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Outer Membrane Proteins and Cell Surface Structure of *Selenomonas ruminantium*

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The protein compositions of the membrane preparations from *Selenomonas ruminantium* grown in glucose or lactate medium were determined by sodium dodecyl sulfate- and two-dimensional (first, isoelectric focusing; second, sodium dodecyl sulfate) polyacrylamide slab gel electrophoresis. The outer membrane from both glucose- and lactate-grown cells contained two major proteins with apparent molecular weights of 42,000 and 40,000. These proteins existed as peptidoglycan-associated proteins in the outer membrane. The critical temperature at which they were dissociated completely into the monomeric subunits of 42,000 and 40,000 daltons was found to be 85°C. The amount of each protein varied considerably depending upon the cultural conditions. The absence of the lipoprotein of Braun in *S. ruminantium* was suggested in our preceding paper (Y. Kamio, and H. Takahashi, J. Bacteriol. **141**:888-898, 1980), and the possible absence of the protein components corresponding to the Braun lipoprotein in this strain was confirmed by electrophoretic analysis of the outer membrane and the lysozyme-treated peptidoglycan fractions. Examination of the cell surface of *S. ruminantium* by electron microscopy showed that the outer membrane formed a wrinkled surface with irregular blebs, some of which pinched off forming vesicles of various sizes. Rapid cell lysis occurred with the addition of a low level of lysozyme to the cell suspension. These findings led us to conclude that the physiological and morphological properties of this strain were similar to those of "deep rough" and *mlp* or *lpo* mutants of *Escherichia coli* K-12, respectively.

In an accompanying paper (16), we have described the isolation procedures and characterization of the outer and inner membranes, especially their lipid compositions, of *Selenomonas ruminantium*. The present paper deals with the protein composition of the outer membrane of this strain.

The cell wall of gram-negative bacteria consists of two layers: the outer membrane layer and the peptidoglycan layer (23). The outer membrane contains lipopolysaccharides, proteins, and phospholipids and has a simple protein composition (3, 25). The well-characterized outer membrane proteins of *Escherichia coli* and *Salmonella typhimurium* are peptidoglycan-associated proteins (matrix proteins). It is well established that these proteins constitute the passive diffusion pores that allow diffusion of low-molecular-weight substances (20-22) and that these proteins are essentially required for assembly of the outer membrane (10, 12, 31, 33). It is of interest to investigate the peptidoglycan-associated proteins in the outer membrane of *S. ruminantium*.

Our previous study revealed that phosphatidyl glycerol and cardiolipin were not present in detectable amounts in either the outer or inner membrane of *S. ruminantium* (16). Recently, Chattopadhyay et al. (8) reported that phosphatidyl glycerol was the effective donor for the glycerol moiety of the Braun lipoprotein, but phosphatidyl ethanolamine was not. These data and our finding that phosphatidyl glycerol is not detected in *S. ruminantium* stimulated us to investigate the occurrence of the Braun lipoprotein in this strain. This paper shows the possible absence of the protein components corresponding to the Braun lipoprotein of enteric bacteria in this strain. The present paper also shows some characteristic physiological and morphological features of the cell envelope of this strain.

MATERIALS AND METHODS

Bacterial strains. *S. ruminantium* subsp. *lactilytica* that was described in an accompanying paper (16) was used. *E. coli* K-12 strain JC1569 (F⁻ *recA1 gal leu his* Str^r), kindly given by H. Uchida, was used.

Media and culture conditions. Media and culture conditions for growth of *S. ruminantium* or *E. coli* were described in the accompanying paper (16).

Isolation of the outer and inner membranes. Isolation of the membranes was carried out by the method described in the accompanying paper (16).

Preparation of peptidoglycan and digestion with lysozyme. Peptidoglycan was prepared from our outer membrane fraction of *S. ruminantium* by the method of Braun and Rehn (7). Lysozyme digestion was carried out in 10 mM Tris-hydrochloride buffer (pH 8.0) at 37°C for 6 h with an enzyme-substrate ratio of 1:22.

Preparation of flagellins. The cells of *S. ruminantium* were grown in 300 ml of the lactate medium at 37°C. At mid-exponential phase, they were harvested by centrifugation at 6,000 × *g* for 15 min at 4°C and suspended in 20 ml of distilled water. Flagellar filaments were prepared by the method of Kamio and Terawaki (17) and disaggregated by 0.01 N HCl, and the flagellins were obtained by the method of Smith and Koffler (27).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) was performed as described previously (14). Two-dimensional (first, isoelectric focusing; second, SDS) PAGE was carried out by essentially the same method as that described by Ames and Nikaido (2). The gels were stained with 0.0025% Coomassie brilliant blue in 25% isopropanol-10% acetic acid. The gels were scanned with a Quick Scan densitometer (Helena Laboratories) with a 595-nm filter.

Effect of EDTA and lysozyme on lysis of *S. ruminantium*. Cells were grown in the glucose medium containing *n*-decanoate (0.01%) at 37°C. At mid-exponential phase, the cells were harvested by centrifugation and washed twice with ice-cold 0.15 M NaCl (pH 8.0) and suspended into the same solution. The optical density of the resulting suspension was adjusted to 0.9 at 660 nm. To this suspension was added either EDTA (at the final concentration of 50 μM) (pH 8.0) or lysozyme (50 μg/ml), or both. The reaction mixtures were incubated at 37°C, and at various intervals the optical densities of these reaction mixtures were followed up at 660 nm with a Coleman Junior II spectrophotometer.

Electron microscopy. For thin sectioning, samples were fixed with 3% glutaraldehyde in 0.1 M veronal buffer (pH 6.5). After 2 h, samples were washed twice with veronal buffer. Samples were postfixed with 1% OsO_4 in 0.1 M Veronal buffer (pH 6.5) for 6 h at 4°C and then dehydrated in ethanol and embedded in Epon 812. Sections were stained with uranyl acetate and lead citrate. Examination was carried out on a JEM 100-B electron microscope (Japan Electron Optics Laboratory Co., Ltd.).

Chemicals. Acrylamide and *N,N'*-methylenebisacrylamide were from Eastman Kodak Co. SDS was obtained from BDH Chemicals Ltd. (Poole, England). Egg white lysozyme was from Sigma Chemical Co., (St. Louis, Mo.). OsO_4 was from E. Merck AG (Darmstadt, Germany). The other chemicals used were of the best grade commercially available.

RESULTS

Protein compositions of the outer and inner membranes. Protein compositions of the outer and inner membrane preparations were examined by SDS-PAGE. Figure 1, samples 2–5) shows the typical SDS-polyacrylamide gel patterns of the outer and inner membrane preparations from *S. ruminantium* cells which were grown in either lactate or glucose medium. Also shown in this figure is the outer membrane prepared from *E. coli* K-12. The major proteins of the outer membrane from the glucose-grown cells were 40K (molecular weight, 40,000) and 42K (molecular weight, 42,000), in which the former was predominant (Fig. 1). The outer membrane proteins in the cells grown in the lactate medium were also composed mainly of 40K and 42K proteins, and the 42K protein was predominant in this case. Thus the ubiquitous presence of 40K and 42K proteins as major proteins in the outer membrane of *S. ruminantium* was confirmed, but the relative amount of each protein varied depending upon the cultural conditions. An appreciable amount of 49K protein was present as a major protein in the lactate-grown cells, but it was a minor constituent in the glucose-grown cells. Thus, we examined whether or not the 49K protein was flagellin, because a large number of flagella was observed

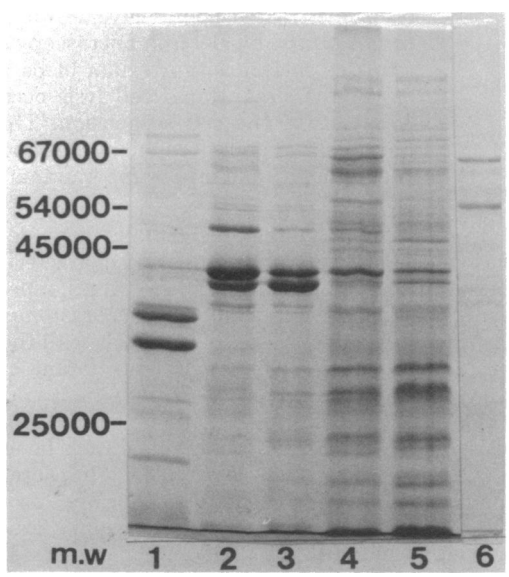

FIG. 1. *SDS-PAGE of the outer and inner membrane proteins of S. ruminantium. 9% polyacrylamide gel was used. The outer and inner membrane fractions were prepared as described in Materials and Methods. Each membrane was solubilized at 100°C for 2 min in sample buffer (1), electrophoresed, and stained. (Sample 1) Outer membrane proteins of E. coli K-12. (Sample 2) Outer membrane proteins of S. ruminantium (lactate-grown cells). (Sample 3) Outer membrane proteins of S. ruminantium (glucose-grown cells). (Sample 4) Inner membrane proteins of S. ruminantium (lactate-grown cells). (Sample 5) Inner membrane proteins of S. ruminantium (glucose-grown cells). (Sample 6) Flagellins of S. ruminantium.*

when *S. ruminantium* was grown in the lactate medium but was absent from the glucose-grown cells. As shown in Fig. 1, the 49K protein did not correspond to the flagellins. Moreover, the 49K protein was detected as one of the major proteins in the outer membrane preparation from the deflagellated cells (data not shown). In both glucose- and lactate-grown cells, various protein bands (68K, 66K, 58K, 55K, 37K, 29K, 26K, 22K, 17.5K, and 15K) were observed as minor components in the outer membrane preparation.

The inner membrane preparation consisted of many protein bands; at least 40 bands could be detected in both glucose- and lactate-grown cells, but there was no significant difference in protein patterns between glucose- and lactate-grown cells.

Characterization of major outer membrane proteins. (i) pI of the major proteins. The pI of the 40K and 42K proteins were determined by two-dimensional PAGE. The 42K protein had a pH of 5.7 and the 40K protein was found to consist of two proteins with pH values of 6.5 and 6.4.

(ii) Effect of temperature on solubilization of the major proteins. We examined the effect of temperature on the solubilization of the major proteins (40K and 42K) and the existence of the peptidoglycan-associated protein in the outer membrane. The outer membrane preparation from the glucose-grown cells was heated for various periods at 65 and 100°C or at various temperatures for 2 min in the presence of 2% SDS and 8 M urea. No difference was observed in the protein patterns between the sample heated at 65°C for 1 min and that heated at 65°C for 30 min (data not shown). In both samples a thick smearing of substances which were stained by Coomassie blue was present at the top of the stacking gel. These substances probably represent oligomeric forms of the major proteins associated with the peptidoglycan. When the outer membrane preparation was heated at 100°C for 10 s or longer, all of the outer membrane proteins were solubilized. This finding indicates that the oligomeric forms of the major proteins are dissociated into the "monomeric forms" at 100°C in the SDS solution. To determine the critical temperature at which the major proteins are dissociated completely into molecular weights of monomeric subunits of 40K or 42K, the following experiment was performed. The outer membrane preparation was heated for 2 min at various temperatures in solubilizing buffer (see legend to Fig. 2), and then the samples were applied to the gel. The amounts of 40K and 42K proteins dissociated were determined densitometrically. As shown in Fig. 2, the critical temperature was 85°C for the two proteins.

The peptidoglycan-associated proteins of the outer membrane are characterized by their strong, but noncovalent linking to peptidoglycan (24). It is well known that the peptidoglycan-associated proteins of *E. coli* can not be released from the peptidoglycan by heating the peptidoglycan-outer membrane complex at 60°C in 2% SDS; however, the proteins are released at 70°C (12). As shown in the accompanying paper (16), our outer membrane consisted of peptidoglycan and outer membrane components, i.e., LPS, proteins, and phospholipids. Therefore, we suggested that both 40K and 42K proteins were so-called peptidoglycan-associated proteins. To ensure this, the following experiment was performed. Our outer membrane prepared from the glucose-grown cells was treated with SDS by the method described in the legend to Fig. 3. As shown in Fig. 3, only 40K and 42K proteins were detected, indicating that both proteins are peptidoglycan-associated proteins in *S. ruminantium*.

SDS-PAGE of the outer membrane proteins by 14% polyacrylamide gel. Braun

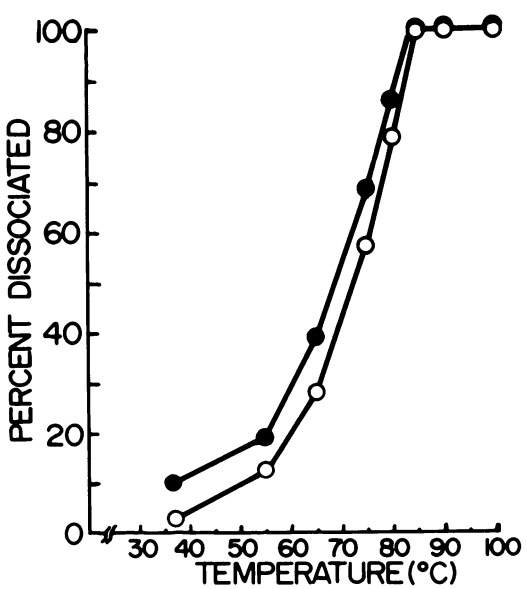

FIG. 2. *Effect of temperature on solubilization of the outer membrane major proteins of S. ruminantium. The samples (20 μl) containing 1 mg of the outer membrane proteins per ml were mixed with a solution of 20 μl of 0.1 M Tris-hydrochloride buffer (pH 6.8), 2% SDS, 2% mercaptoethanol, and 8 M urea and then heated for 2 min at 37, 55, 65, 75, 80, 85, 90, or 100°C. Each sample was applied to 9% gel. The amount of 40K and 42K proteins was determined densitometrically.* (○) *40K protein.* (●) *42 K protein.*

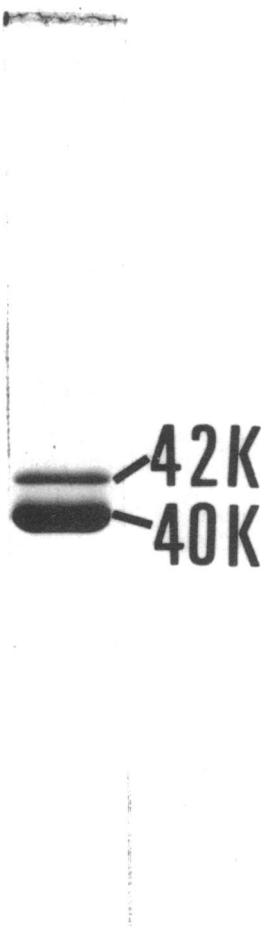

FIG. 3. *Peptidoglycan-associated outer membrane proteins of S. ruminantium. The outer membrane preparation (100 μl) containing 1 mg of protein per ml from the glucose-grown cells was mixed with 100 μl of 10 mM Tris-hydrochloride buffer (pH 7.3) containing 2% SDS and 10% glycerol and heated at 60°C for 30 min. After centrifugation at 50,000 × g for 60 min, the precipitate obtained was washed with distilled water, heated again at 100°C for 2 min in sample buffer (1), and then applied to 9% gel.*

lipoprotein with a molecular weight of 7,200 exists in *E. coli* in two forms: one is a free form which occurs in the outer membrane; the other is a bound form which is covalently linked to the peptidoglycan (7, 13). We used 14% polyacrylamide gel to clarify the existence of the Braun lipoprotein in the outer membrane and peptidoglycan fractions of *S. ruminantium*. Figure 4 shows the SDS slab-gel patterns of the outer membrane proteins from *S. ruminantium* and *E. coli* K-12. As seen in this figure, the 14% gel system resolved the peptides smaller than 14,000 daltons, but no protein band corresponding to the free form of the Braun lipoprotein, designated LPP in this figure, was detected in the outer membrane preparation of *S. ruminantium* cells (samples 2 and 3).

The peptidoglycan fraction was prepared from our outer membrane preparation of *S. ruminantium* and treated with lysozyme. The lysozyme-treated sample was heated at 100°C for 2 min in the presence of 2% SDS and applied to the 14% gel. No protein, except for the lysozyme band, was seen, whereas in the sample prepared from *E. coli*, four bands were observed, one of which migrated near the position where the free form of the Braun lipoprotein appeared (data not shown). These findings indicate that no detectable amount of protein component corresponding to the Braun lipoprotein exists in *S. ruminantium*. This was supported by our preceding observations that neither [^{14}C]decanoate nor [^{3}H]glycerol was incorporated into the peptidoglycan fraction of *S. ruminantium* (16).

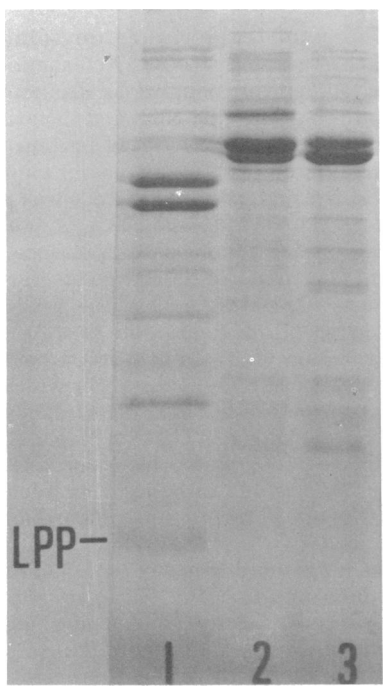

FIG. 4. *SDS-PAGE patterns of the outer membrane proteins from S. ruminantium. Polyacrylamide gel (14%) was used for resolution of peptides smaller than 14,000 dalton. The methods for preparation of the samples were described in the legend to Fig. 1. (Sample 1) Outer membrane proteins of E. coli K-12. (2) Outer membrane proteins of S. ruminantium (lactate-grown cells). (3) Outer membrane proteins of S. ruminantium (glucose-grown cells). LPP, Free form of the Braun lipoprotein of E. coli.*

It is known that the bound form of the Braun lipoprotein is involved in the maintenance of the integrity of the envelope because it connects the outer membrane with the peptidoglycan (5, 6). Our failure to detect the lipoprotein in *S. ruminantium* led us to perform the studies of physiological and morphological characteristics of the envelope of this strain.

Effect of EDTA and lysozyme on lysis of *S. ruminantium*. Cells were grown in the glucose medium supplemented with *n*-decanoate (0.1%), and at mid-exponential phase they were harvested, washed twice with 0.15 M NaCl, and treated with either EDTA or lysozyme, or both. As shown in Fig. 5, the addition of a small amount of lysozyme (50 μg/ml) to the cell suspension caused a rapid decrease in turbidity. A slow cell lysis also occurred after the addition of 50 μM EDTA to the cell suspension. Similar results were obtained in the cells grown in the lactate medium, although the effect of lysozyme was somewhat smaller. The cell lysis could be prevented completely in the presence of 0.4 M sucrose. These findings clearly indicate that *S. ruminantium* cells are hypersensitive to lysozyme.

Electron microscopy. The cell surface structure was examined by electron microscopy with thin-sectioned samples. Cells were grown in the lactate or glucose medium at 37°C, and at mid-exponential phase they were thin sectioned. As shown in Fig. 6A-C, small vesicles were seen outside the outer membrane, and the distribution of small blebs over the surface was observed.

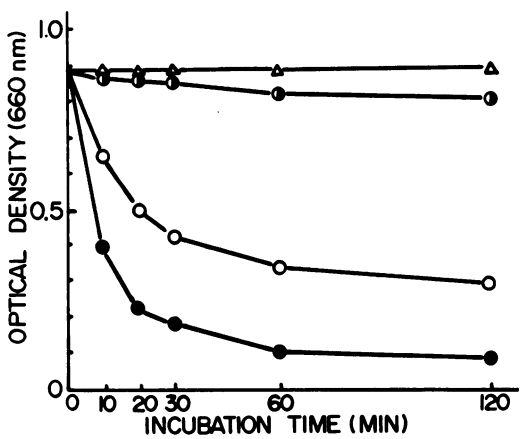

FIG. 5. *Turbidity decrease in the cell suspension of S. ruminantium by lysozyme, EDTA, or both reagents. (△) Control (−lysozyme, −EDTA). (●) (+lysozyme, +EDTA). (○) (+lysozyme, −EDTA). (◑), (+EDTA, −lysozyme). The details of the composition of these reaction mixtures are described in Materials and Methods.*

The thin sections also revealed that the outer membrane and the murein layer formed a wrinkled surface. These observations coincided with those described by Kingsley et al. regarding *Selenomonas* (18). EDTA- or lysozyme-treated cells were also thin sectioned (Fig. 6D-F). Small vesicles with bilayer structures were observed in the periplasmic space. Therefore, it appears that the physiological and morphological properties of the envelope of this strain resemble those of the "deep rough" mutant and the *mlp* or *lpo* mutant of *E. coli* K-12, respectively (28, 29, 32).

DISCUSSION

We have shown in this paper the protein compositions of the outer and inner membranes of *S. ruminantium*. At least two major proteins (40K and 42K proteins) in the outer membrane of this strain were recognized. In addition, the presence of another major protein, the 49K protein, was confirmed in the outer membrane when grown in lactate medium. The relative amount of the 40K and 42K proteins varied considerably, depending upon the culture conditions. Cells grown in glucose medium supplemented with *n*-decanoate contained a smaller amount of 42K protein with a concomitant increase of 40K protein. In contrast, cells grown in lactate medium contained a smaller amount of the 40K protein with a concomitant increase of 42K protein. It is known that the culture conditions, especially the composition of the growth medium, affect markedly the relative amounts of the two peptidoglycan-associated proteins (matrix proteins) Ia and Ib in *E. coli* (4, 12, 19, 26).

The well-characterized major proteins of the outer membrane of *E. coli* and *S. typhimurium* are matrix proteins. These proteins are characterized by their strong, but noncovalent, linking to peptidoglycan (24). We have shown in this paper that 40K and 42K outer membrane proteins of *S. ruminantium* had a strong affinity to the peptidoglycan. These data show that the two major proteins of this strain are similar to the matrix proteins of *E. coli* for the following reasons: (i) both 40K and 42K proteins were not extracted by 2% sodium deoxycholate in the presence of 0.2 M NaCl at 37°C for 30 min (data not shown); (ii) both proteins were completely released from the peptidoglycan when our outer membrane preparation, which is the outer membrane-peptidoglycan complex, was heated in 2% SDS containing 0.5 M NaCl at 37°C (data not shown); and (iii) both proteins migrated on SDS-gel as monomeric forms only when they were heated above 85°C in 2% SDS. The majority of these proteins remained at the top of the stacking gel as oligomeric forms if they were heated

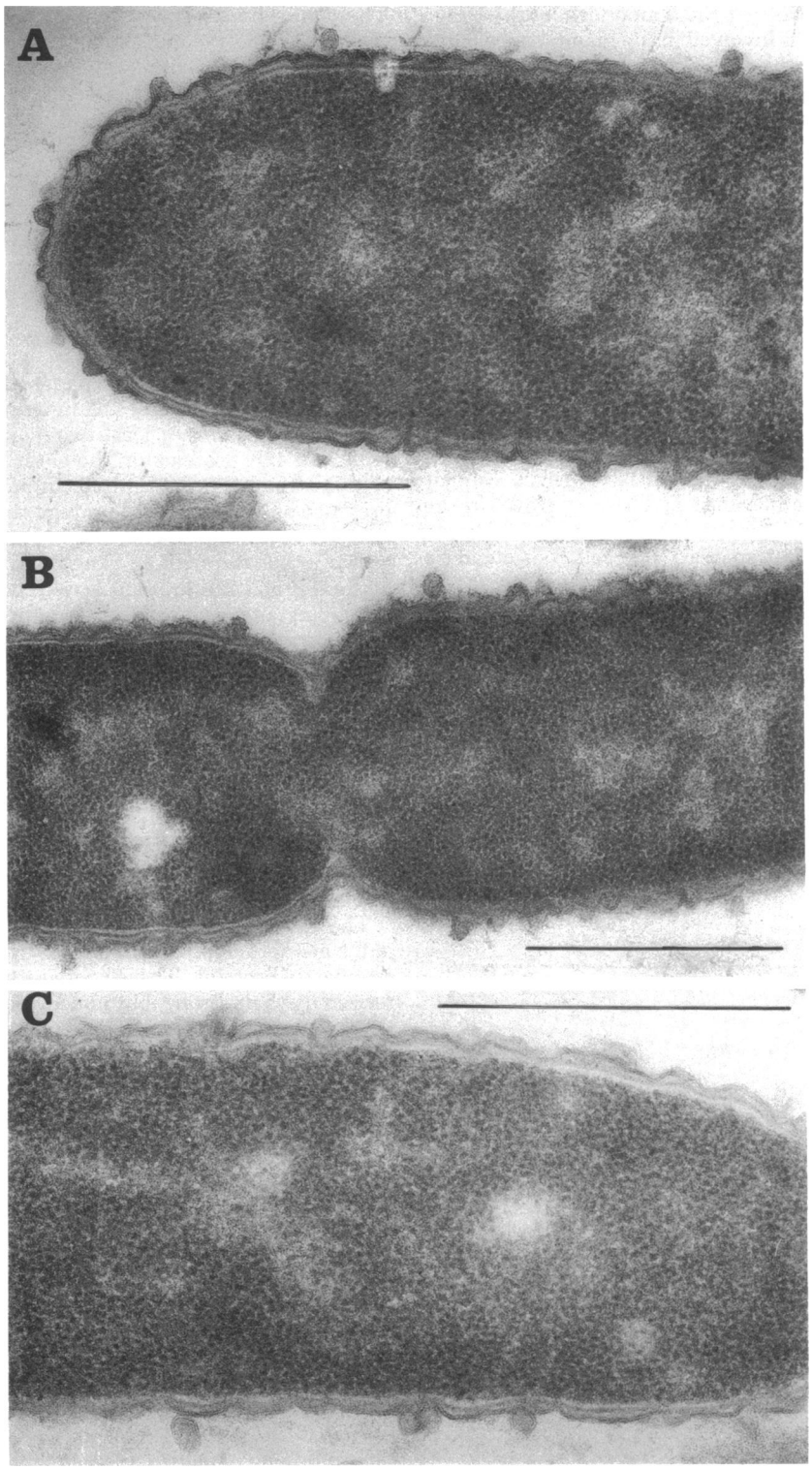

FIG. 6. *Electron micrographs of the ultra-thin sections of S. ruminantium (glucose-grown cells). (A–C) Cells at exponential phase. (D) EDTA-treated cells. (E–F) Lysozyme-treated cells. The bars indicate 0.5 μm.*

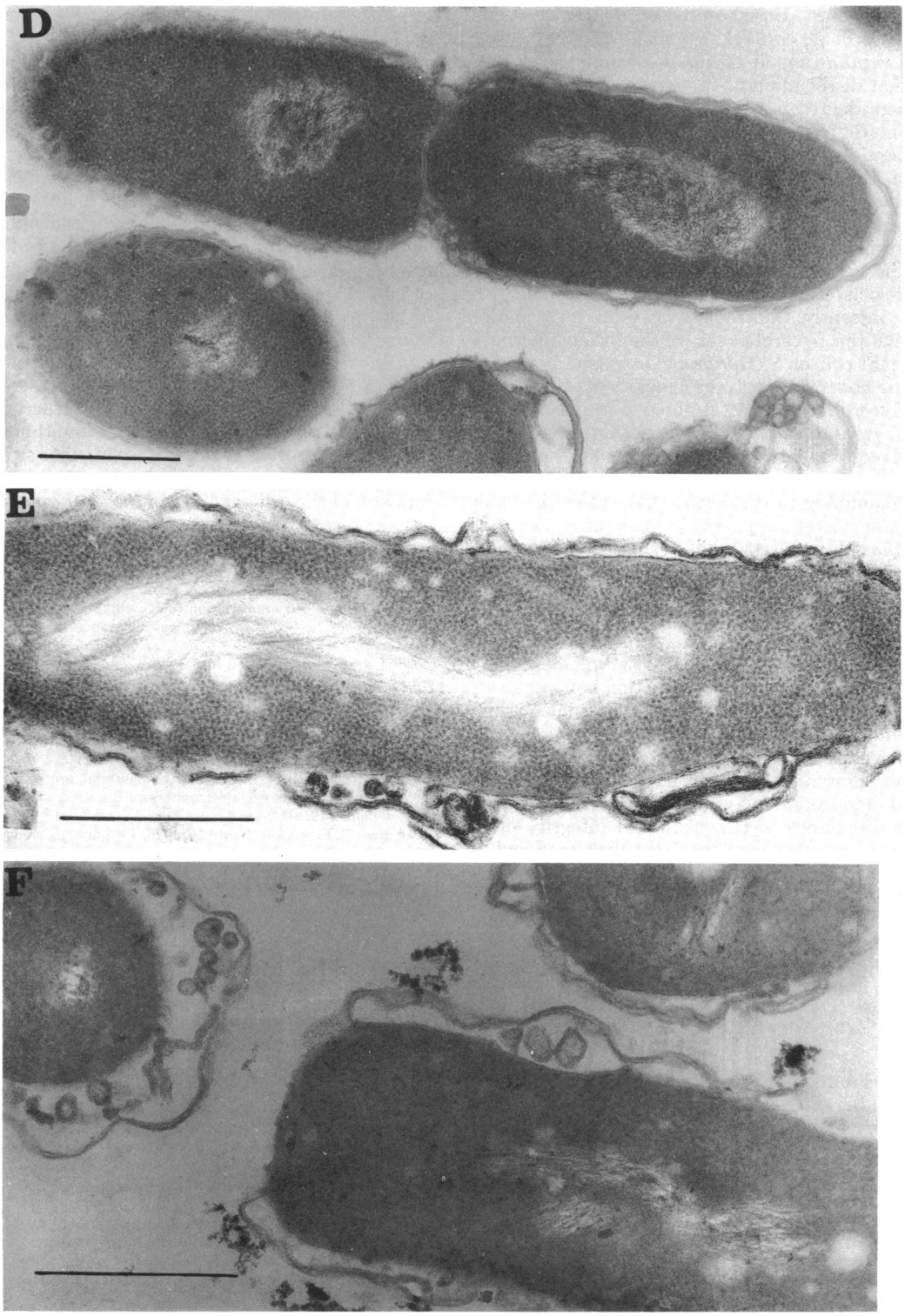

FIG. 6—*D–F*.

below 65°C. Based on these findings, characteristics of *E. coli* (11) might be applicable to *S. ruminantium*. It seems reasonable to consider that dissociation of the oligomeric forms of the peptidoglycan-associated proteins into the monomeric forms, as well as their release from the peptidoglycan, is due to a conformational change of the peptidoglycan-associated protein, and that release of the peptidoglycan-associated protein from the peptidoglycan as the oligomeric forms in the presence of 0.5 M NaCl might be due to an ionic interaction of the peptidoglycan-associated protein with the peptidoglycan.

Recently, Chattopadhyay et al. (8, 9) showed that the glycerol moiety of the Braun lipoprotein in *E. coli* or *S. typhimurium* was derived from the nonacylated glycerol moiety of phosphatidyl glycerol. In contrast to the two strains, however, *S. ruminantium* did not contain phosphatidyl glycerol as its lipid component. In this paper, we presented the evidence that the lipoprotein corresponding to the Braun lipoprotein in the enteric bacteria was not present in *S. ruminantium*. Wu et al. (30) have reported the presence, in one of the lipoprotein mutants (mutant 3) of *E. coli*, of an altered lipoprotein analog which lacks the covalently linked diglyceride; this analog was present in an appreciable amount in the soluble fraction (275,000 × *g* supernatant). Therefore, further investigation is necessary to clarify whether such a lipoprotein analog is present in *S. ruminantium*.

Yem and Wu (32) have shown by using the *mlp* mutant of *E. coli* K-12 that the bound form of lipoprotein plays an important role in the maintenance of the structural integrity of the outer membrane of the cell envelope. Suzuki et al. (28) also reported results similar to those of Yem and Wu from studies of the *lpo* mutant of *E. coli* K-12. Both the *mlp* and *lpo* mutants show hypersensitivity to EDTA, and leakage of the periplasmic enzymes and the formation of the outer membrane blebs by Mg^{2+} starvation were observed. As revealed in this study, wild-type cells of *S. ruminantium* were sensitive, like the *mlp* and *lpo* mutants of *E. coli*, to either EDTA or lysozyme, and the formation of a large number of vesicles and small blebs was observed on the cell surface. In addition, we have shown that the outer membrane and murein layer formed a wrinkled surface. The data reported here coincided essentially with those of Yem and Wu (32) and Suzuki et al. (28). However, the following differences could be pointed out between *S. ruminantium* and the *lpo* or *mlp* mutant of *E. coli*. The former forms the outer membrane blebs despite the presence of Mg^{2+} (1 mM) in the medium (data not shown), but the latter do not undergo such morphological changes under the same conditions (28, 32). In addition, the cell lysis of *S. ruminantium* by EDTA (50 μM or 1 mM) is slow, whereas that of the *lpo* or *mlp* mutants of *E. coli* by 1 mM of EDTA occurs rapidly (28, 32). In our preceding paper (16), 2-keto-3-deoxyoctulosonic acid-lipid A was shown to be present in the outer membrane of *S. ruminantium*. The outer membranes of the deep rough mutants of *E. coli* allow penetration of the lysozyme (29), and those of the deep rough mutants of *S. typhimurium* are accessible to phospholipase C that has been added to the medium (15). These findings and ours strongly suggest that the cell lysis in *S. ruminantium* by exposure to a low concentration of lysozyme and the bleb formation in the presence of Mg^{2+} might be due to both the structural characteristics of lipopolysaccharide and the absence of lipoprotein in this strain. Further studies are desirable to clarify the structure and function of the cell envelope of *S. ruminantium*.

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