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Electron microscopic examination of ultrathin sections and freeze-etched and shadow cast preparations of a bovine prepuce isolate of *Campylobacter fetus* VC119 showed an S layer with subunits in an apparent linear arrangement. Surface radioiodination, enzyme digestion, low-pH extraction, and Western immunoblotting showed that the layer was composed mainly of one protein which is the predominant protein antigen of *C. fetus*. This protein was purified to homogeneity by gel filtration, ion-exchange chromatography, and high-performance liquid chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed an apparent molecular weight of 131,000 for this protein with a pI of 6.35, and no carbohydrate could be detected by a variety of techniques. Amino acid composition analysis showed that the protein contained approximately 1,304 residues per molecule, 41.2% of which were hydrophobic and approximately 22% of which were acidic. Cysteine and histidine were absent. Circular dichroism spectra showed that the prominent structure of the S layer protein was a β-pleated sheet (36%) with aperiodic foldings (31%); a moderate amount of α-helix (28%) and a low amount of β-turn (5%) were also present. The N-terminal amino acid sequence was determined for the first 18 residues. No sequence homology with other S layer proteins was found.

Campylobacter fetus causes venereal genital tract infections in cattle which can lead to infertility and abortion (35, 38). In humans the species has been increasingly recognized as an opportunistic pathogen causing a variety of extraintestinal infections (4, 6, 13, 24). Recent reports suggest that *C. fetus* can also cause an acute diarrheal illness (14, 24). While there have been relatively few studies on the virulence mechanisms of this pathogen, one surface antigen appears to be important to the ability of *C. fetus* to cause disease. In 1971, Myers (28) isolated the postgrowth broth (PGB) antigen, with an apparent molecular weight (M_r) of 135,000, from broth supernatants of cultures of *C. fetus*. McCoy et al. (25, 26) subsequently described a microcapsule on a strain of *C. fetus* which was associated with resistance to phagocytosis. The microcapsule, also called antigen *a*, was shown to contain a protein with an apparent M_r of 98,000, and although it had an apparent M_r different from that of the PGB antigen reported by Myers (28), Winter et al. (43) suggested that the PGB and *a* antigens were identical. Antigen *a* was shown to be associated with carbohydrates by Schiff staining of polyacrylamide gels, and in the study by Winter et al. (43), chemical composition analysis suggested that carbohydrates, including hexose, pentose, and methylpentose, accounted for 4% of the protein on a weight basis. Most recently, this antigen has been shown to contribute to the serum resistance of certain strains of *C. fetus* (4).

Although unable to consistently observe any ordered array of subunits on the surface of *C. fetus*, Winter et al. (43) suggested that, on the basis of amino acid composition and mode of association with the cell envelope, the microcapsule might be analogous to the S layer reported by Buckmire and Murray (7) on *Aquaspirillum serpens*. S layers or paracrystalline surface protein arrays are regular, two-dimensional

assemblies of protein monomers that often constitute the outermost layer of the cell envelope of many bacteria (21, 36, 37, 39). The surface location of these protein structures means that, in pathogens growing in vivo, the S layer must come into close contact with the cells and tissue fluids of its host. S layers are therefore ideally sited to influence the outcome of a host-parasite relationship (18, 27), and not surprisingly, S layers are being increasingly reported on pathogenic bacteria (9, 18, 19).

In this study, we examined the structure of the "glycoprotein microcapsule" of a bovine prepuce isolate of *C. fetus* VC 119 by using electron microscopy of ultrathin sections and freeze-etched and shadow cast preparations. We also identified and purified the S layer subunit protein, and here we report on the purification and biochemical properties, including the amino acid composition, N-terminal amino acid sequence, and predicted secondary structure of that protein.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *C. fetus* subsp. *fetus* VC119 was a bovine prepuce strain and was kindly supplied by P. L. Stovell, Animal Pathology Laboratory, Vancouver, British Columbia, Canada. Stock cultures were maintained at −70°C in tryptic soy broth (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 15% (vol/vol) glycerol. Cultures were grown in an anaerobic jar at 37°C on Mueller-Hinton agar (Difco Laboratories, Detroit, Mich.). An atmosphere containing 5% oxygen and 10% CO_2 was produced with a gas generation kit for campylobacters (Oxoid Ltd., Basingstoke, England).

Electron microscopy. For electron microscopic analysis of cell ultrastructure, cells were washed from plates with 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 8.0; Sigma Chemical Co., St. Louis, Mo.)

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containing 1 mM MgCl₂ and 1 mM CaCl₂ and washed twice. For embedding, cell suspensions were fixed by using a conventional 5% (vol/vol) glutaraldehyde-to-1% (wt/vol) osmium tetroxide fixation regimen, followed by aqueous 2% uranyl acetate in block staining and dehydration with an ethanol-to-propylene oxide Epon 812 series. Silver sections obtained on a Reichert Ultracut OMU 4 ultramicrotome were mounted on Formvar carbon-coated grids and stained with uranyl acetate and lead citrate. For freeze-fracturing and etching, 20% (vol/vol; final concentration) glycerol as a cryoprotectant was added to suspensions of bacteria. This material was freeze-cleaved, etched for 30 to 60 s at −100°C, platinum shadowed, and carbon replicated in a Balzers model BA 360M freeze-etching apparatus. Whole mounts were also platinum shadowed. Electron microscopy was done with either a Philips EM300 or EM400 operating at 60 kV under standard conditions with a liquid nitrogen-cooled anticontamination unit in place.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (22) in a mini-slab gel apparatus (Bio-Rad Laboratories, Richmond, Calif.). Protein solubilized in sample buffer was stacked in 4.5% acrylamide (100 V; constant voltage) and separated by using 7.5% or 12.5% acrylamide (200 V). Protein was stained by Coomassie blue, and glyco compounds were stained by the periodate-Schiff procedure (10).

When required, separated proteins were transferred from the slab gel to nitrocellulose paper by the methanol-Tris-glycine system described by Towbin et al. (40). Electroblotting was performed in a Bio-Rad TransBlot apparatus for 18 h at 60 V. Nondenaturing isoelectric focusing gels were run in tubes as described in the Bio-Rad manual and were stained with Coomassie blue by conventional methods. Denaturing isoelectric focusing gels in the presence of 8 M urea and 2% Nonidet P-40 were run as described by O'Farrell (29).

Isolation of outer membranes. After 48 h of growth on Mueller-Hinton agar, cells were harvested and washed twice in phosphate-buffered saline (pH 7.2). One gram (wet weight) of washed cells was suspended in 10 ml of 20 mM Tris buffer (pH 8.0). DNase and RNase A (Sigma) were added, and the cell suspension was then passed through a French pressure cell three times at 16,000 lb/in². Intact cells were removed by two centrifugations at 6,000 × g at 4°C for 30 min. Envelopes were collected by centrifugation at 40,000 × g at 4°C for 30 min. Cell envelopes were resuspended in the 20 mM Tris buffer (pH 8.0) to give a protein concentration of 1 mg/ml, and outer membranes (OM) were prepared by differential solubilization of the inner membrane with the detergent sodium lauryl sarcosinate (W. R. Grace Co., Nashua, N.H.) as described by Filip et al. (11). The total membrane protein-detergent ratio used was 1:6 (mg/ml). Following shaking at room temperature for 30 min, the preparation was centrifuged at 40,000 × g for 30 min at 4°C and the pellet was washed three times in Tris buffer. Finally the pellet was suspended in Tris buffer for protein concentration determinations and SDS-PAGE analysis.

S layer protein purification. After 48 h of growth on Mueller-Hinton agar, cells were harvested and washed twice in phosphate-buffered saline (pH 7.2). The washed cells were then suspended in 0.2 M glycine hydrochloride buffer (pH 2.2; 3 g of cells per 100 ml) as described by McCoy et al. (25), and the suspension was stirred for 20 min at room temperature. Whole cells were then removed by centrifugation at 12,000 × g for 15 min. The supernatant was neutralized with

NaOH and filter sterilized (0.22-μm-pore-size filter; Millipore Corp., Bedford, Mass.). Phenylmethylsulfonyl fluoride as a protease inhibitor (PMSF; Sigma) was added to the filtrate (0.1 mM) and stirred for 30 min at room temperature. The solution was then dialyzed against distilled water and lyophilized. The lyophilized sample was rehydrated in 1/10 of the initial volume of Tris hydrochloride (0.05 M; pH 7.0) and applied to a Sephadex G-75 column (bed volume, 180 ml) equilibrated in the same buffer. The protein of interest eluted in the first peak between 60 and 80 ml. The pooled protein sample was then ultracentrifuged at 160,000 × g for 2 h at 4°C. The supernatant was collected and loaded onto the 25-ml bed volume of a Fractogel TSK DEAE-650 S column (E M Reagents, Gibbstown, N.J.). The column was then washed with 2 bed volumes of equilibration buffer (Tris hydrochloride [0.05 M; pH 7.0]) and eluted with a linear gradient of 100 ml of 0 to 0.25 M NaCl in the same buffer. The peak eluting around 0.18 M NaCl was pooled, dialyzed, and lyophilized. The protein of interest was then purified to homogeneity by high-pressure liquid chromatography (HPLC) with a Beckman HPLC system along with a Mono-Q column (Pharmacia, Uppsala, Sweden) and a gradient from 0 to 1.0 M NaCl. The A_{230} and A_{280} of the effluent from the Mono-Q column were monitored with a Beckman model 165 variable wavelength detector.

LPS analysis. Lipopolysaccharide (LPS) was determined by a modification of the procedure of Hitchcock and Brown (15). Cells were boiled in the SDS-PAGE solubilization buffer for 5 min and then digested with proteinase K at 60°C for 1 h at a ratio of 10 mg of bacterial cell mass to 1 mg of proteinase K. Samples were loaded onto 12.5% SDS-PAGE gels at a concentration of 50 μg of original cell mass per lane. After electrophoresis, gels were silver stained by the procedure of Tsai and Frasch (41).

Antibody production. Antiserum was raised in an adult New Zealand White rabbit by intramuscular injections. The rabbit was immunized with a Formalin-killed bacterial suspension of 10⁹ cells of a 24-h culture of C. fetus VC119 per ml. One milliliter of this suspension was emulsified with an equal volume of Freund complete adjuvant. A booster dose was given in Freund incomplete adjuvant on days 14 and 28. On day 42, the rabbit was exsanguinated and the serum was collected and stored at −20°C. Control nonimmune serum was obtained before the first injection.

Amino acid composition analysis. The purified S layer protein was dialyzed extensively against distilled water, lyophilized, and then hydrolyzed in 6 N HCl at 100°C for 18 h. Amino acid composition was determined on a Beckman 119CL amino acid analyzer. The method used was as described by the manufacturer for a 90-min single-column procedure. Cysteine was analyzed as cysteic acid after performic acid oxidation, and tryptophan was determined by the method of Penke et al. (30), involving hydrolysis of the protein with mercaptoethanesulfonic acid.

N-terminal sequence analysis. The amino acid sequence analysis was performed on a model 470A gas phase sequenator (Applied Biosystems, Foster City, Calif.) by running a standard operating program. Phenylthiohydantoin derivatives were separated on an IBM cyanocolumn (4.6 by 250 mm), as described by Hunkapiller and Hood (16), fitted to a Beckman model 332 HPLC equipped with a 3390A integrator (Hewlett-Packard Co., Palo Alto, Calif.).

Radioiodination of surface proteins. Cells were washed and suspended in cold phosphate-buffered saline. A 1:10 dilution gave an A_{550} of 1.0. A 100-μl sample of the suspension was radioiodinated with the New England Nuclear Corp.

(Boston, Mass.) radioiodination system (^{125}I), which uses immobilized lactoperoxidase and glucose oxidase. Enzyme beads were removed by brief low-speed centrifugation, and the ^{125}I-labeled cells were then washed five times and suspended in 20 mM Tris hydrochloride buffer (pH 7.4). The iodinated whole cells were then mixed with solubilization buffer, boiled for 5 min, and separated on SDS-PAGE gels. After migration, the gels were dried and submitted to autoradiography.

Western blotting (immunoblotting). After electroblotting, antigenic polypeptides were detected by reaction with antisera, followed by incubation with ^{125}I-radiolabeled *Staphylococcus aureus* protein A by the procedures previously described (17, 23). Detection of bound radiolabeled protein A was accomplished by autoradiography of washed and dried nitrocellulose sheets.

Enzyme digestion. Proteinase K (Merck & Co., Inc., Rahway, N.J.), trypsin, or α-chymotrypsin (Sigma) was added to 50 mg of bacterial cells in a 100-μl reaction volume and incubated for 30 min at 37°C. Reactions were stopped by placing the tubes on ice, adding solubilization buffer, and boiling the mixture for 10 min.

Lectin affinity chromatography. Purified protein (10 μg) was applied to a 1.0-ml-volume column of concanavalin A bound to agarose (Dimension Laboratories, Inc., Mississauga, Ontario, Canada). The column was then closed and left for 1 h at room temperature. Elution was done first by using 10 ml of the starting buffer as recommended by the manufacturer and then with 10 ml of starting buffer to which 0.2 M α-D-methylmannopyranoside (Sigma) was added. Fractions of 1.0 ml were collected. The presence of the protein was monitored by SDS-PAGE.

Radiolabeling of carbohydrates. The sodium borohydride reduction reaction was used to determine whether carbohydrates were associated with the S layer protein (5). HPLC-purified protein (12 μg) was oxidized with 10 mM sodium periodate for 5 min on ice. Glycerol was then added (0.1 M) to quench the oxidation reaction, and reduction was performed with tritiated sodium borohydride (0.5 mCi; New England Nuclear Corp., Boston, Mass.) for 15 min at room temperature. Hen egg white ovalbumin (5 μg; Sigma) was used as the positive control. The treated samples were diluted with solubilization buffer, boiled, and subjected to SDS-PAGE. After migration, the gel was soaked in scintillation liquid (En^{3}Hance; New England Nuclear), dried, and subjected to autoradiography.

CD measurements. Circular dichroism (CD) measurements were effected on a Jasco J-500 C spectropolarimeter interfaced with a DP 500N data processor. The cell was maintained at 25°C with a Lauda RM6 circulating water bath. Near UV (320 to 250 nm) scans were performed in a micro cell with a path length of 1 cm, which required only 90 μl of solution. Far UV (250 to 190 nm) runs were done in either a 0.01-cm or a 0.05-cm cell. Concentrations of *C. fetus* VC119 S layer protein ranged from 0.28 to 1.07 mg/ml. The computer-averaged trace of either four or eight scans was used in all calculations. Signal due to solvent was subtracted. The instrument was routinely calibrated with $d(+)$-10-camphorsulfonic acid at 290 nm and pantoyllactone at 219 nm as outlined by the manufacturer. The data were normally plotted as mean residue weight ellipticity (units: −degrees cm^2 dmol^{-1}) versus the wavelength in nanometers. The mean residue weight was taken to be 100.7.

Computer-assisted analyses. In addition to plotting the far UV CD data in terms of mean residue ellipticity versus wavelength, we determined the secondary structure of the S layer protein under a variety of conditions by using the mainframe-driven FORTRAN program CONTIN developed by Provencher and Glöckner (32), which analyzes CD spectra as a sum of data collected from 16 proteins whose structures are known from X-ray crystallography. The input to the program was the mean residue weight ellipticities in 1-nm intervals from the minimal value measured to 240 nm.

Fluorescence studies. Fluorescence emission and excitation spectra for the S layer protein were measured on a Perkin-Elmer MPF 44B recording spectrofluorometer equipped with the DCSU-2 corrected-spectrum accessory, which allows for automatic subtraction of fluorescence due to solvent. The instrument was operated in the ratio mode, and thermostat-equipped cells were maintained at 20°C. Detection of fluorescence was effected at 90° to the excitation beam. The emission and excitation slits were usually set at 5 nm. The optical densities of the solutions at the excitation wavelength of 280 nm never exceed 0.1, thus obviating the need to make corrections for self-quenching due to the inner-filter effect.

Absorption spectrum. The absorption spectrum for the S layer protein was measured on a Cary 118C recording spectrophotometer in a solvent system of 100 mM NaCl-25 mM Tris hydrochloride (pH 7.5).

Protein concentration determination. Concentrations of S layer protein in solution were measured by the A_{278} with an extinction coefficient established by using the refractometric method of Babul and Stellwagen (1), in which the absorbance of a protein sample is correlated with its weight concentration, the latter determined from synthetic boundary experiments in the analytical ultracentrifuge. An average refractive index increment of 4.1 fringes per mg per ml was used in these calculations.

Sedimentation equilibrium. A Beckman Spinco model E analytical ultracentrifuge equipped with an rotor temperature indicator and control (RITC) temperature control unit and electronic speed control was operated at 20°C. Cells with charcoal-filled Epon centerpieces (12 nm) and sapphire windows were used. Photographs from the Rayleigh interference optical system were analyzed on a Nikon model 6C microcomparator. The weight average molecular weight of the *C. fetus* VC119 S layer protein was measured by conventional low-speed sedimentation equilibrium techniques (33). Equilibrium photos were taken at intervals after 20 h until no further change was apparent. Before these measurements, the S layer protein sample was dialyzed for 48 h at 4°C against 1,000 volumes of 100 mM NaCl-25 mM Tris hydrochloride (pH 7.5) to ensure complete equilibration. A value of 0.73 ml g^{-1} was used for the partial specific volume.

RESULTS

Electron microscopy. When cells of *C. fetus* VC119 were examined by electron microscopy after negative staining with phosphotungstic acid, ammonium molybdate, or uranyl acetate, no surface detail was visible. It is possible that this S layer is not easily wettable (i.e., hydrophobic); however, when platinum-shadowed replicas were examined, the presence of a delicate S layer was revealed (Fig. 1A). Shallow (ca. 15 to 25°) shadowing angles were best, and the array was most commonly seen when the short axis of the cells was ca. 90° to the direction of the shadowing substance. Higher magnifications (Fig. 1B) showed the array to be linear. Ultrathin sections confirmed the cell coverage by the S layer and allowed the individual subunits of the S layer to be visualized (Fig. 2A and B). Freeze-fracture replicas of the
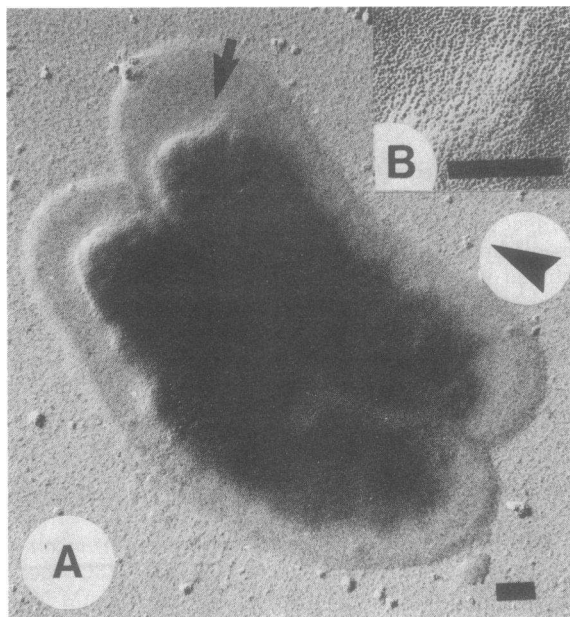
FIG. 1. (A) Shadowed replica of two *C. fetus* VC119 cells which have the array on their surface. The spacing of the array is so small that higher magnification is necessary to make it more apparent. The more highly magnified image (B) is the area pointed to by the arrow in panel A. The shadow direction is denoted by the arrowhead in the white circle. The bars are 100 nm.

outer (Fig. 2C) and inner surfaces (Fig. 2D) of the S layer of *C. fetus* VC119 also showed the subunits in an apparent linear arrangement. Analysis of the images from freeze-etched specimens revealed a center-to-center spacing of 8.75 ± 0.75 nm for the subunits.

Identification of the S layer protein. SDS-PAGE analysis of whole-cell lysates and isolated OM fraction of *C. fetus* VC119 showed that the predominant cell protein had an M_r of approximately 131,000 (Fig. 3, lanes 2 and 4). To demonstrate the surface exposure of this protein, cells were surface radiolabeled with ^{125}I by using the immobilized-lactoperoxidase-glucose oxidase procedure. Autoradiography of whole-cell lysates fractionated by SDS-PAGE revealed that the 131,000-M_r protein was strongly radiolabeled (Fig. 3, lane 3). SDS-PAGE analysis also indicated that the protein was removed from the cell surface and from the isolated OM fraction by a low-pH extraction procedure (0.2 M glycine hydrochloride [pH 2.2]) (Fig. 3, lane 1). Western immunoblotting of whole-cell lysate with antibodies raised against whole cells showed that the predominant cell protein antigen was the 131,000-M_r protein (Fig. 3, lanes 6 and 7). Further evidence for the surface exposure of this protein was provided by the susceptibility of the native protein to proteinase K digestion. When intact cells of *C. fetus* VC119 were exposed to 5 µg of the nonspecific protease proteinase K per ml, the 131,000-M_r protein was the first of the cell proteins to be hydrolyzed (data not shown). In contrast, trypsin at a concentration as high as 5 mg/ml had no effect on the native S layer, and the native S layer received only a minor clip by chymotrypsin at this concentration (data not shown).

Purification of the S layer protein. The S layer protein was removed from the cell surface by the 0.2 M glycine hydrochloride (pH 2.2) extraction procedure of McCoy et al. (25). The S layer protein was the predominant protein present in the 0.2 M glycine hydrochloride extract when the procedure

was performed on a preparative scale, although a number of other minor cell proteins and LPS were also present in the glycine fraction. PMSF was added at this step to minimize the possibility of degradation of the S layer protein by proteases. The concentrated sample was then applied to a molecular sieve (Sephadex G-75) to remove glycine, salts, and low-molecular-weight proteins. The proteins that eluted in the voided volume were then ultracentrifuged to pellet contaminating OM protein-LPS complexes. The supernatant obtained in this way contained only a few minor contamination proteins, and silver staining of SDS-PAGE showed that the preparation was free of LPS (data not shown). The supernatant was then loaded onto a Fractogel TSK DEAE-650 S ion-exchange column, and the protein of interest eluted as a single peak at 0.18 M NaCl. The protein was then purified to apparent homogeneity by high-pressure liquid chromatography with a Mono-Q column (Pharmacia) (Fig. 4). The S layer protein eluted at around 0.5 M NaCl. No LPS was detected by silver staining, and no carbohydrate was detected by Schiff staining after the purified sample was run on an SDS-PAGE gel. Radiolabeling of carbohydrates with tritiated sodium borohydride of HPLC-purified protein gave a negative result. Purity was confirmed by the presence of a single N-terminal amino acid sequence with a terminal methionine residue. The protein was completely soluble after purification.

Biochemical characterization. SDS-PAGE analysis of the purified protein indicated an apparent M_r of 131,000. Amino acid composition analysis showed that it contained approximately 1,304 residues per molecule, with Asx (asparagine or aspartic acid), threonine, leucine, valine, alanine, and glycine present in the highest molar concentrations (Table 1). Composition analysis also indicated that histidine and cysteine were absent and that only a single residue of methionine was present. It had a calculated relative hydrophobicity of 41.2% (assuming V, M, I, L, A, F, W, and P) and was predominantly acidic, as shown on a molar basis by its content of approximately 22% acidic amino acid residues, compared with approximately 6% basic residues. Isoelectric focusing on polyacrylamide rods showed that under nondenaturing conditions the protein focused into one band with a pI of 6.35, whereas under denaturing conditions (2% Nonidet P-40 and 8 M urea) the band focused at a pI of 8.00 (data not shown). N-terminal amino acid sequence analysis was performed, and the first 18 residues of the *C. fetus* VC119 S layer protein are shown in Table 2. The single methionine of the protein was shown to be the N-terminal residue, and no homology was shown with the N-terminal sequences reported for the *Aeromonas salmonicida* and *Bacillus brevis* S layer proteins.

The possible association of carbohydrate with the *C. fetus* VC119 S layer protein was also investigated. Schiff staining of the protein in isolated OM fractions (Fig. 3, lane 5) and HPLC-purified S layer protein (data not shown) both gave a negative result. Lectin affinity chromatography was also used to determine whether carbohydrates were associated. HPLC-purified protein did not bind to concanavalin A, suggesting that no carbohydrates with alpha anomers of manno- and glucopyranose residues were present. Furthermore, the protein was oxidized with sodium periodate and then reduced with tritiated sodium borohydride to radiolabel any carbohydrates present. No incorporation of radiolabel was observed at the position occupied by the protein when SDS-PAGE gels were submitted to autoradiography (data not shown).

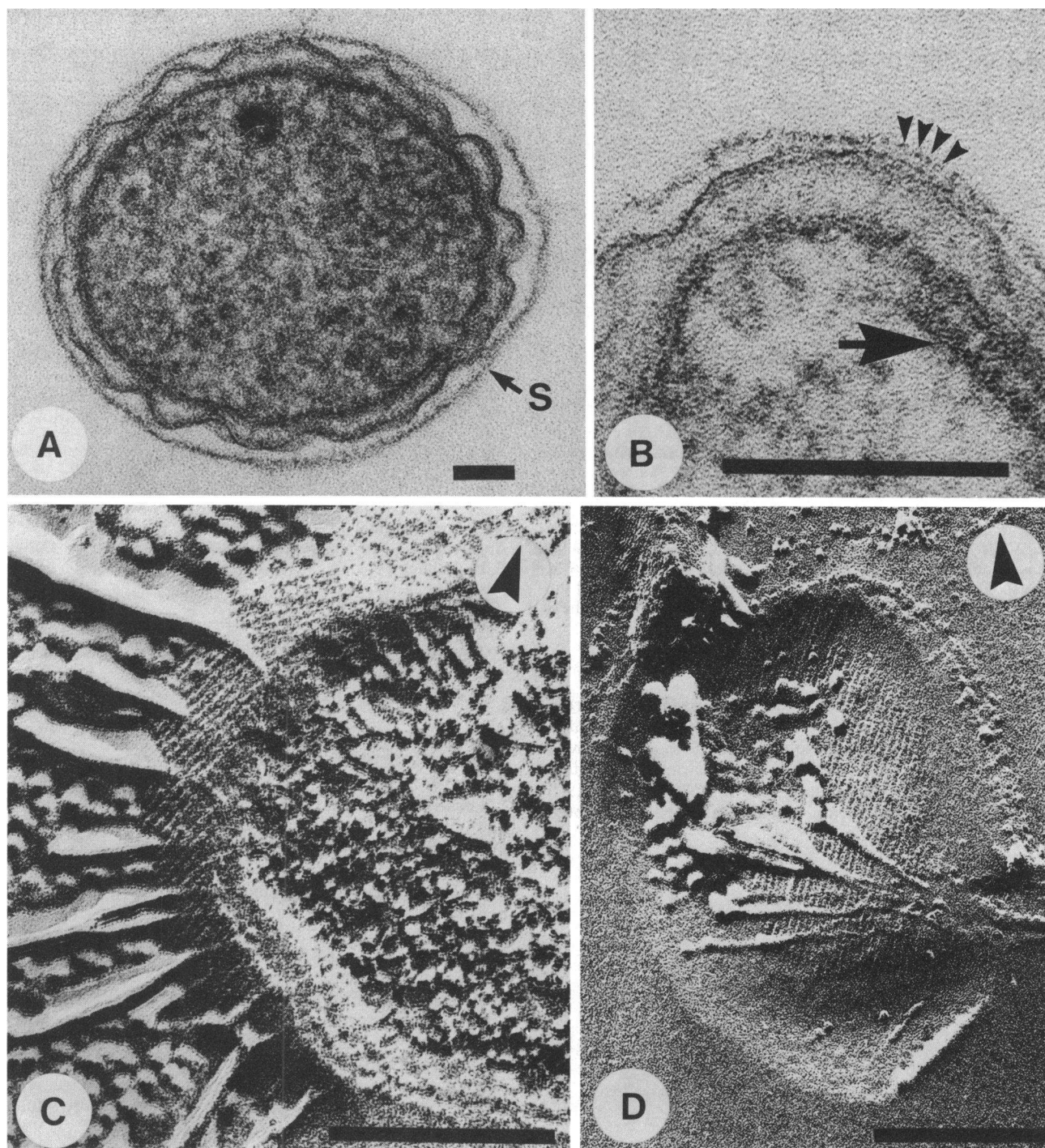**CD.** CD was chosen to measure conformational changes in

FIG. 2. Ultrastructural analysis of the cell surface of *C. fetus* VC119. (A and B) Ultrathin sections showing the surface array (S). A cross-section is shown in panel A, and a portion of a polar end is shown in panel B. Individual subunits are visible in panel B (small arrowheads), as is the additional inner polar membrane structure near the flagellum-based basal body insertion site (large arrow) characteristic of *C. fetus*. (C and D) Surface array and cell envelope freeze-fractured and -etched in the presence of 20% glycerol. The convex view in panel C shows the individual array subunits as a linear array. The concave view shown in panel D illustrates the overall basket weave appearance of the inner surface of the array. The shadow direction in the freeze-etch images is shown by the arrowhead in the white circle. The bars are 100 nm.

the protein and to predict the secondary structure of the S layer protein. Figure 5 shows the far UV CD spectra for the *C. fetus* VC119 S layer protein under three different sets of conditions. The spectrum of the native protein in 100 mM

NaCl–25 mM Tris hydrochloride at pH 7.5 showed a broad minimum centered at 216 nm ($[\theta]_{216nm} = -11,500°$). Since this protein was extracted in a glycine buffer at a pH near 2, it was felt appropriate to look at the structure at this low pH.
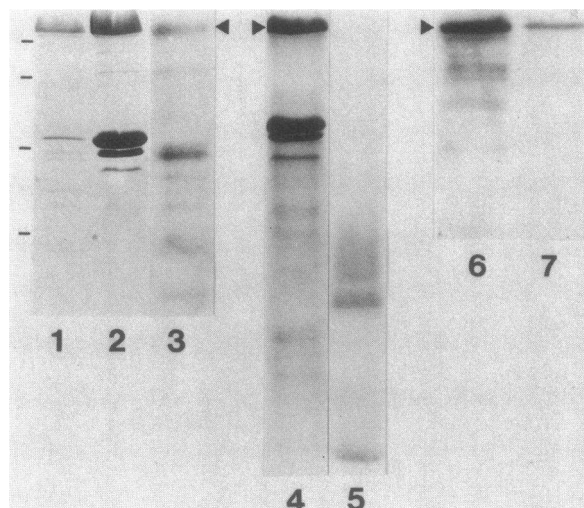
FIG. 3. (A) 12.5% SDS-PAGE analysis of *C. fetus* VC119. Lanes: 1, glycine extract of whole cells stained by Coomassie blue; 2 and 4, OM fraction stained by Coomassie blue; 3, autoradiogram of a whole-cell lysate of cells subjected to surface radioiodination; 5, OM fraction stained for carbohydrate by the Schiff procedure; 6 and 7, autoradiographs of a Western blot of a whole-cell lysate of *C. fetus* VC119 reacted with a 1:500 dilution of antiserum prepared against whole cells of *C. fetus* VC119 exposed for 48 and 16 h, respectively. Positions of M_r standards are marked on the left of the gel at 97,400 (top), 66,200, 42,699, and 31,000. The position of the S layer subunit protein is indicated by arrowheads.

A reduction in signal was noted; $[\theta]_{216nm}$ dropped from $-11,500°$ to $-8,900°$, and there was a 1-nm shift to 215 nm of the broad minimum. Acidic solutions of SDS have proved useful to induce helix structure in a variety of protein systems (44). This was the case with the *C. fetus* VC119 S layer protein as the spectrum noted in this medium adopted
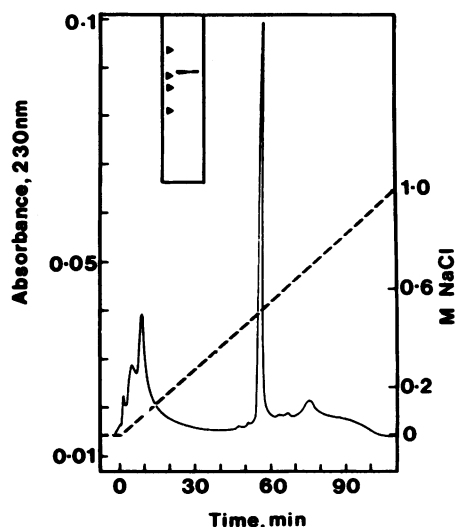

FIG. 4. HPLC elution profile of purification of *C. fetus* VC119 S layer protein by Mono-Q ion-exchange column chromatography. The inset shows 7.5% SDS-PAGE stained by Coomassie blue of the 131,000-M_r S layer protein contained in the major elution peak. Arrowheads indicate M_rs of 200,600 (top), 116,250, 94,400, and 66,200.

TABLE 1. Amino acid compositions of S layer proteins from *C. fetus* and other species

Amino acid or molecular composition parameter	Mol% in S layer protein of:			
	C. fetus		*A. serpens*[b]	*A. salmonicida* A450[c]
	VC119	23D[a]		
Asx	18.4	17.2	18.3	14.9
Thr	11.4	10.3	13.7	8.9
Ser	6.9	5.8	4.6	4.0
Glx	7.6	5.8	5.2	9.5
Pro	0.7	3.1	1.4	2.3
Gly	5.8	7.0	7.6	4.8
Ala	8.4	10.1	10.1	8.1
Val	9.8	7.4	7.0	8.5
Met	0.1	1.7	0.3	1.0
Ile	7.1	7.0	5.2	5.2
Leu	11.3	7.7	8.6	10.1
Tyr	1.2	2.1	1.7	2.0
Phe	2.9	4.4	2.8	7.3
His	0	0.9	0.4	1.1
Lys	7.7	8.2	4.0	6.1
Arg	0.3	0.7	1.9	4.4
Cys	0	0	ND[d]	0
Trp	0.5	0.8	7.1	1.9
No. of residues/mol	1,304	985	472	473
Apparent M_r	131,400	98,700	47,800	49,200
% Hydrophobic residues (V, M, I, L, A, F, W, P)	41.2	42.6	41.3	45.2

[a] Calculated from Winter et al. (43).
[b] Calculated from Buckmire and Murray (7).
[c] Calculated from Kay et al (20).
[d] ND, Not done.

the bimodal pattern of moderately helical proteins: $[\theta]_{220nm} = -12,900°$ and $[\theta]_{206nm} = -16,800°$.

These data were subjected to computer analyses, and the amounts of the various conformers present in the protein under the three sets of solvent conditions that were used are presented in Table 3. The *C. fetus* VC119 S layer protein had a moderate amount of α-helix (28%) at pH 7.5. The prominent structures appeared to be β-pleated sheets (36%) and aperiodic folding (31%). The protein had a rather low figure for β-turns (5%). At pH 2, the α-helix content dropped to 17% and the β-sheet value increased to 45%. In acidic SDS, the helical content was elevated to 34% at the expense of aperiodic structure, showing the stability of the formed β-sheet.

The near UV CD spectrum of the *C. fetus* VC119 S layer protein was also measured because this is the spectral region where contributions are found from asymmetrically situated

TABLE 2. N-terminal amino acid sequences of *C. fetus* VC119 S layer protein and S layer proteins of other bacteria

Organism	Residue[a]
	10 20
C. fetus VC119	M I S K S E V S E L F I V L F G R P
A. salmonicida A450[b]	D V V I G P N D N T F V T N S L A S V T
B. brevis 47[c]	A P K D G I Y I G G N I K K Y Y S Y D V

[a] Amino acid residues are designated by the single-letter nomenclature.
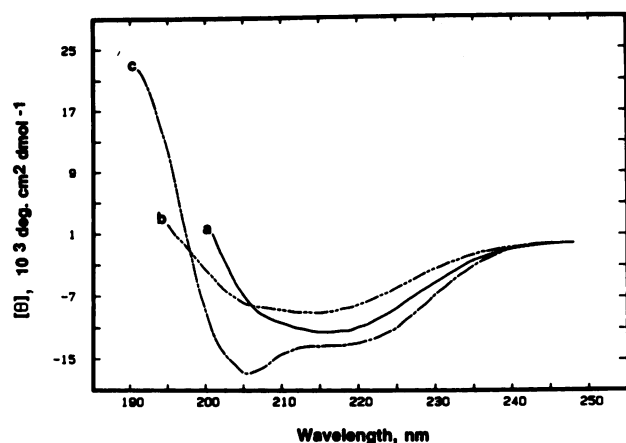[b] Phipps et al. (31).
[c] Tsuboi et al. (42).

FIG. 5. Far UV CD spectra for S layer protein in three different solvent systems. Curve a represents the native protein in 100 mM NaCl–25 mM Tris hydrochloride (pH 7.5). Protein concentration was 0.61 mg/ml, and a 0.05-cm path length cell was used. Curve b shows a fivefold dilution of the stock protein solution into 0.1 M H_3PO_4 (pH 2). The protein concentration was 0.28 mg/ml. The cell path length was 0.05 cm. Curve c shows the spectrum obtained for the protein in 2.5% (vol/vol) SDS in 75 mM H_3PO_4 at pH 2. The protein concentration was 1.066 mg/ml, the cell path length was 0.01 cm, and the temperature in all cases was 25°C.
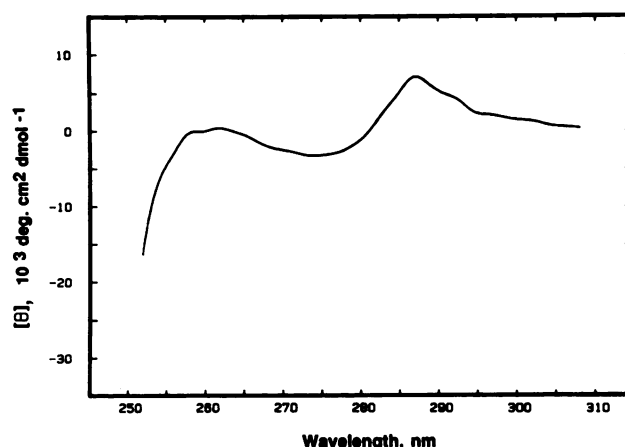

FIG. 6. Near UV CD spectra for *C. fetus* VC119 S layer protein in 25 mM Tris hydrochloride (pH 7.5). The path length was 1 cm, the temperature was 25°C, and the protein concentration was 1.422 mg/ml.

aromatic amino acids. The data in Fig. 6 showed that the spectrum was characterized by a maximum near 278 nm. The signal was rather weak in this region, indicating a paucity of suitably disposed aromatic amino acids; however, the following points emerged. The small positive peak situated near 290 nm was probably due to tryptophan contributions, while the weak broad minimum centered near 275 nm may be attributed to tyrosine residues. The fine structure noted between 270 and 250 nm was attributed to phenylalanine residues. Since the signals were so weak, the effects of acid and acidic SDS were not explored.

Fluorescence. Fluorescence emission and excitation spectra provide information on the nature and disposition of certain aromatic chromatophores, such as tryptophan. Therefore, as an additional aid to confirming the presence of tryptophan residues in the *C. fetus* VC119 S layer protein, the excitation and emission spectra were determined at pH 7.5. The emission maximum was noted near 324 nm, a wavelength position indicative of rather buried tryptophan fluorophores, since this was too high a wavelength to be due solely to emission from tyrosine residues.

Molecular weight. Sedimentation equilibrium analysis is the standard technique for providing the best resolution of molecular weight. Sedimentation equilibrium analysis was therefore performed to estimate the M_r in solution of the VC119 S layer protein in 100 mM NaCl–25 mM Tris hydro-

chloride (pH 7.5). Figure 7 shows a typical plot obtained for ln *y* (the protein concentration in fringe displacement units versus r^2 (the square of the distance from the center of rotation). The plot shows continuous upward curvature, and weight average M_rs obtained from such plots varied from approximately 60,000 near the meniscus to approximately 110,000 near the cell bottom. This is a reflection of some degradation in the sample which occurred during the 2-day preliminary dialysis phase or, more likely, during the time (~30 h) taken to reach equilibrium during the sedimentation equilibrium run. Inclusion of the proteolysis inhibitor PMSF did not affect the molecular weight distribution.

SDS-PAGE was performed on the actual samples used in the centrifuge experiments (data not shown). Although a band in the vicinity of 131,000 was quite prominent, the bulk
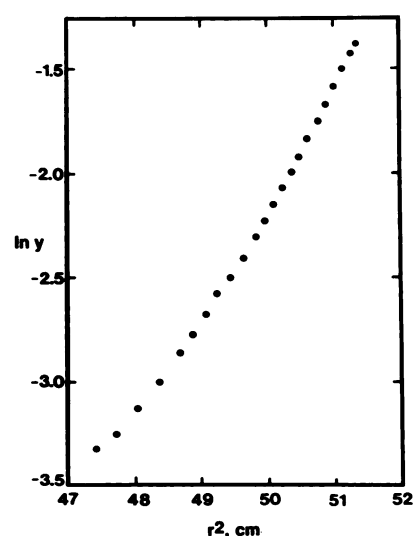
TABLE 3. Far UV CD data for *C. fetus* VC119 S layer protein

Solvent conditions	Secondary structure content (%)			
	α-Helix	β-Sheet	β-Turn	Remainder
100 mM NaCl–25 mM Tris hydrochloride (pH 7.5)	28	36	5	31
75 mM H_3PO_4–20 mM NaCl (pH 2)	17	45	8	30
75 mM H_3PO_4–20 mM NaCl 2.5% (vol/vol) SDS (pH 2)	34	43	2	21

FIG. 7. A plot of ln *y* (the concentration of protein in fringe displacement units) versus r^2 (the square of the distance from the center of rotation) when *C. fetus* VC119 S layer protein in 100 mM NaCl–25 mM Tris hydrochloride (pH 7.5)–1 mM PMSF was subjected to sedimentation equilibrium. The temperature was 20°C, the rotor speed at equilibrium was 10,000 rpm, and the initial protein concentration was approximately 1.0 mg/ml.

of the material was present in a band with an apparent M_r near 110,000. There was little evidence for lower-molecular-weight species being present.

DISCUSSION

This study clearly established that the microcapsule of *C. fetus*, first reported by McCoy et al. (25), is an S layer. Electron microscopic examination of both freeze-etchings and shadow casts showed the array to be linear, although this may be an artifact of the shadowing direction and angle; it is possible that the array is tetragonal. Like Winter et al. (43), we were unable to resolve the subunit structure of the *C. fetus* S layer by using negative staining, although Winter and colleagues did record observing structures with lateral striations in some negatively stained preparations. It is important to note, however, that most of the S layers studied to date have been hexagonal or tetragonally arrayed, and arrays with linear symmetry are rare. P2 symmetries have been found on eubacteria (e.g., *Aquaspirillum putridoconchylium*) and archaebacteria (34). P1 symmetry has been reported for *Bacillus anthracis* (R. J. Doyle, T. Beveridge, M. Stewart, and J. Ezzell, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, J18, p. 191).

In *C. fetus* VC119, the subunit protein of the array was the predominant cellular protein and also the immunodominant cell protein antigen. When present in its native form, the S layer protein of *C. fetus* VC119 was readily surface radio-iodinated by the lactoperoxidase technique. This contrasts with the findings with tetragonal S layer proteins of other gram-negative bacteria that indicate a general absence of surface-exposed tyrosine residues in the native S layer (2, 9, 18). Indeed, in these other cases this property has prevented S layer protein identification by techniques which rely on transfer of ^{125}I to tyrosine. The native S layer of *C. fetus* VC119 was susceptible to digestion by the nonspecific protease proteinase K, and we used this property to confirm the identity of the S layer subunit. However, incubation of cells with other proteolytic enzymes showed that the native S layer of *C. fetus* VC119 was generally resistant to proteases, a finding in keeping with the general resistance of surface layer proteins to proteolytic digestion when folded into their native conformation (21).

The S layer protein of *C. fetus* had an apparent M_r of 131,000 by SDS-PAGE. Unfortunately when the protein was subjected to sedimentation equilibrium analysis to determine the solution M_r, degradation of the parent 131,000-M_r particle was noted, and this degradation was not prevented by the presence of the protease inhibitor PMSF. The M_r figure of 131,000 is similar to the 135,000 reported by Myers (28) for the PGB antigen, although it is higher than the 98,000 reported by Winter et al. (43) for the *a* antigen. It is likely, however, that these M_r differences simply reflect strain-to-strain differences, since we have noticed that the predominant surface protein on different *C. fetus* isolates can vary from 100,000 to 150,000 (T. J. Trust, unpublished data). Blaser et al. (4) have also reported strain-to-strain differences from 100,000 to 125,000 in the M_rs of major surface proteins of human isolates of *C. fetus*.

The glycine extraction procedure we used to remove the S layer protein from the cell provided a high yield of S layer protein, with minimal disruption of the cell. Importantly, no major difference in the secondary structure content of the purified S layer protein was found at low or neutral pH, indicating that the low-pH treatment did not produce any drastic conformational change in the S layer protein during

its removal from the cell surface. Purification of this major surface protein of *C. fetus* was simply achieved with the aid of molecular sieving and ultracentrifugation to remove OM fragments, and the soluble S layer protein was then purified to homogeneity by first binding to the matrix with a net negative charge at its surface and elution at 0.18 M NaCl, followed by binding to the stronger Mono-Q (Pharmacia) anion-exchange column and elution at around 0.5 M NaCl.

We could find no evidence of carbohydrate associated with the purified *C. fetus* VC119 S layer protein by using a variety of techniques. The major contaminant during the purification procedure was LPS, and we believe that previous preparations of the *C. fetus* protein which were reported to be glycoproteins were likely contaminated with LPS. Certainly, Winter et al. (43) made the observation that the *a* antigen in vesicles derived from sheared cells appeared to exist in a complex with LPS, and we found much S layer protein associated with the LPS-rich OM fragments in glycine extracts of whole cells. In addition, the pI value of 4.2 determined by Myers (28) could also be explained by LPS bound to the protein, since in nondenaturing conditions this LPS would remain attached and supply an extra negative charge to the complex, resulting in an apparently lower pI. We found that although the protein was acidic, it was not as acidic as that reported by Myers (28). The VC119 S layer protein focused in one band at a position corresponding to a pI of 6.35 in nondenaturing conditions. In denaturing conditions (8 M urea–2% Nonidet P-40), the protein focused into a single band at a pI of 8.00. These differences are interpretable as a conformational change brought about by the absence of urea-Nonidet P-40 (12).

The amino acid composition also revealed approximately 22% acidic amino acid residues, compared with approximately 6% basic residues. The overall amino acid composition of the VC119 S layer protein was similar to that reported by Winter et al. (43) for the 98,000-M_r microcapsule protein of strain 23D and is consistent with the amino acid compositions of other S layer proteins examined to date (3, 8, 20, 37), including those of *Aquaspirillum serpens* and *Aeromonas salmonicida* (Table 1). For example, the absence of cysteine is characteristic of S layer proteins (37). Unlike with the VC119 protein, however, Winter et al. (43) reported the presence of both histidine and 14 methionine residues in the protein they studied, compared with the single N-terminal methionine we detected. The VC119 protein also contained significantly less proline than the protein from strain 23D (43). In contrast to the similarity in the overall amino acid compositions of the S layer proteins, virtually no identity has been seen in their N-terminal amino acid sequences (8, 31, 42). This was certainly also the case with the N-terminal sequence obtained for the *C. fetus* VC119 S layer protein.

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