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Outer Membrane Protein Composition of *Yersinia pestis* at Different Growth Stages and Incubation Temperatures

RICHARD P. DARVEAU, WILLARD T. CHARNETZKY,* AND RONALD E. HURLBERT

Department of Bacteriology and Public Health, Washington State University, Pullman, Washington 99164

The protein composition of the outer membrane of *Yersinia pestis* grown at 26 and at 37°C was examined. The outer membrane was isolated by isopycnic sucrose density centrifugation, and its degree of purity was determined with known inner and outer membrane components. Using two-dimensional gel electrophoresis, we identified a large number of heat-modifiable proteins in the outer membrane of cells grown at either incubation temperature. One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis of heated preparations indicated five proteins in the outer membrane of 37°C-grown cells not evident in 26°C-grown cells. Differences in the protein composition of the outer membrane due to the stage of growth were evident at both 26°C and 37°C, although different changes were found at each temperature. When cell envelopes were examined for the presence of peptidoglycan-associated proteins, no differences were seen as a result of stage of growth. Envelopes from 26°C-grown cells yielded two peptidoglycan-associated proteins, E and J. Cells grown at 37°C, however, also contained an additional protein (F) which was not found in either the bound or free form at 26°C. The changes in outer membrane protein composition in response to incubation temperature may relate to known nutritional and antigenic changes which occur under the same conditions.

The cell envelope complex of gram-negative bacteria consists of three layers: an inner (cytoplasmic) phospholipid membrane containing a large variety of proteins, a peptidoglycan layer external to the inner membrane, and an outer phospholipid membrane containing a smaller variety of proteins and a unique glycolipid, lipopolysaccharide (3). Since the outer membrane is usually available for direct contact between a pathogen and its host, one might suspect it to play a role in pathogenesis. The properties of lipopolysaccharide have been extensively investigated, and much is known about its structure, synthesis, and biological properties (17). Lipopolysaccharide is often toxic (1) and has been shown to provide some protection from lysosomal enzymes (20, 21). Relatively little is known, however, about the function, if any, which the outer membrane proteins play in pathogenesis.

Some of the major outer membrane proteins of a number of organisms have been shown to transverse the outer membrane and to function as porins, permitting the flow of small-molecular-weight compounds from the external milieu to the periplasmic space and vice versa (3). Environmental factors such as carbon source (23), incubation temperature (14), and medium osmolarity (25) affect the protein composition of the outer membrane of *Escherichia coli*. Based on the limited information available, it is not yet possible to predict how other bacteria might alter their outer membrane protein composition in regard to environmental change. One might suspect, however, that in a bacterium which has altered physiological capabilities or antigenic components or both in different environments, outer membrane proteins might be altered to a significant extent. This is a particularly relevant consideration with regard to pathogenic bacteria, many of which alternate between host-associated and free-living forms or between mammalian hosts and insect vectors. *Yersinia pestis*, the causative agent of bubonic plague, alternates in nature between a mammalian host and its flea vector. The two hosts represent significantly different environments with regard to temperature and other growth conditions. *Y. pestis* grows in the flea gut at a comparatively low temperature, whereas in mammals it grows both intra- and extracellularly at a higher temperature. *Y. pestis* differs both antigenically and physiologically depending upon the temperature of incubation, and two virulence determinants (for mammals) are present in cells grown at 37°C, but absent in cells grown at 26°C (2). Furthermore, additional nutrients including vitamins, amino acids, and the cation Ca^{2+} are required for growth at 37°C. One would suspect that such changes might result from, or be ac-

companied by, alterations in the outer membrane. This investigation was undertaken to see whether the differences in *Y. pestis* antigenic composition and nutritional requirements, seen as a result of different growth conditions, are reflected in alterations in the proteins of the outer membrane, since such knowledge might contribute to an understanding of the role of the outer membrane in virulence. We have isolated the outer membrane of *Y. pestis* by isopycnic sucrose gradient centrifugation. We show here that *Y. pestis* responds to changes in the incubation temperature by altering the protein composition of the outer membrane. Incubation at 37°C results in the appearance of several bands not seen, or barely detectable, in cells grown at 26°C, one of which is a major peptidoglycan-associated protein at 37°C. We also show that changes in the outer membrane protein composition which occur after the onset of the stationary phase of growth at 26°C are not found at 37°C under similar conditions, but rather that a new pattern of changes in the outer membrane proteins is evident at the elevated temperature.

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MATERIALS AND METHODS

Bacterial strain, medium, and culture conditions. *Y. pestis* EV76 was obtained from R. R. Brubaker, Michigan State University. *Y. pestis* was grown in broth consisting of: N-Z amine (3%), lactic acid (10 mM), $MgCl_2$ (2 mM), K_2HPO_4 (25 mM), citric acid (10 mM), $MnCl_2$ (0.01 mM), and $FeCl_2$ (1 mM). After autoclaving, xylose (10 mM), $Na_2S_2O_3$ (25 mM), and potassium gluconate (10 mM) were added. Where specified, $CaCl_2$ (2.5 mM) was added. The medium was adjusted with 5.5 N NaOH to pH 7.0. When calcium was to be omitted from the growth medium, the N-Z amine was decalcified by the following procedure. A 9% solution was heated to boiling, sodium oxalate was added to a final concentration of 0.02 M, and the solution was placed at 4°C overnight. The solution was centrifuged at 13,200 × *g* for 20 min at 4°C, filtered, and stored at room temperature.

All cultures were started aerobically at 26°C. Cell growth was monitored by following the absorbance at 620 nm. Cells were grown with shaking (200 rpm) in 1-liter Erlenmeyer flasks containing 250 ml of medium. Logarithmic-phase cells were harvested at an optical density of 1.0, and stationary-phase cultures were harvested after 24 h of growth at an absorbance between 3.0 and 4.0. Cells to be harvested in the logarithmic and stationary phases of growth at 37°C were shifted from 26 to 37°C at an absorbance of 0.15. All cells were collected by centrifugation at 13,200 × *g* for 30 min at 4°C and stored at −74°C until used.

Extraction of cell envelopes and separation of membrane fractions from *Y. pestis*. Frozen cells with a wet weight between 2 and 3 g were resuspended in 10 ml of buffer containing 10 mM Tris-hydrochloride buffer (pH 8.0), 20% sucrose (wt/vol), and 1 mM DL-dithiothreitol. EDTA was added to 1 mM where specified. Pancreatic RNase and DNase were added to the suspension, which was then passed three times through a French pressure cell at 15,000 to 20,000 lb/in^2. This and all subsequent steps were carried out at 4°C. After centrifugation of the lysate at 1,300 × *g* for 10 min, the pellet, containing unbroken cells, was discarded. A 2-ml amount of lysozyme (1 mg/ml) was added to the supernatant. After incubation for 30 min, the suspension was centrifuged at 210,000 × *g* for 2.4 h, and the resultant pellet of the cell envelopes was resuspended in the breakage buffer. Cell envelopes (8 mg of protein) in 0.5 ml of the breakage buffer were applied to the top of a sucrose gradient containing 1.7 ml of 65% sucrose, 2 ml each of 50, 45, 40, and 35% sucrose, and 2.3 ml of 30% sucrose in buffer containing 1 mM dithiothreitol and 5 mM Tris-hydrochloride buffer, pH 8.0. Where specified, the gradient buffer also contained 5 mM EDTA. The gradients were run to equilibrium for 16 h at 225,000 × *g* or 12 h at 286,000 × *g* in an SW41 rotor. Fractions from each tube were collected, and absorbances at 280 and 260 nm were determined. A correction was then made for the presence of nucleic acids by multiplying absorbance at 280 nm by (absorbance at 280 nm/absorbance at 260 nm) (8). Fraction peaks were pooled, centrifuged for 2.5 h at 210,000 × *g*, and resuspended in buffer containing 1 mM dithiothreitol and 10 mM Tris-hydrochloride buffer, pH 8.0. Where specified, this buffer also contained 1 mM EDTA. Ribosomes were isolated by the method of Kurland (9).

Chemical analysis. Proteins were determined by the method of Lowry et al. (12) as modified by Herbert et al. (6). 2-Keto-3-deoxyoctonate was determined as described by Droge et al. (4).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the discontinuous buffer system of Laemmli (10). Membrane samples were dissolved at ambient temperature or at 100°C for 5 min immediately before electrophoresis in slab gels prepared and run as described by Hui and Hurlbert (7). For two-dimensional analysis of heat-modifiable proteins in the outer membrane, the procedure described by Hui and Hurlbert (7) was used. Molecular weight standards used were bovine serum albumin (molecular weight, 67,000), ovalbumin (molecular weight, 43,000), aldolase (molecular weight, 37,000), trypsinogen (molecular weight, 24,000), and lysozyme (molecular weight, 14,000).

Enzyme assays. NADH oxidase (EC 1.6.99.3) and D-lactate dehydrogenase (EC 1.1.2.4) activities were measured by following the decrease in absorbance at 340 nm after the addition of 20 to 100 μg of protein. The reaction mixture for NADH oxidase contained 50 mM Tris-hydrochloride (pH 8.0), 0.2 mM dithiothreitol, and NADH sufficient to yield an absorbance of 1.8. For D-lactate dehydrogenase assays, 45 nM sodium pyruvate was added, and the absorbance change resulting from NADH oxidase activity was subtracted.

Peptidoglycan-associated proteins. Peptidoglycan-associated proteins were detected as originally

described by Rosenbusch (22) and modified by Lugtenberg et al. (14), except that bromophenol blue was present in the extraction buffer.

RESULTS

Extraction of cell envelopes and separation of membrane fractions from *Y. pestis*. Initial attempts to separate the outer membrane from the cytoplasmic membrane were carried out with EDTA present at all extraction steps. The protein profile obtained after sucrose gradient centrifugation showed the presence of two peaks (Fig. 1A). The lower peak had a specific density of 1.21 g/cm^3 and the same protein composition as purified outer membranes from *Y. pestis* as described below (data not shown). When the upper peak was centrifuged at 210,000 × *g* for 3 h, a small quantity of material was recovered. This material contained 20 times the specific activity of NADH oxidase and lactate dehydrogenase found in the lower peak (data not shown). However, the yields of the upper peak were quite low, and we were unable to obtain an accurate density determination or reasonable quantitation for purity criteria.

When cell envelopes were subjected to sucrose gradient centrifugation without EDTA present, three peaks were observed (Fig. 1B). The data presented in Table 1 are averages obtained from all culture conditions. Based on three independent extractions from each culture condition, no difference in the degree of purity of the outer membrane was observed. Peak c was enriched in both NADH oxidase and lactate dehydrogenase activities and was depleted in 2-keto-3-deoxyoctonate when compared to peak a. Peak b displayed intermediate specific activities for both enzymes (Table 1). Each peak presented a unique protein composition when subjected to SDS-PAGE. Peak c contained many bands typical of cytoplasmic membranes (Fig. 2, lanes 1

TABLE 1. *Composition of membrane fractions*

Sucrose gradient peak	Density[a]	NAD ox[b]	LDH[b]	KDO[c]
a	1.21	0.06	<0.01	75
b	1.16	0.21	0.04	13
c	1.14	1.10	0.23	6

[a] Expressed as grams per cubic centimeter.

[b] NAD ox, NAD oxidase; LDH, lactate dehydrogenase. Activity is expressed as micromoles of NADH oxidized per minute per milligram of protein.

[c] KDO, 2-Keto-3-deoxyoctonate. Amount expressed as nanomoles per milligram of protein. Obtained from stationary-phase extracts (see text).

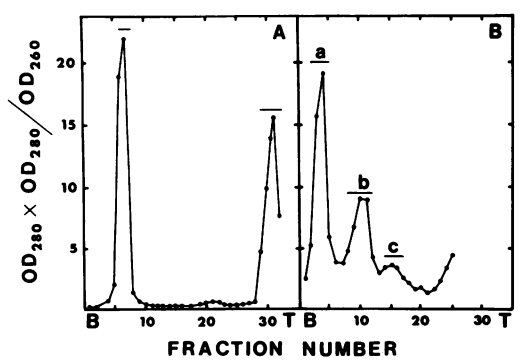

FIG. 1. *Protein profiles of Y. pestis membranes obtained after sucrose gradient centrifugation of cell envelopes. (A) Gradient consisted of 3 ml of 65% sucrose, 2 ml each of 50, 45, and 40% sucrose and 3 ml of 35% sucrose (wt/vol) in the buffer described in the text containing 5 mM EDTA. (B) Gradient prepared as described in the text without EDTA. Bars indicate the fractions which were pooled and characterized. Letters T and B indicate the top and bottom of the gradients, respectively.*

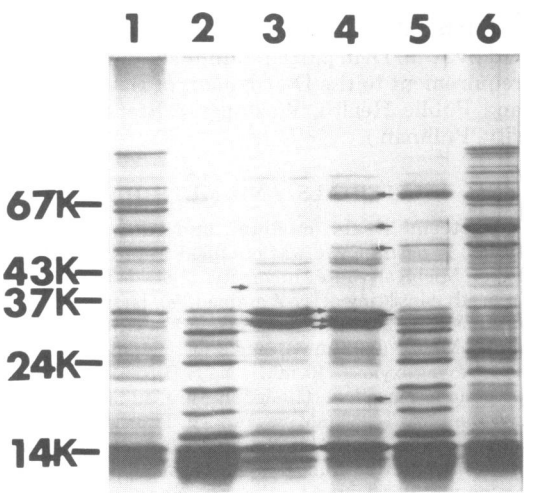

FIG. 2. *SDS-PAGE of sucrose gradient peaks obtained from stationary-phase cells grown at 26 and 37°C. Envelopes obtained from cells grown at 26°C (lanes 1 to 3) or 37°C (lanes 4 to 6) were separated in sucrose density gradients in the absence of EDTA as described in the text (Fig. 1B). The peaks were analyzed in 9 to 15% acrylamide continuous SDS-gradient gels as described in the text. Peak a material, lanes 3 and 4; peak b, lanes 2 and 5; peak c, lanes 1 and 6. A 30-μg amount of protein was applied to each lane. Arrow between lanes 2 and 3 indicates protein D, arrows between lanes 3 and 4 indicate, from top to bottom, E, G, H, and J. Arrows between lanes 4 and 5 indicate proteins which appear in the outer membrane of 37°C-grown cells, but not of 26°C-grown cells. From top to bottom these are A, B, C, F, and I. Positions of molecular weight standards are indicated on the left.*

and 6); peak a had relatively few polypeptides, with a few bands quantitatively predominating (Fig. 2, lanes 3 and 4), and peak b contained some bands present in peaks a or c or both and, in addition, some bands not present in either peaks a or c (Fig. 2, lanes 2 and 5). From these data, we conclude that peak c is cytoplasmic membrane and peak a is outer membrane that has been effectively separated from the cytoplasmic membrane. All further separations were performed with samples isolated in the absence of EDTA. Only those changes in outer membrane composition which were consistantly obtained and were readily apparent (or relate directly to readily apparent bands) are discussed. Protein bands discussed are designated A through J in order of increasing relative mobilities seen in heated outer membrane samples.

Effect of growth temperature and growth stage on the protein composition of the outer membrane. Analysis by SDS-PAGE showed that the protein pattern of the outer membrane obtained from stationary-phase cells grown at 37°C was significantly different from that obtained with the outer membrane of 26°C-grown stationary cells. Five minor bands present in the outer membrane of 37°C cells (Fig. 2, lane 4) were absent or greatly diminished in the outer membrane of 26°C cells (Fig. 2, lane 3). These bands, designated A, B, C, F, and I, have apparent molecular weights of 72,000, 57,000, 50,000, 32,000, and 18,000, respectively (Fig. 2, lane 4). Although B is clearly visible in this figure, significantly lesser quantities were present in other extractions. One minor band (designated D) with an apparent molecular weight of 38,000 was present in the outer membrane of 26°C stationary cells, but was absent in 37°C stationary-phase cells (Fig. 2, lane 3). The quantities of two major outer membrane polypeptides with apparent molecular weights of 33,000 and 30,000 (designated E and H, respectively) were altered by incubation temperature. Protein E decreased and H increased at 37°C relative to the 26°C sample (Fig. 2, lanes 3 and 4). The relative concentrations of two other major outer membrane proteins with apparent molecular weights of 31,500 (G) and 15,000 (J) did not change significantly in response to incubation temperature (Fig. 2, lanes 3 and 4).

A number of changes occur in the protein composition of the outer membrane in response to stage of growth. At 26°C, stationary-phase cells were enriched in polypeptides D and E and had an additional protein, H, when compared with logarithmic-phase cells (Fig. 3, lanes 1 and 2). When outer membrane samples containing 20 to 100 μg of protein were subjected to electrophoresis, H was seen in all extracts except those from cells harvested in log phase at 26°C. Two low-molecular-weight polypeptides also changed their relative intensities depending upon the stage of growth at 26°C (Fig. 3, lanes 1 and 2).

The changes in outer membrane protein composition of 37°C cells in response to the stage of growth were distinct from those found at 26°C. Although the changes in the two low-molecular-weight polypeptides found at 26°C were also found at 37°C, the changes in H, E, and D described for 26°C-grown cells were not evident in 37°C-grown cells. Two proteins seen only at 37°C, B and I, increased in the stationary phase (Fig. 3, lane 4) when compared with the logarithmic phase (Fig. 3, lane 3).

Since four bands (E, F, G, and H) migrated to apparent molecular weight positions between 30,000 and 33,000, their presence on the gels was difficult to resolve. To clarify their positions in the gels and to confirm the presence or absence of these bands due to cultural conditions, mixing experiments were performed. When *Y. pestis* was grown at 26°C and harvested in the logarithmic phase of growth, proteins F and H were not evident (Fig. 4, lane 1). When an extract containing F was mixed with this extract, F was

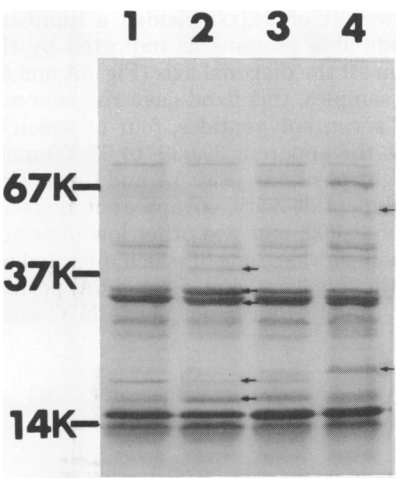

FIG. 3. *SDS-PAGE of outer membrane proteins of logarithmic- and stationary-phase cells incubated at 26 and 37°C. Outer membrane obtained from sucrose density gradients as described in the text. Lane 1, growing cells at 26°C; lane 2, stationary-phase cells at 26°C; lane 3, growing cells at 37°C; lane 4, stationary-phase cells at 37°C. Arrows between lanes 2 and 3 indicate those proteins which show changes due to stage of growth at 26°C, from top to bottom; D, E, H and two low-molecular-weight proteins. Arrows on lane 4 indicate proteins showing changes due to stage of growth at 37°C, from top to bottom; B and I. All other conditions are as described for Fig. 2.*

clearly resolved between E and G (Fig. 4, lane 2). Similarly, when a 26°C stationary-phase extract containing H (Fig. 4, lane 3) was mixed with the 26°C logarithmic-phase extract (Fig. 4, lane 1), the resultant electrophoretic pattern clearly resolved H as a distinct band (Fig. 4, lane 4). This indicated that H was absent or barely detectable in the 26°C logarithmic-phase preparations and not merely obscured by band G. Clearly, then, *Y. pestis* contains at least four electrophoretically distinct polypeptides migrating in this molecular weight range, and at least two of these are dependent upon incubation temperature and stage of growth.

Heat-modifiable proteins in the outer membrane of *Y. pestis*. When outer membrane samples of both 26°C and 37°C stationary-phase cells were solubilized at room temperature and subjected to SDS-PAGE analysis, two major bands at apparent molecular weights of 24,000 and 18,000 were seen (Fig. 5, lane 1 and Fig. 6, lane 1). A diffuse, lightly staining region (which appeared as a series of sharply defined bands in some gels) with an apparent molecular weight of between 70,000 and 80,000 was also present (Fig. 5 and 6, lane 1). When the first dimension gels were heated and subjected to SDS-PAGE in the second dimension, the band at an apparent molecular weight of 24,000 yielded a number of heat-modifiable proteins as indicated by their migration off the diagonal axis (Fig. 5A and 6A). In both samples, this band gave rise to a minimum of seven polypeptides, four of which migrated to the apparent 30,000- to 33,000-molecular-weight positions (Fig. 5A and 6A, position 2), a polypeptide with an apparent molecular weight of 44,000, and two other low-molecular-weight polypeptides, one of which migrated with or near the dye front (Fig. 5A and 6A, positions 3 and 4). Finding four bands in the 26°C extract was unexpected, since we consistantly resolve only three bands in this region with one-dimensional analysis. At present, we are unable to correlate bands E, G, and H to polypeptides seen in two-dimensional analysis. Such correlations for proteins from 37°C-grown cells are also impossible at this time. The band at an apparent molecular weight of 18,000 yielded, upon heating, material migrating close to, or with, the dye front which often appeared as a streak (Fig. 5A and 6A, band 5). Upon heating, the band at an apparent molecular weight between 70,000 and 80,000 yielded one band that migrated to a po-

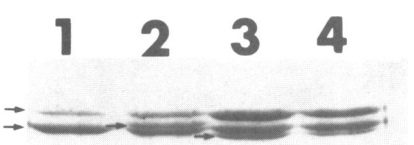

FIG. 4. *SDS-PAGE gel demonstrating the resolution of the major outer membrane proteins. Arrows indicate, respectively, in lane 1 (top to bottom) E and G; in lane 2, F; and in lane 3, H. Lane 1, outer membranes from logarithmic-phase 26°C cells; lane 2, preparation as in lane 1 to which preparation containing F was added; lane 3, outer membrane proteins from stationary-phase 26°C; and lane 4, a mixture of outer membranes from logarithmic- and stationary-phase 26°C cells. Only that portion of the gel of interest is shown. Gels were 10% acrylamide and 25 μg of protein was applied to each lane.*

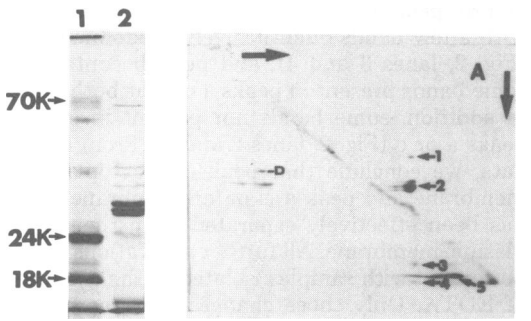

FIG. 5. *Two-dimensional analysis for heat-modifiable behavior of outer membrane proteins from 26°C-grown cells. Lane 1, unheated one-dimensional gels. Lane 2, heated one-dimensional gels. (A) Two-dimensional figure: initial migration left to right (unheated). Second dimension, top to bottom (after heating at 100°C, 5 min). Arrow 1, 44,000 protein; arrow 2, E, G, and H (for discussion, see text); arrows 4 and 5, two low-molecular-weight proteins. Numbers on the far left indicate the molecular weight of the major bands of the unheated material. A 30-μg amount of protein was applied to lanes 1 and 2, whereas 60 μg of protein was used for the two-dimensional gel. Ten percent acrylamide was used for both one- and two-dimensional gels.*

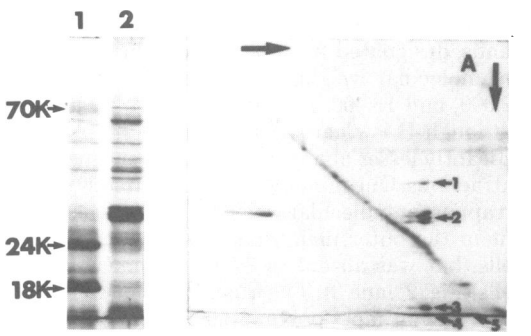

FIG. 6. *SDS-PAGE of outer membrane of 37°C-grown cells dissolved at room temperature and at 100°C. All conditions and symbols are as described in Fig. 5.*

sition similar to D (molecular weight, 38,000) and a second protein which migrated with an apparent molecular weight between 30,000 and 33,000 (Fig. 5A). The 37°C sample yielded similar results, except that protein D was absent and a faint streak (not discernible in Fig. 6A) at the position expected for A was seen which extended left from the diagonal axis to a position in line with the origin. When two-dimensional gels were run with unheated strips, no proteins migrated off the diagonal axis with the exception (in 37°C preparations) of a streak in the approximate position of A, which appeared as previously described for the heated gel. No proteins became fixed in the first-dimensional gels upon heating. The data reported here for 37°C-grown cells was obtained after growth in the presence of 2.5 mM calcium, which is required for optimal growth at this temperature. After incubation at 37°C in the absence of calcium, protein patterns identical to those presented here were obtained.

Effect of incubation temperature on peptidoglycan-associated proteins of *Y. pestis*. Dependent on the culture conditions, several proteins remained associated with the peptidoglycan after exposure of the cell envelopes to SDS at 60°C. Cells grown at 26°C contained two peptidoglycan-associated proteins, E and J (Fig. 7, lane 1). Cells grown at 37°C also contained E and a decreased amount of J, as well as another protein (F), which was absent or barely detectable at 26°C (Fig. 7, lane 2). There was no change in the peptidoglycan-associated proteins due to stage of growth at either temperature.

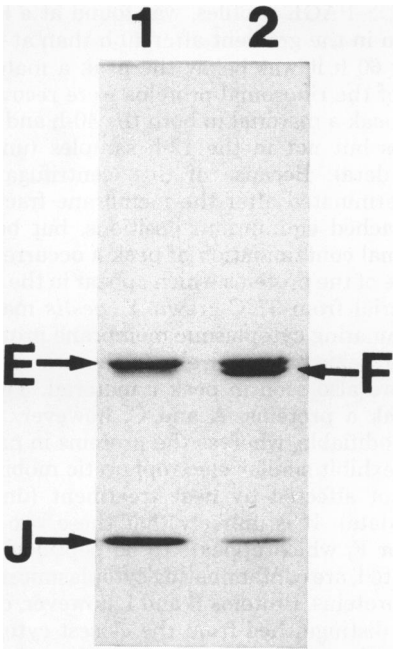

FIG. 7. *SDS-PAGE of peptidoglycan-associated proteins from 26 and 37°C cells. Lanes 1 and 2 contain proteins obtained from 26 and 37°C-grown cells, respectively. In both cases, 10 μg of protein was applied to a 10% acrylamide gel.*

DISCUSSION

The results of this study show that although *Y. pestis* outer membrane can be isolated by isopycnic gradient centrifugation in the presence or absence of EDTA, it was not possible to isolate typical cytoplasmic membrane when EDTA was present. In the absence of EDTA, we obtained three distinct bands on the sucrose gradients. Based on the density and the chemical and enzymatic analysis of the three peaks, we concluded that the most dense peak represented outer membrane, whereas the least dense peak contained cytoplasmic membrane. When the purity of the preparations obtained without EDTA was based on enzymatic activity, no pattern was found which suggested any difference in purity between inner and outer membrane due to any of the cultural conditions employed. We did observe, however, that independent of incubation temperature, stationary-phase cells yielded cytoplasmic membrane fractions containing 6% of the total 2-keto-3-deoxyoctonate recovered, whereas cells from logarithmically growing cultures yielded cytoplasmic membrane fractions containing 16% of the total 2-keto-3-deoxyoctonate recovered. We believe that this indicates not a lesser degree of separation of outer and inner membranes in logarithmically growing cells, but rather the presence of nascent lipopolysaccharide in the inner membranes (22).

When ribosomes were isolated and subjected to the same sucrose gradient centrifugation procedure, a single peak was found in the position of peak b. When the SDS-PAGE ribosomal protein profiles were compared to those of peak b protein profiles, each unique peak b protein was found to have a mobility identical to a ribosomal protein, although no proteins in the ribosomal preparations displayed relative mobilities similar to any protein in the outer membrane preparation (unpublished data). The recovery of ribosomes was not unexpected, since EDTA was not present at any step in our procedure. Although a high ratio of absorbance at 260 nm to that at 280 nm (1.8) was found in peak b, as expected for ribosomes, the apparent density (1.17 g/cm^3) was less than expected. Subsequently we subjected cell envelopes to prolonged sucrose density centrifugation. Peak b material, as determined by the high ratio of absorbance at 260 nm to that at 280 nm and the character-

istic SDS-PAGE profiles, was found at a lower position in the gradient after 40 h than at 12 h, and by 60 h it was below the peak a material. Some of the ribosomal proteins were recovered in the peak a material in both the 40-h and 60-h samples but not in the 12-h samples (unpublished data). Because of this, centrifugations were terminated after the membrane fractions had reached equilibrium positions, but before ribosomal contamination of peak a occurred.

Some of the proteins which appear in the peak a material from 37°C-grown *Y. pestis* may be contaminating cytoplasmic membrane proteins, since proteins of similar electrophoretic mobilities were also seen in peak c material. Two of the peak a proteins, A and C, however, were heat modifiable, whereas the proteins in peak c which exhibit similar electrophoretic mobilities were not affected by heat treatment (unpublished data). It is unlikely that these two proteins, or F, which appears to be peptidoglycan associated, are contaminating cytoplasmic membrane proteins. Proteins B and I, however, could not be distinguished from the closest cytoplasmic membrane counterparts by either of these criteria. Since other major cytoplasmic membrane proteins were not evident in the outer membrane preparations, the appearance of B and I in the outer membranes would have to result from a selective contamination. Although we cannot rule out this possibility, we chose to classify them tentatively as outer membrane proteins.

The changes in the outer membrane composition which occur as a result of incubation temperature are significant in two respects. First, there was a significant change in protein composition between cells harvested at 26 and 37°C. Second, the alteration of the stage of growth had a different effect on the protein composition of 37°C-grown cells compared with those incubated at 26°C. At 26°C, a new major protein (H) appeared in the outer membrane of stationary-phase cells. At 37°C, however, no changes in major outer membrane proteins occurred. Reports describing changes in protein composition due to these factors in other organisms are limited (14, 23), but, in general, describe varying quantitative changes in certain major outer membrane proteins. One report, however, shows that the appearance of major outer membrane protein a in *E. coli* is temperature dependent (14). We cannot at this time relate the changes in outer membrane protein composition seen in *Y. pestis* to known nutritional and antigenic changes, which include capsule production, that occur between 26- and 37°C-incubated cells. It appears likely that such relationships exist, since an outer membrane protein in *E. coli* has been identified tentatively as being associated with encapsulated strains (18).

Previous studies of heat-modifiable membrane proteins have shown that such proteins can migrate either to higher or lower apparent molecular weights after heating (5, 11, 15, 19). A gain in apparent molecular weight may be due either to heat-induced aggregation (7) or to changes in tertiary structure (19), whereas an apparent decrease in molecular weight may be due to changes in the aggregation state of the protein (11) or protein-lipid or lipopolysaccharide complexes or both (24). In *Y. pestis* at least five outer membrane proteins show an increase and at least four show a decrease in apparent molecular weight upon heating. It is interesting that seven heat-modifiable proteins originate from the apparent 24,000-molecular-weight region seen in SDS-PAGE of unheated material. The significance of this, as well as the exact nature of the changes that occur upon heating, remains to be determined.

Y. pestis outer membrane contains three peptidoglycan-associated proteins, two of which predominate in 26°C cells (E and J) and an additional one (F) which is seen only with 37°C samples. The recovery of J was significantly lower from 37°C-grown cells than from 26°C-grown cells. The correlation of the relative amounts of peptidoglycan-associated proteins on growth conditions has been well documented for *E. coli* (3), although the nature of the factors controlling these variations are largely unknown. Lugtenberg et al. (13) have proposed that all peptidoglycan-associated proteins found in the family *Enterobacteriaceae* span the outer membrane and form, or play a role in the formation of, hydrophilic channels. If the peptidoglycan-associated proteins of *Y. pestis* are also porins, the acquisition of an additional such protein to the outer membrane of 37°C-grown cells may be significant in relation to changes in permeability, in nutritional capabilities, and in the attachment of the outer membrane to the peptidoglycan sacculus. A porin role for the peptidoglycan-associated proteins of *Y. pestis* has not been demonstrated.

It was our intent to determine whether *Y. pestis* altered its outer membrane composition in response to changes in incubation temperature or stage of growth or both. The results demonstrate that this organism responds in both cases with changes in the outer membrane protein composition. The continuation of this work will be directed at investigating the possible relationships between the changes in the outer membrane proteins and the changes in the phys-

iology and antigenic determinants which are known to occur between cells grown at 26 and 37°C, some of which are reflected in the virulence determinants of this bacterium. We report here that the presence or absence of calcium in the growth medium does not affect the resultant outer membrane protein patterns. The peptidoglycan-associated proteins at 37°C presented in this study were obtained from cells grown in the presence of 2.5 mM calcium. In Fig. 7, approximately equivalent amounts of proteins F and D are evident. This is characteristic of cells grown at 37°C with 2.5 mM calcium. Cells incubated at 37°C without added calcium yield a significantly lower recovery of protein F relative to protein D. This indicates that a relationship between the calcium dependence of *Y. pestis* and outer membrane protein composition of this organism may exist.

Further consideration of the peptidoglycan-associated proteins will be considered in a subsequent report.

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