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

SHUI FUBJIMOTO,1 AKEMI TAKADE,2 KAZUNOBU AMAKO,2 and MARTIN J. BLASER2

Department of Bacteriology, Faculty of Medicine, Kyushu University, Maidashi, Fukuoka, Japan,1 and Department of Medicine, Vanderbilt University School of Medicine and Veterans Affairs Medical Center, Nashville, Tennessee 372322

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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 expressed and produced 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, 15, and 24).

Since the S layer of C. fetus was described as an anti-phagocytic 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 (17) 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 strain TK and M were clinical isolates in Japan and were kindly donated by T. Morooka (Fukuoka University Hospital, Fukuoka, Japan) and by S. Maek (Hokkai Hospital, Fukuoka, Japan), respectively. Strains 84-112 and 82-40LP were clinically isolated in the United States. C. fetus 82-40LP-J3, a spontaneous variant of 82-40LP-J, was obtained during in vitro passage on trypticase soy agar with 5% sheep erythrocytes (BHI, Microbiological 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 (BHI, 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 milliliter of bacterial suspension was inoculated into a stomach of a guinea pig (ddY, 5 to 6 days old); through a fine polyethylene tube attached to a small injection syringe with a 23-gauge...
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 \textit{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 200 ml of 0.2 M glycine-hydrochloric buffer (pH 2.2) at a concentration of 5 x 10^8 CFU/ml. After gently rocking on a shaker for 20 min at room temperature, cells were removed by centrifugation at 12,000 rpm for 30 min. The pH of the supernatant was 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 PMS membrane filter (Amicon Corp., Lexinton, Mass).

### Anti-S-protein antiserum

Six 6-8Y mice imvune (4 weeks) were intranasally immunized 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 with a Balzers' device (BAP 301, Balzers Union, Liechtenstein) as well as with a JED-1 device (JET 1000, Jodel Co., Ltd., Tokyo) as described previously [9]. A specimen was frozen in Freon 225 cooled by liquid nitrogen. After fracturing at -110°C at a pressure of less than 10^-6 Torr, 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 a Lucan 3 (Nikon, Tokyo, Japan), an image processor and selected for subsequent computer image analyses. Digitized data (1,024 x 1,024 pixels) were preprocessed and Fourier transformed.

### SDS-PAGE

SDS-PAGE was performed with the method of Laemmli ([24]) with modifications as described [9].

### Immunoblotting

Electrophoresed whole-cell lysates were electrotransferred to a nitrocellulose sheet (membrane filter, 0.45-μm pore size; Schleicher & Schuell, Dassel, Germany) by the method of Towbin et al. [68]. After transfer, the sheet was blocked by soaking in 5% (v/v) bovine serum albumin-containing PBS-Tween (phosphate buffered saline, pH 7.5, 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 then 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 (IgG) (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-chloro-1-napthol 148 mg of substrate in 100 ml of 50 mM Tris-HCl buffer (pH 7.5), to which 35 μl of 10% (v/v) hydrogen peroxide had been added.

### RESULTS

#### Antigenic and molecular-size variations among \textit{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). The S protein of strain TK formed a hexagonal S layer with a 24-nm center-to-center space. In contrast, there was a tetragonal arrangement of a 127 kDa protein on the surface of \textit{C. fetus} strain M. The major S protein of strain TK was recognized by both the anti-84-112 and the anti-TK serum.

#### Two morphological types in \textit{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 did not react with either the anti-84-112 or the anti-TK serum.

#### Immunoblot analysis

It has been observed that during passage in animals or in vitro, \textit{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 structures [1]. In contrast, the morphology of the S layer of strain TK (98 kDa) was morphologically and antigenically different.

To verify this hypothesis, we then examined S layers of two variants which differ from the parents only by the MW of the S protein. Strain 84-112 transformed into a spontaneous variant after mouse passage of strain 84-112. The 98-kDa S protein expressed in the variant showed a 127 kDa band (Fig. 3A). Anti-sera raised against the 98-kDa protein of strain TK, reacted with both the 98- and 149-kDa S-protein bands (Fig. 3b). Anti-sera raised against the 127-kDa protein of strain M, reacted only with the 127-kDa S-protein bands of strains M (lane 2) and TK (lane 3). Sera were diluted 1:100.
parent strains (84-112AP3, 82-40LP3) showed essentially the same profile except for the S-protein bands. In panel b, the antiserum reacted with a 127-kDa band in 82-40LP3 but not with the 98-kDa band in 84-112AP3. There was a minor band in 82-40LP3 migrating at 127 kDa, which reacted with the antiserum M-SF (lane 1).

arrangement (Fig. 4b) whereas the parent, 82-40LP, had a 127-kDa band in 82-40LP3 (lanes 2). In SDS-PAGE, the mutants (84-112, 82-40LP) and the parent strains (84-112AP3, 82-40LP3) showed essentially the same profile except for the S-protein bands. In panel b, the antiserum reacted with a 127-kDa band in 82-40LP3 but not with the 98-kDa band in 84-112AP3. There was a minor band in 82-40LP3 migrating at 127 kDa, which reacted with the antiserum M-SF (lane 1).

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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 antigens (98-kDa and 127-kDa proteins, Fig. 1b and c, 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 microscopy 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) and the pattern (hexagonal, tetragonal, 9, and oblique) in 71 types of the S layers, and that varieties with different molecular sizes of proteins can be isolated by passage of a C. fetus strain through an animal or on a culture medium. Studies of these varieties show that the change in molecular size is usually associated with changes in antigenicity (98, 127, and 149 kDa), 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 proteins. 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 within these three molecular sizes.

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