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Major Outer Membrane Protein of *Campylobacter fetus*: Physical and Immunological Characterization

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The outer membrane proteins of *Campylobacter fetus* have been isolated by extraction of cell envelopes both in Triton X-100 and ethylenediaminetetraacetate (EDTA) and in sodium dodecyl sulfate (SDS). Each method yielded a major protein component, which migrated identically in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and proved comparable in electrophoretic and molecular weight characteristics to the analagous protein from *Escherichia coli*. In addition, the surface of SDS-extracted *C. fetus* cells displayed a subunit structure similar to that observed in *E. coli*. The major envelope protein isolated with SDS appeared antigenically identical with one of the proteins isolated with Triton-EDTA on the basis of immunodiffusion reactions with specific antisera. Antibodies directed to the major envelope protein were not reactive in agglutination, immobilization, bactericidal, or opsonization reactions. Strains of *C. fetus* belonging to each of the three O serotypes possessed major envelope proteins comparable in SDS-PAGE but distinguished antigenically in a fashion paralleling the O serotype classification.

The cell envelope of gram-negative bacteria is a complex structure consisting of the outer membranous layer, the murein layer, and the cytoplasmic membrane. The outer membrane contains substantial amounts of protein, phospholipid, and lipopolysaccharide (LPS) and appears to be heterogeneous with respect to structural organization (14, 17, 28). In *Enterobacteriaceae*, a low-molecular-weight lipoprotein is covalently bound to the murein layer and extends into the outer membrane (2, 3).

The results of comparative studies of cell envelopes of a variety of gram-negative organisms have also revealed the presence of a major protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (21). This protein, which represents approximately 70% of the total outer membrane protein, is an integral structural component and is difficult to solubilize without dissolution of the membrane (23). Based on the results of chromatographic separation and cyanogen bromide cleavage, Schnaitman (24) has reported that the 42,000-molecular weight major envelope protein in *Escherichia coli* O111 is a complex consisting of at least four distinct polypeptide species (proteins 1, 2, 3a, and 3b). In a recent investigation, Rosenbusch (18) reported that separation of a major envelope protein ("matrix protein") from the murein layer in *Escherichia coli* BE by SDS treatment yielded a homogeneous polypeptide with an apparent molecular weight of 36,500 on SDS-PAGE. Schnaitman et al. (25) have concluded that the "matrix protein" corresponds to "protein 1" of Schnaitman as well as to "protein I," "protein A_1," and "protein B" defined by other investigators.

The location of the major envelope protein within the cell envelope has not been fully defined. Using electron microscope and serological techniques, Rosenbusch suggested that the major envelope protein in *E. coli* BE is localized at the outer surface of the murein layer and differs from lattices observed on the cell surface in gram-positive and gram-negative species (8). However, evidence that the major envelope protein may extend to the outer cell surface is afforded by studies on *Neisseria meningitidis* of group B, in which the major protein is accessible to antibodies (7), and studies on *E. coli* K-12, in which "protein 1" may serve as a phage receptor (25).

The present report is part of an investigation of antigens composing the outer membrane of *Campylobacter fetus*. This is particularly relevant in that recent studies (5, 26) have shown that the persistence of *C. fetus* in the reproductive tract is associated with alterations in surface antigens. Emphasis has been placed on characterizing the major envelope protein and determining possible biological functions of its specific antibody.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. fetus* subsp. *intestinalis* 23D was principally utilized in this study. The medium and method for cultivation have been described in a previous report (11). For the preparation of large numbers of cells, the flask method was employed; when fewer cells were required, organisms were grown in overlay bottles. Other strains were obtained from John Bryner (National Animal Disease Center, Ames, Iowa), with the exception of strain 13017, provided by Burton Firehammer (Veterinary Research Laboratory, Montana State University, Bozeman). Classification of the strains according to their serological and biochemical characteristics, utilizing the scheme proposed by Berg et al. (1), is presented in Table 1.

Isolation of cell envelopes. Cells grown in flasks were collected in the exponential (9-h) or stationary (24-h) phases of growth by centrifugation at 12,000 × *g* for 30 min at 4 C. The method employed for the preparation of cell envelopes was that of Schnaitman (19), using a Ribi cell fractionator (Ivan Sorvall, Inc.) at 12,000 lb/in² and 4 C for cell disruption. Crude envelope preparations were separated on sucrose gradients (20).

Extraction of cell wall-enriched envelope fractions. Cell wall-enriched fractions from the density gradients, identified by using succinic dehydrogenase and 2-keto-3-deoxy sugars as markers, were extracted sequentially with Triton X-100 and Triton X-100 with ethylenediaminetetraacetate (EDTA) (22). Ninety-five percent ethanol (1:1, vol/vol) was added to the supernatants from the Triton X-100 and Triton X-100–EDTA extractions with stirring, and the mixtures were allowed to stand overnight at −20 C. Precipitates from the ethanol fractionation were separated by centrifugation and suspended in 0.01 M HEPES buffer (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) (pH 7.4).

Crude envelope fractions after gradient centrifugation were also extracted in the SDS extraction buffer of Rosenbusch (18). Mixtures were incubated at 60 C for 30 min and then centrifuged at 45,000 × *g* for 30 min. The pellets obtained were washed twice with distilled water and employed as the source of major envelope protein. For small-scale preparations, cell disruption was omitted, and water-washed cell pellets were extracted directly with the extraction buffer and then treated in a fashion similar to that used for the envelope preparations.

Chemical and enzyme analyses. Protein was determined by the method of Lowry et al. (10), with bovine serum albumin (Miles Laboratories) as the standard.

The assay for 2-keto-3-deoxy sugars described by Weissbach and Hurwitz (29) was performed, using Osborn's modification (16). LPS extracted as described previously (30) from *C. fetus* strain 23 by the method of O'Neill and Todd (15) was used as a standard, and the assumption was made that detection of this class of sugars in envelope fractions reflected the presence of an LPS component, presumably 2-keto-3-deoxyoctonate.

Succinic dehydrogenase (EC 1.3.99.1) was measured by the method of King (9) by coupling the enzyme via phenazine methosulfate to the reduction of 2,6-dichlorophenolindophenol and following the reaction spectrophotometrically at 600 nm.

SDS-PAGE. Tube gel electrophoresis in 0.2% SDS was carried out essentially as described by Weber and Osborn (27). The preparations were incubated in tightly stoppered tubes for 2 h at 37 C. In most instances, samples were heated at 100 C for 1 min before gel application. Standards used for determination of molecular weight included: bovine serum albumin, ovalbumin, trypsin, pepsin, myoglobin, and ribonuclease (obtained from G. D. McCoy, Biochemistry Department, Cornell University). Selected protein bands were removed from gels by comparing the R_f of stained and unstained gels. Immunological identification of protein bands was determined by placing gel columns after electrophoresis on slides and overlaying with agar employed for immunodiffusion. After gel solidification, antiserum troughs were cut parallel to the embedded gel column. Slides were incubated with antiserum at room temperature in a moist chamber for band development (2 to 4 days).

Production of antisera. Male New Zealand white rabbits were immunized subcutaneously and into the footpads of each hind leg with extracts that had been emulsified in complete Freund adjuvant. Two-milliliter volumes of emulsion containing approximately 4 mg of protein per ml were administered to each animal. Two weeks later each rabbit was in-

TABLE 1. *Classification of C. fetus strains*

Strain	O serotype	Biochemical type[a]	Subspecies
1016	A	1	*C. fetus* subsp. *venerealis*
481	A	Subtype 1	*C. fetus* subsp. *venerealis*
23D	A	2	*C. fetus* subsp. *intestinalis*
437 (7) 3852	B	2	*C. fetus* subsp. *intestinalis*
1083 2255	B	2	*C. fetus* subsp. *intestinalis*
652-Minturn	C	2	*C. fetus* subsp. *intestinalis*
958-E74	C	2	*C. fetus* subsp. *intestinalis*
13017	C	2	*C. fetus* subsp. *intestinalis*

[a] Based upon growth in medium containing 1% glycine and production of H_2S in cysteine-containing medium. Type 1: Glycine (−), H_2S (−). Subtype 1: Glycine (−), H_2S (+). Type 2: Glycine (+), H_2S (+). Glycine tolerance of serotype C strains is variable.

jected intramuscularly with 1 ml of the same preparation. Rabbits were exsanguinated approximately 1 week after the final immunization. Two rabbits were immunized with each preparation.

An O antiserum (313) produced previously with a trichloroacetic acid extract of strain 23D (11) was employed in bactericidal assays.

Serological techniques. Agglutination, immobilization, immunodiffusion, and immunoelectrophoresis were performed as described previously (6, 11, 12). When the demonstration of serological relatedness of antigens on immunoelectrophoresis was desired, an additional antigen well was cut after electrophoresis and before the antiserum troughs were filled. The second antigen was placed in this well, and the reaction was allowed to develop in a moist chamber at room temperature.

Bactericidal assays were performed with cells harvested in the exponential and stationary phases of growth. The assay mixture consisted of: 0.1 ml of a cell suspension adjusted to an optical density of 0.3 at 525 nm (approximately 10^7 cells), 0.1 ml of guinea pig complement (Grand Island Biological Co.), 0.1 ml of the test antiserum previously inactivated at 56 C for 30 min, and Albimi broth (Difco) to a final volume of 2.0 ml. The mixtures were incubated at 37 C for 1 to 3 h in a shaker incubator (New Brunswick) at 175 rpm. Viable counts were determined by the drop method (13) on Albimi medium. Plates were incubated for 3 days in vacuum desiccator jars in an atmosphere of 87.5% N_2, 10% CO_2, and 2.5% O_2.

Opsonization tests were performed by Lynette B. Corbeil. Bovine macrophages were employed in the method described previously (11).

Electron microscopy. Methods for negative staining have been described previously (12). Negative stains were viewed in a Phillips E.M.-300 electron microscope at 80 kV.

RESULTS

Separation of cytoplasmic and outer membrane fractions. Sucrose density gradient centrifugation of crude envelope preparations resulted in gradient patterns similar to those described by Schnaitman (20); an upper yellow band appeared at the 0.77 to 1.44 M sucrose interface, and a heavier band appeared at the 1.44 to 2.02 M sucrose interface, less intensely colored than the upper fraction. Identification of the upper band as cytoplasmic membrane was made by determination of succinic dehydrogenase activity (Table 2). The heavier band, designated the crude outer membrane fraction, contained a low proportion of succinic dehydrogenase activity and most of the 2-keto-3-deoxy sugars (Table 2). It was used as starting material for the extraction of the major envelope protein.

Extraction of crude outer membrane fraction. A comparison of gel electrophoresis patterns of the Triton X-100-soluble (TS) and the Triton X-100-EDTA-soluble (TES) membrane proteins showed the presence of many distinct bands in the TS fraction but relatively few in the TES fraction. When TES was applied to gels after 2 h of incubation at 37 C, four principal bands were observed on gel electrophoresis with apparent molecular weights of approximately 100,000, 45,000, 38,000, and 36,000, respectively (Fig. 1A). Brief heating of TES at 100 C after incubation at 37 C resulted in the development of a major protein band with an apparent molecular weight of 41,000 and a smaller band with a molecular weight of approximately 39,000 (Fig. 1A). SDS-extracted membranes contained a major band that appeared to be of identical mobility with the 41,000-molecular-weight protein from the TES fraction (Fig. 1B). The supernatants of SDS-extracted membranes also contained substantial quantities of the major envelope protein.

Serological comparison of TES and SDS-solubilized proteins. Antisera were prepared against the TES fraction (serum 365), the TES major protein band that developed upon heating TES at 100 C and was eluted from SDS-polyacrylamide gels (serum 367), and the washed pellet after extraction with SDS buffer (serum 369). In immunodiffusion reactions with TES, each antiserum developed a single, broad band migrating halfway between antigen and antibody wells. Immunoelectrophoresis of TES with serum 367 revealed a heavy band migrating cathodally (Fig. 2). A combination of immunoelectrophoresis of TES and immunodiffusion with SDS extracts produced reactions of iden-

TABLE 2. *Chemical and enzyme analyses of cell envelope fractions*[a]

Fraction	Membrane protein recovery (%)	Sp act of SDH[b]	2-Keto-3-deoxy sugar (mg/mg of protein)
Crude envelopes	100	0.115	0.114
Top bands of sucrose gradient	18.8	5.56	0.013
Bottom bands of sucrose gradient	51.5	0.342	0.184

[a] Results represent averages of three experiments.

[b] SDH, Succinic dehydrogenase. Expressed as micromoles of succinate oxidized per minute per milligram of protein $\times 10^{-3}$.

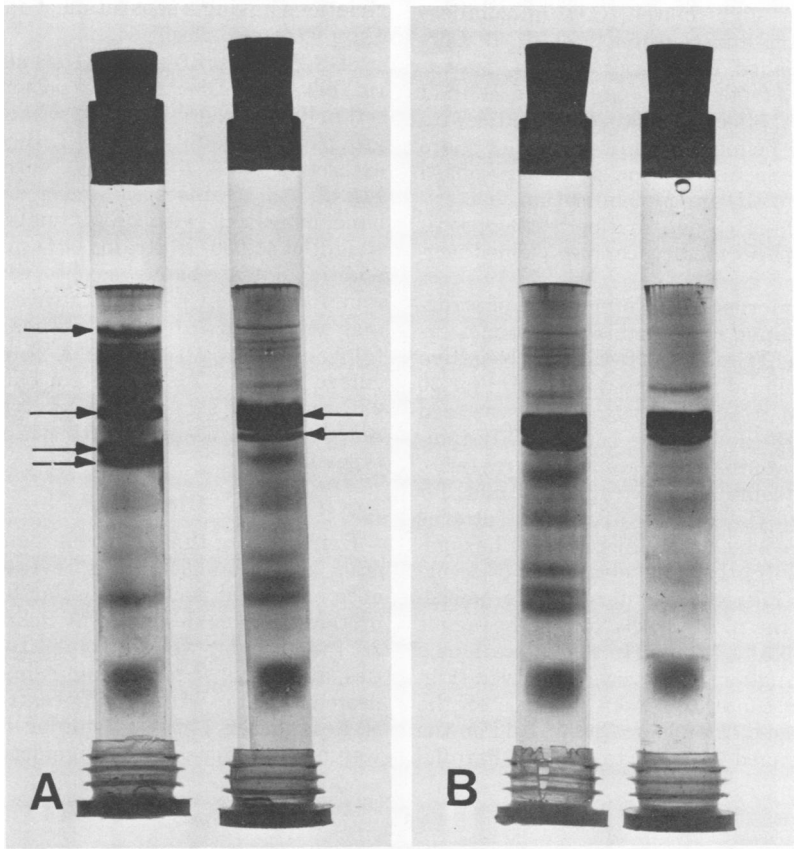

FIG. 1. *SDS-PAGE of outer cell membranes of C. fetus 23D extracted with Triton X-100-EDTA and SDS. (A) TES membrane proteins not heated (left) and heated (right) to 100 C before application. Arrows at left correspond to bands with molecular weights estimated at 100,000, 45,000, 38,000, and 36,000; those on right correspond to bands of molecular weights of 41,000 and 39,000. (B) Electrophoresis after boiling of TES membrane proteins (left) and SDS-extracted membranes (right). The major band in each gel has an estimated molecular weight of 41,000.*

tity with serum 367 (Fig. 2) as well as serum 369, indicating that the same antigen, designated antigen [g] and presumably corresponding to the major envelope protein, was present in each preparation. This was substantiated further in immunodiffusion reactions on SDS gel columns after electrophoresis of TES or SDS antigens. In each case the precipitin arc produced with serum 367 corresponded exactly with the location of the major envelope protein. An artifact band, as judged by its development with several normal rabbit sera, occurred consistently in immunodiffusion reactions employing antigens in SDS (Fig. 2). This has been observed previously (4).

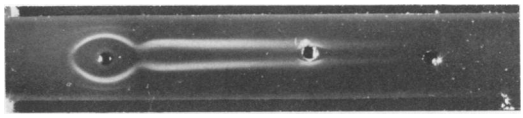

FIG. 2. *Combined immunoelectrophoresis and immunodiffusion of C. fetus 23D major envelope protein. A sample of TES membrane protein (center well) was subjected to electrophoresis and developed with antiserum 367 (both troughs) produced against the major envelope protein. A sample of SDS-extracted outer membrane, placed in the left well after electrophoresis, produced a reaction of identity with TES. The faint lines nearer the serum troughs are artifacts produced by SDS. Anode is to the right.*

Biological activity of [g] antibodies. Antiserum 367 contained O antibodies, even though LPS was present in TES extracts in concentrations lower than 20 μg/ml, as estimated by the limits of sensitivity of the template immunodiffusion test in the detection of *C. fetus* LPS (A. J. Winter, unpublished data). This serum was used to test the biological activity of [g] antibodies since it was known that homologous O antibodies (serum 313) were not reactive with

strain 23D in agglutination (11), immobilization (12), or opsonization reactions (L. B. Corbeil, unpublished data) and it was demonstrated in two trials that O antiserum 313 had no bactericidal effect on strain 23D. Antiserum 367 produced negative results in two or more trials with exponential- or stationary-phase cells of strain 23D in immobilization, bactericidal, or whole-cell agglutination tests. Opsonization tests, performed twice, also yielded negative results.

Electron microscopy. No evidence of structural organization remained in membranes extracted with Triton X-100–EDTA. Negative stains of whole cells extracted with SDS for 30 min revealed a surface pattern in scattered sites (Fig. 3B), and a time course study indicated that organized surface structures were best demonstrable after extraction at 60 C for 10 min (Fig. 4). Several structural patterns were noted, of which the majority were hexagonal subunits, tightly or loosely packed or occurring singly (Fig. 4C). Center-to-center measurements of the most closely packed hexagons varied from 6.3 to 7.4 nm. In a few locations a distinct rectangular pattern was observed (Fig. 4B) in which center-to-center spacings on the long axis ranged from 6.3 to 7.4 nm and on the short axis ranged from 4.2 to 5.2 nm. Parallel striations having a separation of approximately 4 nm were also observed.

SDS-PAGE profiles of different *C. fetus* serotypes. The SDS-PAGE gel patterns of whole-cell pellets of representative strains of *C. fetus* groups A-1, A-subtype 1, A-2, B, and C, after extraction in 2% SDS at 60 C, are depicted in Fig. 5. All strains possessed a distinct major band migrating with an estimated molecular weight of 41,000. In strains of O serotype C, an additional dense band was observed (Fig. 5). In immunodiffusion reactions using gel columns and antiserum 367, prepared against antigen [g] from strain 23D (group A-2), reactions occurred with the major protein bands of strain 23D and other strains of O serotype A, but no reactions were observed with strains of O serotypes B and C.

DISCUSSION

Extraction of the outer cell membrane of *C. fetus* by Triton-EDTA has revealed the presence of several proteins similar to the *E. coli* protein complex described by Schnaitman (23–25) in molecular weight, solubility characteristics, and response to heating. A major envelope protein isolated with the SDS extraction buffer of Rosenbusch (18) was similar in molecular weight and appeared antigenically identical

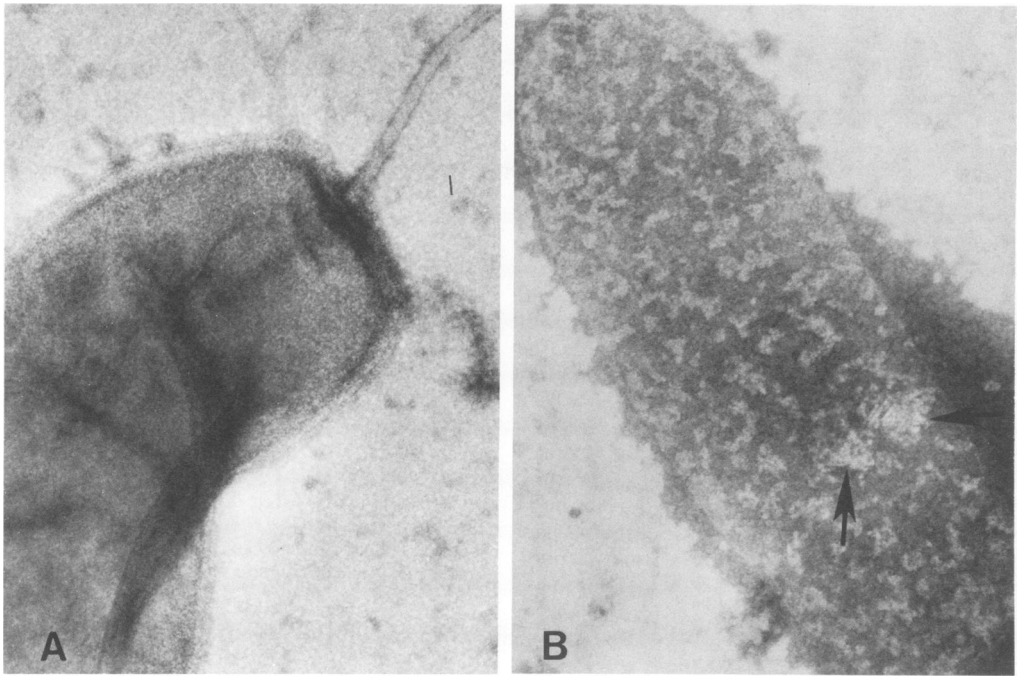

FIG. 3. *C. fetus 23D. (A) Untreated cell. ×138,000. (B) Cell extracted with SDS for 30 min. ×66,000. Regular surface structure remains at only a few sites (arrows) after treatment. Uranyl acetate stain.*

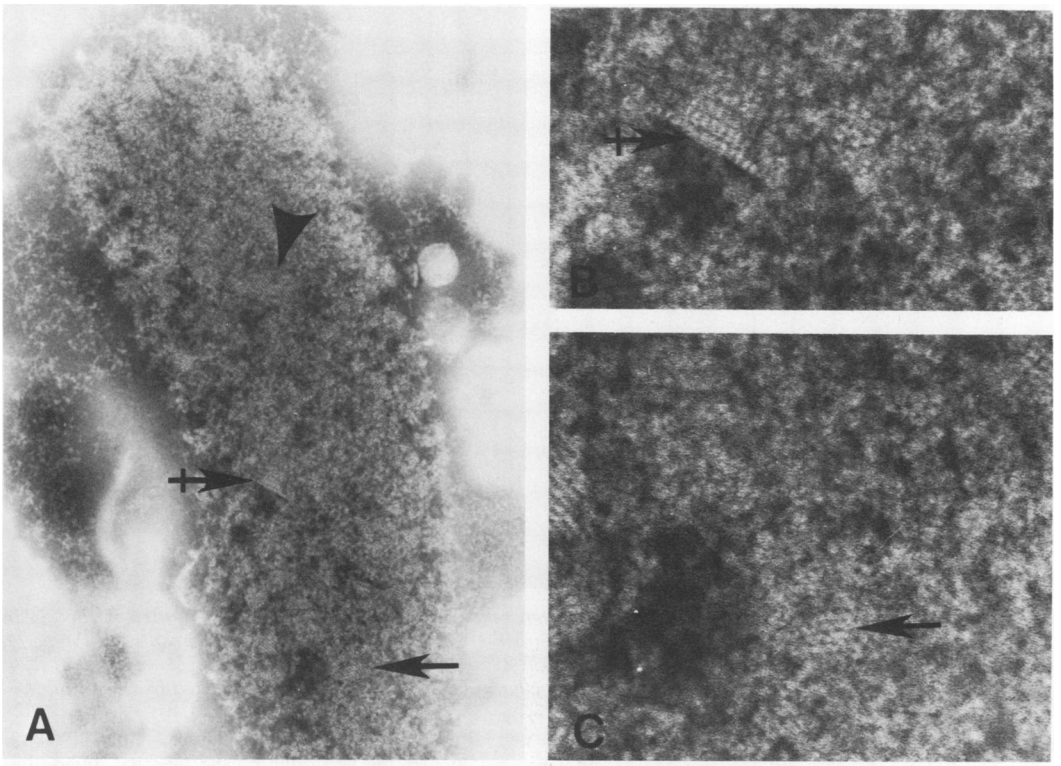

FIG. 4. *(A) C. fetus 23D extracted with SDS for 10 min. Hexagonal surface structures are apparent over much of the surface, closely packed in places (→). Parallel striations (►) and rectangular structures (↔) are present in some areas. ×75,000. (B) Enlargement of rectangular structures in Fig. 5A (↔). ×250,000. (C) Enlargement of area in Fig. 5A containing closely packed hexagonal structures (→). Separated hexagon units are also evident. ×250,000.*

with one of the proteins isolated with Triton-EDTA. We hypothesize that the antigenically related proteins derived by each of the two extraction procedures correspond to the "matrix protein" (18, 25) of *C. fetus*.

Rosenbusch identified the major envelope protein in SDS-extracted cells of *E. coli* as a symmetrical hexagonal structure lying on the outer surface of the murein, having a periodicity of 7.5 ± 0.5 nm (18). *C. fetus* cells extracted in this fashion also displayed a hexagonal subunit structure which, in its most closely packed arrangement, approximates the same periodicity. The presence of loosely grouped and single hexagonal structures is consistent with a progressively disruptive effect of the extraction process. The appearance and dimensions of the rectangular structures suggest that they were composed of hexagon subunits tipped on end. However, it could not be established definitely whether the rectangular and striated structures represented altered aspects of the hexagon subunit or other components lying deeper in, or more superficially on, the outer membrane. The identity of any of these structures with the lipoprotein covalently linked to murein (2, 3) can be excluded since *C. fetus* is known to lack this protein (31).

SDS-PAGE of solubilized cell pellets from strains of the representative O serotypes of *C. fetus* resulted in the demonstration of a major envelope protein band in all preparations. The additional dense band noted in each group C strain may reflect a significant serotype characteristic. The limited data available suggest that *C. fetus* may be classified by the major envelope protein antigen in a manner paralleling the O serotype classification.

Frasch and Gotschlich have shown that the serotype antigen of *Neisseria meningitidis* group B is identical with the major envelope protein and that antibodies specific for this antigen function as bactericidins (7). Our findings with *C. fetus* fail to indicate a protective effect of antibodies to the major envelope protein. The lack of biological reactivity of [g] antibodies

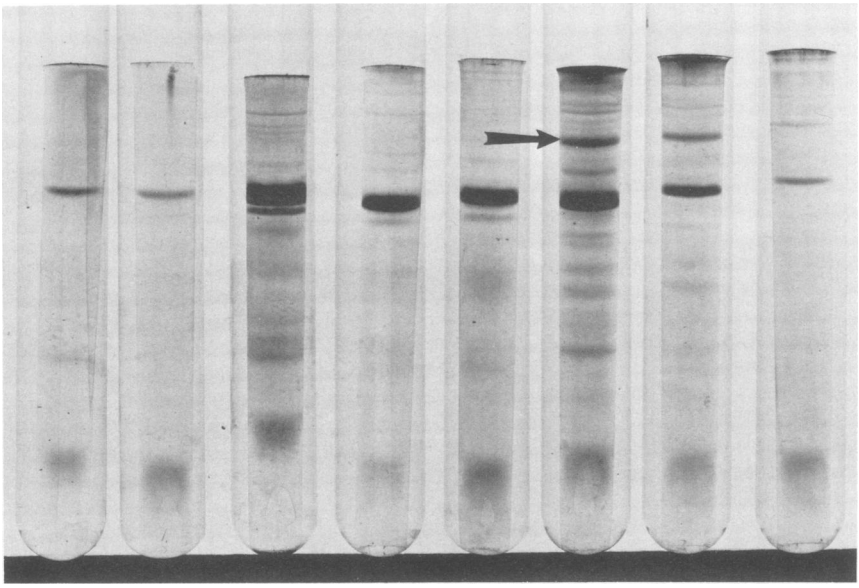

FIG. 5. *SDS-PAGE of major envelope proteins from representative strains of C. fetus, after extraction in SDS. From left to right, extracts were derived from strain 1016 (group A-1); strain 481 (group A-subtype 1); strain 23D (group A-2); strains 437(7) 3852 and 1083 2255 (group B); and strains 652-Minturn, 958-E74, and 13017 (group C). The location of the second dense band in group C strains is indicated by the arrow.*

indicates inaccessibility of antibodies to the major envelope protein. This may have been due to a blocking effect of the superficial antiphagocytic glycoprotein (11) or to the restriction of the major envelope protein to a deep position in the cell envelope.

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