S layers with an 8-nm center-to-center space (Fig. 2b).

S proteins of spontaneous variants. It has been observed that during passage in animals or in vitro, *C. fetus* may express an S protein with a MW different from that of the inoculated strain. The individual S protein must contain the information for assembly, because S layers are self-assembly systems (13); however, it is possible that the variant's S protein forms an S layer that differs from that of the parent, both morphologically and antigenically.

To verify this hypothesis we then examined S layers of

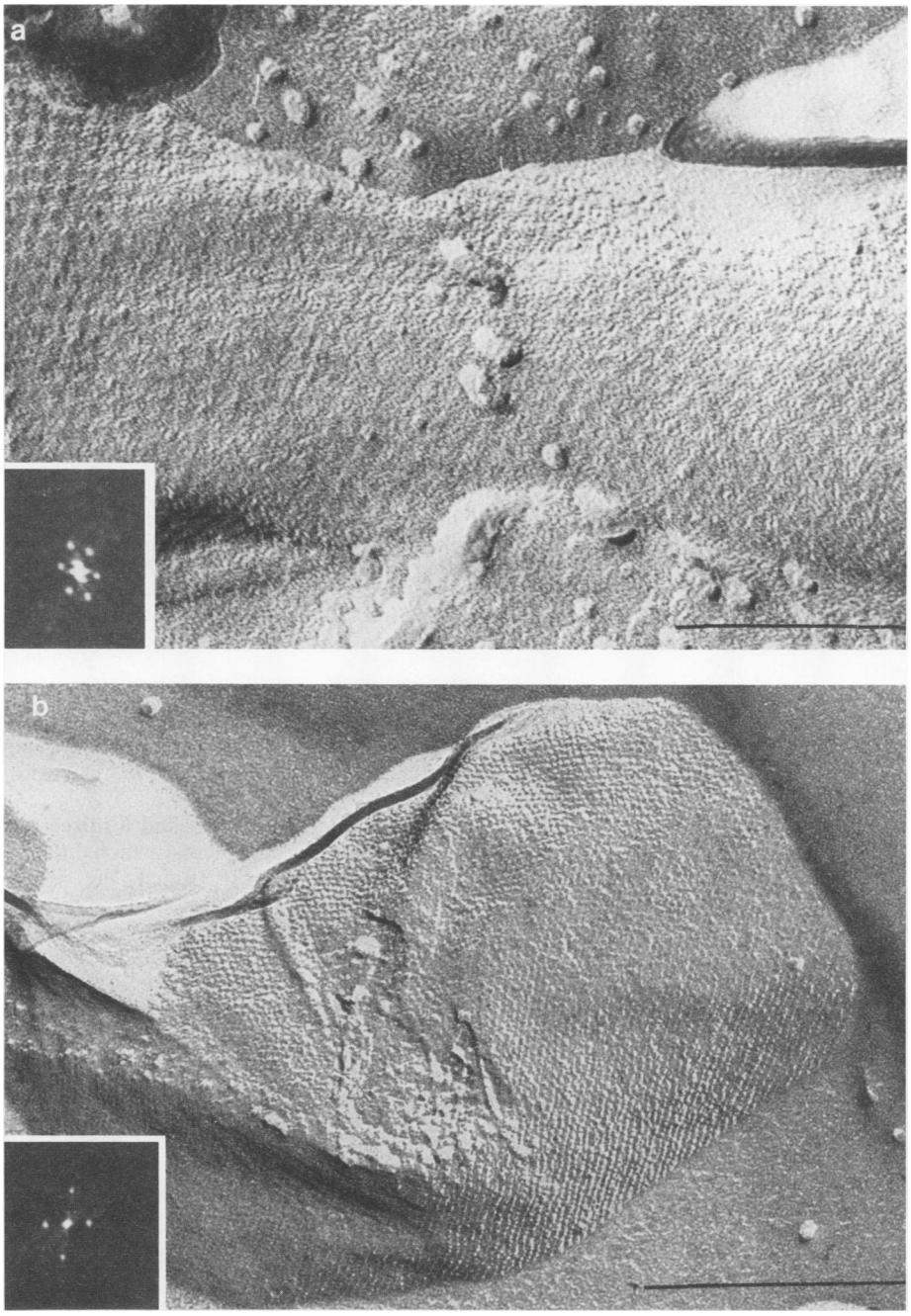

FIG. 2. Hexagonal and tetragonal S layers of *C. fetus* and their optical diffractions. (a) Freeze-etched preparation of *C. fetus* TK showing a hexagonal S layer with a 24-nm center-to-center space. In contrast, there was a tetragonal arrangement of S proteins (b) with an 8-nm center-to-center space on the surface of *C. fetus* strain M. Bar, 200 nm. Each optical diffraction pattern from panels a and b (insets) showed the hexagonal or tetragonal pattern.

two variants which differ from the parents only by the MW of the S protein. Strain 84-112AP3, a spontaneous variant isolated after mouse passage of strain 84-112 (149-kDa S protein), expressed a 98-kDa S protein (Fig. 3A). Antisera raised against the 149-kDa protein or the 98-kDa protein each recognized the 149- and 98-kDa bands as expected (data not shown). Strain 82-40LP3, which was derived from strain 82-40LP (98-kDa S protein) during in vitro passage, possessed a 127-kDa S protein (Fig. 3B).

Figure 3B, panel b, shows the antigenic difference between the S protein of strain 82-40LP3 and that of the parent strain, 82-40LP. The major 98-kDa band of strain 82-41LP was not recognized by the antiserum to the 127-kDa S protein of an unrelated strain, but the minor 127-kDa band of 82-40LP and the major 127-kDa band of 82-40LP3 both were recognized.

Freeze-etching analysis of the variants revealed that the 127-kDa S protein of 82-40LP3 consisted of a tetragonal

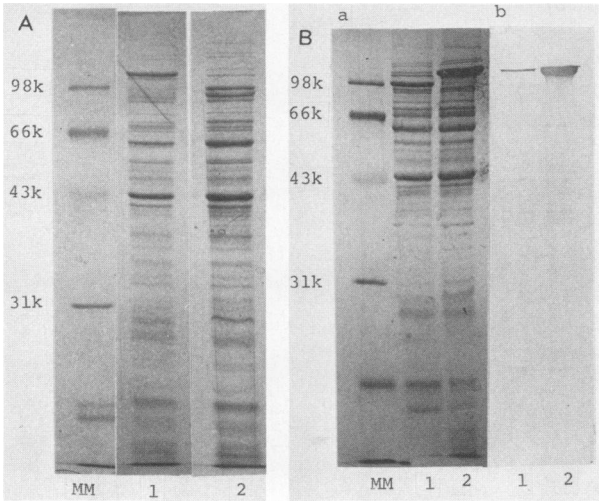

FIG. 3. (A) SDS-PAGE (12.5% acrylamide) and Coomassie blue staining of whole-cell preparations of strain 84-112 (lane 1) and 84-112AP3 (lane 2). (B) SDS-PAGE (12.5% acrylamide and Coomassie blue staining) (panel a) and immunoblot (panel b) of whole-cell preparations of strains 82-40LP (lanes 1) and 82-40LP3 (lanes 2). MM, molecular weight markers. By SDS-PAGE the mutants (84-112, 82-40LP) and the parent strains (84-112AP3, 82-40LP3) showed essentially the same profiles except for the S-protein bands. In panel b, the antiserum M-SP reacted with a 127-kDa band in 82-40LP3 but not with the 98-kDa band in 82-40LP. There was a minor band in 82-40LP migrating at 127 kDa, which reacted with the antiserum M-SP (lane 1).

arrangement (Fig. 4b) whereas the parent, 82-40LP, had a hexagonal S layer (Fig. 4a). In contrast, the S protein of strain 84-112AP3 formed a hexagonal S layer which differed from the tetragonal S layer of its parent, 84-112. These observations suggest that the S proteins of larger molecular size (127 and 149 kDa) are in the tetragonal arrangement and that the smaller molecule (98 kDa) arranges the hexagonal S layer.

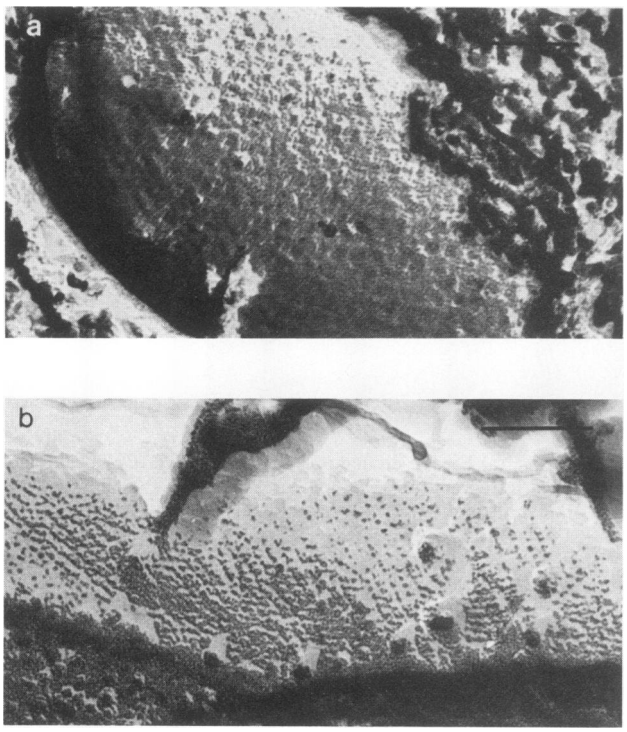

FIG. 4. Freeze-etching preparations of *C. fetus* 82-40LP and its spontaneous mutant, 82-40LP3. (a) Freeze-etched preparation of 82-40LP showed the hexagonal S layer with a 24-nm center-to-center space. (b) In contrast, 82-40LP3 had a tetragonal S layer with an 8-nm center-to-center space. Bar, 100 nm.

The minor S protein and a mixed-type S layer. The presence of minor S proteins, which differ from the major one by MW, in SDS-PAGE (Fig. 1, Fig. 3A and B) suggests that a single *C. fetus* strain could express several kinds of S proteins which showed different antigenicities (98-kDa and 127-kDa proteins, Fig. 1b and 1c, lanes 3). Alternatively, the population of cells sampled could include a majority of cells expressing one S protein and a minority expressing others. However, by electron micrography we occasionally noted the presence of two different arrangements of S proteins on a single cell as shown in Fig. 5. This observation indicates that even a single cell can produce different S proteins expressed together on its surface. Alternatively, reattachment of another type of S protein produced by a different cell could have occurred.

DISCUSSION

Recent research on the S layer of *C. fetus* showed the diversity of the molecular size of the S protein (98, 127, and 149 kDa) (20) and the pattern (hexagonal, tetragonal [9], and oblique [6, 7] type) of the S layers, and that variants with S proteins of different MW can be isolated by passage of a *C. fetus* strain through an animal or on a culture medium. Studies of these variants showed that the change in molecular size was usually associated with changes in antigenicity (6, 26), but the relationship between the size of the S protein and the morphology of the S layer has not been determined.

In this study, we used clinical strains isolated in widely separated places (Japan and the United States) and their spontaneous variants differing from the parents only by MW of the S protein. In our examination of these strains, we could identify S proteins with only three different molecular sizes (98, 127, and 149 kDa), and the MW change in the variants occurred only within these three molecular sizes.

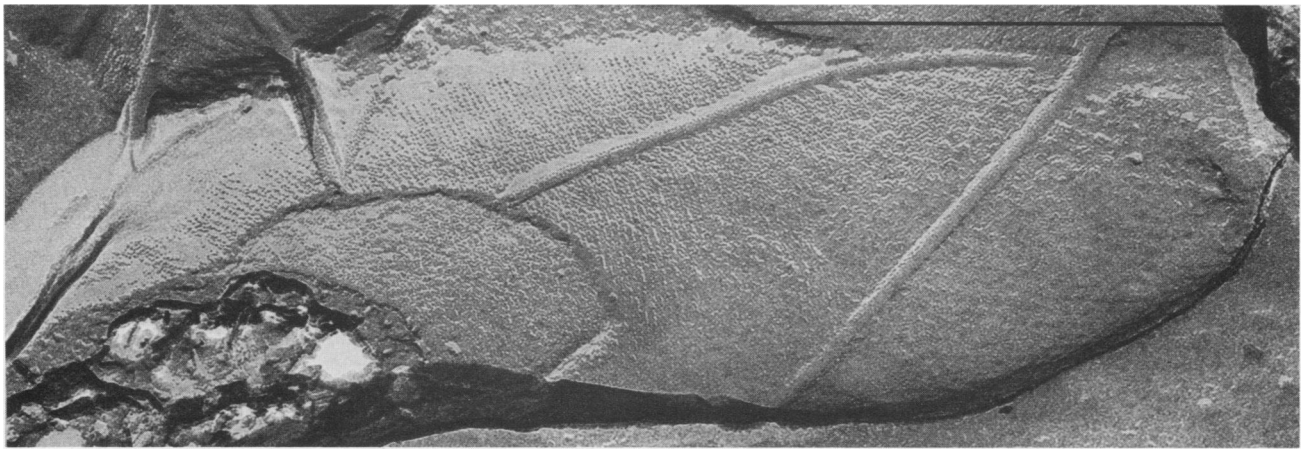

FIG. 5. Two different types of S layers on a single cell of *C. fetus* strain M as revealed by freeze-etch electron microscopy. There is a tetragonal (on the left side) and a hexagonal (on the right side) pattern on the surface of a single *C. fetus* cell. Bar, 500 nm.

These observations indicate that the molecular size variation in *C. fetus* S proteins is not random, and they suggest that the repertoire for size variation is highly limited.

The three types of S proteins had different antigenicities and formed different S-layer patterns (Table 1). In this analysis, using polyclonal antisera, the variability in the antigenicity was not related to molecular size or the array pattern, since the 98- and 149-kDa S proteins shared the same antigenicity although they had different S-layer patterns. However, our data strongly suggest a correlation between S-layer pattern and MW of S protein, with the 98-kDa S protein forming a hexagonal S layer and the larger S proteins (127 or 149 kDa) forming tetragonal ones. This also implies that the pattern of S layer is variable for an individual strain and is affected by the MW of the S protein.

Antigenic analysis with monoclonal antibodies directed against the S protein of strain 82-40LP suggest that the antigenic change in the S protein of 82-40LP3 might be induced by peptide changes toward the carboxy terminus of the protein (26). Since the N-terminal amino acid sequence of *C. fetus* S protein is highly conserved (20), it may be an attachment site to an underlying bacterial cell. It is possible that the carboxy terminus extends to the outside of the S layer and contributes to the form and antigenicity of the S layer. These data suggest that the family of *C. fetus* S proteins possibly represent a single protein that is processed to various degrees in the carboxy-terminal region. This hypothesis is also supported by the fact that *C. fetus* strains sometimes express S proteins with different molecular sizes as minor proteins (20; our data). Furthermore, as observed in this study, the presence of two different patterns on a single cell indicates that more than one S protein can be produced by a single cell. To evaluate the alternative hypothesis, we plan to determine whether reattachment (28) of more than one S protein to an individual cell can occur.

S layers act as virulence factors in bacterial infections. *Aeromonas salmonicida* becomes less virulent for fish after loss of its S layer (11, 12.). In *C. fetus* infection, the S layer also has an important role as a protective barrier against phagocytes and complement-mediated serum bactericidal activity (2, 3, 15). Since specific antibody for the S protein acts as an opsonizing antibody (3), the antigenic variability in the S protein might help *C. fetus* to evade host defense mechanisms (4). A parallel phenomenon has been recognized in *Borrelia hermsii* (22).

Although genetic studies of the 98-kDa S protein of *C. fetus* strain 84-32 (1) have been done, we do not know how *C. fetus* can alter its S protein. Further studies of the synthesis of the *C. fetus* S protein and its regulation are warranted.

TABLE 1. *C. fetus* strains and characteristics

Strain	Source	10^3 MW of major S protein	S-layer pattern	Reactivity of antisera[a]		
				TK-SP	M-SP	84-112-SP
TK	Human	98	Hexagonal	+	−	+
M	Human	127	Tetragonal	−	+	−
84-112	Bovine	149	Tetragonal	+	−	+
84-112AP3	Variant of 84-112	98	Hexagonal	+	−	+
82-40LP	Human	98	Hexagonal	+	−	+
82-40LP3	Variant of 82-40LP	127	Tetragonal	+	+	−

[a] +, antisera react with S protein in immunoblot; −, antisera do not react with S protein in immunoblot.

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