INFECTION AND IMMUNITY, Dec. 1983, p. 1092–1101
0019-9567/83/121092-10$02.00/0

Vol. 42, No. 3

Effects of Growth Temperature, 47-Megadalton Plasmid, and Calcium Deficiency on the Outer Membrane Protein Porin and Lipopolysaccharide Composition of *Yersinia pestis* EV76

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Received 20 May 1983/Accepted 20 September 1983

The expression of several virulence determinants of *Yersinia pestis* is known to be dependent on the in vitro growth temperature. One of these, calcium dependence, is associated with the presence of a 47-megadalton plasmid. We have examined the effects of incubation temperature, calcium in the growth medium, the presence of the 47-megadalton plasmid on the outer membrane protein, and the lipopolysaccharide composition of *Y. pestis* EV76. When cells were grown at 37°C as opposed to 26°C, a change in lipopolysaccharide composition and a decrease in the amount of an outer membrane protein (protein E) were observed. The lipopolysaccharide obtained from cells incubated at 37°C had a lower proportion of 2 keto-3-deoxyoctanate, a lower phosphate to 2-keto-3-deoxyoctanate ratio, and an increased gel mobility upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis when compared with lipopolysaccharide obtained from cells grown at 26°C. Because of its growth temperature-related abundance, we investigated the nature of protein E. This protein had physical properties similar to those of other enterobacterial porins, including apparent formation of an oligomer on sodium dodecyl sulfate-polyacrylamide gels when solubilized at low temperature, acidic isoelectric point, and strong noncovalent association with the peptidoglycan. Protein E was purified and shown to form an aqueous channel in planar lipid membranes with a conductance of 1.1 nS in 1 M KCl. In addition to growth temperature-related alterations in the lipopolysaccharide and porin components of the outer membrane, the amount of three spots in two-dimensional polyacrylamide gels was shown to be related to the temperature or the presence of calcium during growth. One of these spots was shown to contain residual unmodified portions of two major heat-modifiable proteins which failed to shift to their heat-modified positions on gels, despite solubilization at 100°C for 10 min before electrophoresis. The other two spots were the heat-modified and unmodified forms of another outer membrane protein (J) which did not appear in the isoelectric focusing gel of cells grown at 37°C. It is proposed that the appearance of these spots in two-dimensional analyses is related to the lipopolysaccharide composition of the cells from which the outer membrane is derived and reflects lipopolysaccharide-protein interactions or calcium-protein interactions.

Yersinia pestis, the causative agent of bubonic plague, is a facultative intracellular parasite (9). In nature, the organism alternates between mammalian and flea hosts, which present significantly different environments with regard to temperature and other growth conditions. One requirement for the maintenance of virulent isolates in vitro at 37°C is the presence of exogenously added calcium in the growth medium (8, 9). When cells are incubated at 37°C under calcium-deficient conditions for extended periods of time, mutants which are calcium independent and avirulent become predominant (8). Evidence has been reported to suggest that this is caused by the selection of strains which have lost all or part of a 47-megadalton plasmid associated with the virulence of this organism (17, 41). Calcium deprivation of *Y. pestis* strains harboring the 47-megadalton plasmid causes a variety of effects, including bacteriostasis after two generations (20, 55), increased production of two virulence antigens designated V and W (8), pleiomorphism (10, 20), and sensitivity to lysis under certain culture conditions (10). It has

been proposed that incubation under these conditions may reflect adaptive changes that the organism must undergo to survive after phagocytosis (9, 10, 54). A difference in the viability of calcium-dependent and independent strains after phagocytosis has been reported (W. W. Shuford and W. T. Charnetzky, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, D61, p. 50).

Calcium dependence is observed when cells are incubated at 37°C but not after incubation at 26°C (9). The in vitro incubation temperature of *Y. pestis* has also been shown to be an important factor in the expression of cell invasiveness (29, 50), nutritional requirements (26), and capsule production (9).

Biochemical analysis has revealed that some virulence properties of *Y. pestis* may be associated with the outer membrane (47). We have examined the outer membrane of *Y. pestis* to describe those changes which are due to growth temperature, the 47-megadalton plasmid, and the presence or absence of calcium during growth. We describe changes in the amount of porin protein and the composition of the lipopolysaccharide (LPS) which occur due to variation in the growth temperature. In addition, we present preliminary evidence that the 47-megadalton plasmid is associated with other changes in outer membrane composition.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Y. pestis* EV76 was obtained from R. R. Brubaker, Michigan State University. Cells of this isolate are avirulent due to a presumed lesion in iron metabolism unrelated to calcium dependence (51). A calcium-independent isogenic derivative of this strain, EV76PL$^-$, was isolated on magnesium oxalate agar (27). The calcium-dependent strain EV76 contained the 47-megadalton plasmid, which has been associated with this phenotype in *Yersinia* sp. (17, 41), whereas the calcium-independent isogenic derivative EV76PL$^-$ did not (D. Portnoy, personal communication). The medium was prepared as previously described (14). The culture conditions used in this study for both derivatives were incubation at 26 and 37°C with and without exogenously added $CaCl_2$ (2.5 mM). Cells were grown with shaking (200 rpm) in 1-liter Erlenmeyer flasks containing 250 ml of medium. All cells were harvested in the stationary phase of growth. When cells of the calcium-dependent *Y. pestis* EV76 were incubated at 37°C under calcium-deficient conditions, the cultures were harvested after 11 h at 37°C, by which time they had undergone two doublings. It should be noted that these cells were not in a stationary phase brought about by nutrient or oxygen depletion, but rather were in a unique state, some parameters of which have been described previously (10, 55).

Isolation of outer membrane and porin protein. Outer membranes were isolated as described before for strain EV76 (14). Separations nearly identical to those observed before were obtained for strain EV76PL$^-$ (R. Darveau, Ph.D. thesis, Washington State University, Pullman, 1981). In the outer membrane preparations, there was an 18- and a 23-fold decrease in the specific activities of the inner membrane markers NAD oxidase (EC 1.6.99.3) and D-lactate dehydrogenase (EC 1.1.2.4), respectively, compared with inner membranes. In addition, outer membrane preparations showed a 12-fold increase in the amount of 2-keto-3-deoxyoctanate (KDO) per milligram of protein (14). Porin protein was isolated as described by Nikaido (39) with the following exceptions: lyophilized cells instead of a wet cell pellet were used, and after extraction in NaCl buffer, the preparation was dialyzed overnight against buffer without NaCl; the preparation was then passed over a Sepharose 6B column in column buffer, and the porin was eluted with the void volume.

Two-dimensional electrophoretic analysis of outer membrane samples. The two-dimensional analysis was performed as originally described by O'Farrell (40) with the modifications described by Ames and Nikaido for use with membrane samples (3), except that 8% (final concentration) Triton X-100 was used instead of Nonidet P-40 and ammonium persulfate was used to achieve polymerization. The total concentration of ampholines (LKB) was 2% and was comprised of a 2:2:1 ratio of the pH ranges 4 to 6, 6 to 8, and 3.5 to 10, respectively. Gels (2 mm by 11 cm) were focused at 0.15 W per tube for 9 h with constant cooling. All samples were adjusted to 3 mg of protein per ml, and 21 μl (63 μg) was applied to each tube. Regardless of the samples analyzed, a linear pH gradient from 4.0 to 7.2 was obtained routinely. The agarose solution used to embed the tube gel on top of the second-dimension gel was kept at 45°C before use. The second-dimension gel contained 11% (wt/vol) acrylamide. Membrane samples were suspended in isoelectric focusing (IEF) buffer immediately after isolation and subjected to electrophoresis the same day. When samples had been stored at −20°C in either IEF buffer or deionized water before electrophoresis, all of proteins L, G, and J ran as their heat-modified forms after electrophoretic analysis of heated samples. All two-dimensional analyses described in this paper were performed on at least three separate occasions with different membrane samples.

Black lipid bilayer experiments. Black lipid bilayer experiments were performed as described previously (5, 22). Optically black lipid bilayer membranes were obtained from a 1 to 2% (wt/vol) solution of oxidized cholesterol (a kind gift from R. Benz) in *n*-decane. The chamber used for bilayer formation was made of Teflon. The circular holes in the wall between the two aqueous compartments had an area of either 2 mm^2 (for macroscopic conductance measurements) or ca. 0.1 mm^2 (for the single-channel experiments). The temperature was kept at 25°C.

Isolation of LPS. LPS was extracted from 10 to 50 g of frozen cell pellets by the hot aqueous phenol procedure of Westphal and Jann (52). After additional ribonuclease (Sigma R-4875) and pronase (Sigma P-5147) treatments of the aqueous phase, the pellet obtained after ultracentrifugation contained less than 1% protein by weight. In addition, no peaks or shoulders were detected at a wavelength of 260 nm (OD_{260}) or OD_{280} when scans (OD_{300} to OD_{190}) were performed on the LPS preparations, indicating there was no significant nucleic acid or protein contamination. The

LPS preparations were extracted by the method of Folch et al. (18) to remove contaminating phospholipids. We did not lose any LPS in the chloroform phase, as judged by KDO assays, with any of the preparations used in this study. The LPS yields were between 1 and 2% of the bacterial cell dry weight. LPS was also isolated by the procedure described by Darveau and Hancock (15).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of LPS. One-dimensional SDS-PAGE was carried out by using the discontinuous buffer system of Laemmli (32); analysis of proteins was performed as described previously (14). For analysis of LPS, samples of LPS were mixed 1:1 with solubilization mix containing 0.1 M Tris-hydrochloride (pH 6.8), 2% SDS (wt/vol), 1% 2-mercaptoethanol (vol/vol), 0.001% bromophenol blue (wt/vol), 10% sucrose (wt/vol), and 40 mM EDTA (pH 6.8), and were heated at 100°C for 5 min before electrophoresis. Running gels were made from a stock solution consisting of 44% (wt/wt) acrylamide (Eastman) and 1.6% (wt/wt) *N,N'*-methylene bisacrylamide, to a final concentration of 18% acrylamide. Gels were stained as described by Tsai and Frasch (49).

Chemical analyses. Protein was determined by the method of Lowry et al. (34) as modified by Herbert et al. (25). KDO was estimated as described by Droge et al. (16). Phosphate was determined as described by Ames and Dubin (2). Heptose was detected as described by Wright and Rebers (53).

RESULTS

Two-dimensional analysis of outer membrane preparations. We have previously shown that the in vitro incubation temperature of *Y. pestis* affects the appearance of several outer membrane proteins (14). Others have shown in *Yersinia enterocolitica* (42) and *Yersinia pseudotuberculosis* (46) that certain other outer membrane proteins may be involved with the virulence of these organisms. To continue our study on the outer membrane of *Y. pestis*, we have employed two-dimensional analysis.

We examined outer membrane preparations obtained from *Y. pestis* EV76 and EV76PL$^-$ incubated at 26 and 37°C in the presence and absence of calcium in the culture medium. In agreement with Straley and Brubaker (46), we found that *Y. pestis*, unlike *Y. enterocolitica* and *Y. pseudotuberculosis*, does not contain new outer membrane polypeptides whose appearance is associated with the presence of the 47-megadalton plasmid. However, the use of two-dimensional analysis permitted us to extend some of our previous observations on the outer membrane of *Y. pestis* and revealed differences in the outer membrane—due to incubation temperature and calcium—which we did not detect before (14).

One difference not previously observed in the outer membranes of cells grown at 37°C was a substantial reduction in a major outer membrane protein (designated E) relative to other major outer membrane proteins (Fig. 1). This reduction was not observed before due to a comigrating major outer membrane protein, L*, which appears to be present at relatively the same amounts at the two incubation temperatures.

A second difference evident in the two-dimensional analysis was the appearance of a major polypeptide spot (labeled L,G in Fig. 1) in the outer membrane preparations obtained from cells grown at 26°C but not from those grown at 37°C (see Fig. 1). This spot was consistently observed in nine independent experiments and represented residual unmodified portions of two major heat-modifiable major outer membrane proteins which migrated to two different apparent molecular weight positions after two-dimensional analysis. Thus, in cells grown at 26°C, but not in cells grown at 37°C (with one exception; see below and Table 1), a portion of proteins L and G did not show altered mobility and thus did not run as the heat-modified forms L* and G*, despite treatment at 100°C for 10 min before electrophoresis. In contrast, when solubilized at 30°C before electrophoresis, proteins L and G from cells grown at both temperatures comigrate as spot L,G (R. Darveau, Ph.D. thesis). Additional evidence that the spot observed in gels of outer membrane preparations from cells grown at 26°C represented alternate (unmodified) forms of these major outer membrane proteins—despite the high solubilization temperature—was obtained by cutting the spot out of the two-dimensional gels and subjecting it to SDS-PAGE after solubilization at 100°C for 5 min. When this analysis was performed, spot L,G yielded two spots at the apparent molecular weights of the heat-modified forms L* and G* (data not shown). The molecular mechanism for the retention of unheated forms of heat-modifiable proteins after two-dimensional analysis is unknown. LPS has been shown to be involved in the retention of the OmpA protein in its unmodified form (24), and it is possible that the presence of urea in the solubilization buffer promotes the association of this protein with LPS (4, 45). The observation that protein spot L,G migrated to the heat-modified forms L* and G* upon subsequent heat treatment after electrophoresis may be due to the fact that these components (urea and LPS) or some other unidentified components were absent during the second heat treatment. Another outer membrane protein (H) migrated to two different apparent molecular weights (designated H and H*) when outer membranes were examined from cells grown in either 26 or 37°C (Fig. 1).

Another difference observed was that protein J appeared in all two-dimensional gels of outer membranes of cells grown at 26°C in both heat-modified (J*) and heat-unmodified (J) forms, but

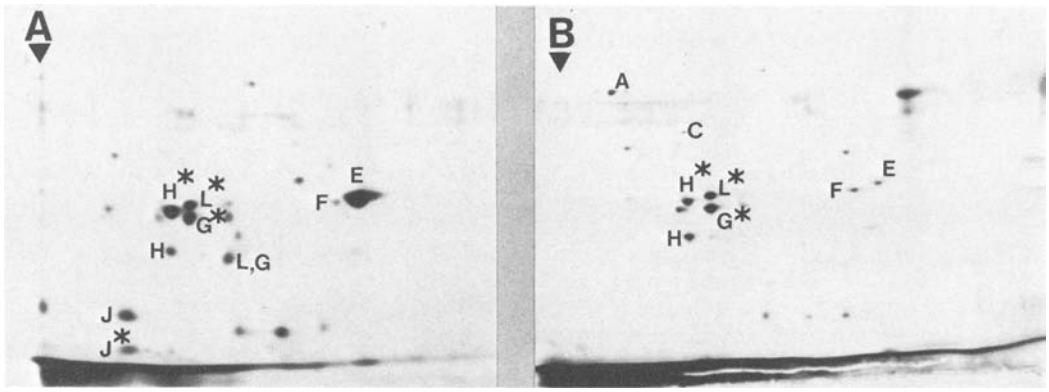

FIG. 1. Two-dimensional (IEF and SDS-PAGE) analysis of outer membrane protein preparations obtained from *Y. pestis* EV76PL cells grown at 26°C (A) and 37°C (B). The first dimension (horizontal) was IEF, and the second dimension (vertical) was SDS-PAGE (11% acrylamide). Samples were prepared as described in the text. Spots labeled with an asterisk represent the heat-modified forms of selected major outer membrane proteins (see the text for explanation). Protein E displayed an isoelectric point of 4.34, after accounting for changes in the gel size due to fixing and drying procedures. The spot in the upper right hand corner of B is an artifact of staining which appeared in this experiment and does not represent an outer membrane protein. The arrow below either A or B indicates the origin of the IEF gel.

did not appear in gels of outer membranes from cells grown at 37°C (with one exception; see below and Table 1) when samples were solubilized at 100°C for 10 min. The absence of spots equivalent to protein J (J/J*) in the outer membranes of cells grown at 37°C was in contrast to our previous observations on these outer membranes analyzed by one-dimensional SDS-PAGE (14), and presumably was due to the loss of protein J/J* from cells grown at 37°C during either IEF or during the fixing and staining procedures. Similar problems have been encountered with the lipoprotein from *Escherichia coli* (24).

Results that clarified our previous observations include (i) proteins A and C have been tentatively identified as being present in outer membrane preparations obtained from cells incubated at 37°C but not those incubated at 26°C (Fig. 1); (ii) protein F, reported earlier as being found only at 37°C, was detected in outer membrane preparations from cells grown at 26°C. This protein was not previously observed in these preparations due to the large amount of protein E present and due to a comigrating major outer membrane protein H. Finally, two other proteins, B and I, which we tentatively concluded as being outer membrane proteins, were not identified in the two-dimensional gels.

The appearance of two separate spots corresponding to a single protein after two-dimensional analysis is not unique (3, 24, 30). However, the observation that the appearance of spots L,G and J/J* was dependent upon the tempera-

TABLE 1. Presence of the unmodified forms of specific heat-modifiable major outer membrane proteins

Strain	Growth temp	Ca^{2+} present during growth	Presence of specific unmodified major outer membrane protein spots after two-dimensional protein analysis[a]		
			H	L, G	J
EV76PL	26	−	+	+	+
		+	+	+	+
	37	−	+	−	−
		+	+	−	−
EV76	26	−	+	+	+
		+	+	+	+
	37	−	+	+	+
		+	+	−	−

[a] These spots represented residual unmodified portions of major heat-modifiable major outer membrane proteins which migrated to two different apparent molecular weight positions after two-dimensional analysis (see the text for explanation).

ture at which the cells were grown in EV76PL$^-$ and both incubation temperature and calcium in EV76 (see Table 1) was unusual. In *E. coli*, Henning et al. (24) have shown that LPS is involved in the retention of unheated forms of the OmpA protein during two-dimensional analysis, despite heating before electrophoresis. We therefore isolated LPS from cells incubated at 26 and 37°C and compared its composition.

LPS analyses. SDS-PAGE of LPS has proved to be useful for examining different types of LPS (19, 49). This procedure allows detection of differences in the mobility of the LPS of defined rough mutants of *Salmonella typhimurium* (28, 38). LPS obtained from cells grown at 26°C displayed a lower relative mobility than did preparations obtained from cells grown at 37°C (Fig. 2). This difference in relative mobility occurred in both the calcium-dependent and independent strains and was independent of calcium addition to the medium. Although this difference in relative mobility was small, the difference could be consistently observed when the amount of LPS added to the gel was varied from 50 ng to 5 μg, using three independently isolated LPS preparations. In addition, and in agreement with another study (23), the LPS of *Y. pestis* appeared rough since we could not detect, even in overloaded gels, the presence of LPS of higher apparent molecular weight (lower relative mobility) which one would expect if smooth-type LPS was present (19, 28, 49). The LPS obtained from cells grown at 26°C had a distinct red to brown color when visualized by the silver staining procedure of Tsai and Frasch (49), whereas the LPS obtained from cells grown at 37°C was stained gray. Differences in the color of different LPS preparations have been noted before (15, 28), although the reason for this phenomenon is unknown.

FIG. 2. SDS-PAGE of LPS from *Y. pestis* EV76. LPS (100 ng) from cells grown at 37°C (lane 1) or 26°C (lane 2) was analyzed on an 18% polyacrylamide gel and visualized with the silver staining technique described by Tsai and Frasch (49). The apparent difference in the amount of sample added is due to the fact that LPS obtained from cells incubated at 26°C displayed a reddish-brown color, whereas LPS obtained from cells incubated at 37°C stained gray.

Chemical analysis of the LPS preparations revealed additional differences in the composition. The results shown in Table 2 are means obtained from at least three different LPS preparations. There was a 30 to 40% increase ($P < 0.01$ by Student's unpaired t test) in the percent dry weight of KDO in the LPS from cells grown at 37°C. In addition, the ratio of phosphate to KDO in the LPS was significantly ($P < 0.01$) lower in cells grown at 37°C compared with cells grown at 26°C. The difference in the phosphate-to-KDO ratio was also observed in LPS preparations isolated by a technique which has been shown to obtain a more representative sample of the cellular LPS with certain bacteria (15).

We also compared the amounts of heptose in these LPS preparations. Since we did not have a suitable standard for this assay, we compared the intensity of the heptose peak (OD_{505} to OD_{545}) with the amount of KDO used in the assay. We did not detect a significant difference in the ratio of heptose to KDO in any of the preparations.

Major outer membrane protein E is a porin. Two-dimensional analysis of outer membranes revealed that the amount of protein E was consistently reduced relative to the amounts of other major outer membrane proteins when cells were grown at 37°C (Fig. 1). Protein E had properties similar to those of the porin proteins of *E. coli*, including strong noncovalent association with the peptidoglycan layer of the cell envelope (14), an apparent oligomeric state after pretreatment with SDS at low temperature (Fig. 3), an apparent monomeric molecular weight of 33,000, and an acidic isoelectric point (4.2 to 4.5) (Fig. 1). Making use of some of these properties, we were able to purify this protein from *Y. pestis* incubated at 26°C by a method described by Nikaido (39) for the isolation of other enterobacterial porins.

When purified protein E was added to the aqueous phase of a solution bathing a planar

TABLE 2. Partial analysis of LPS obtained from *Y. pestis*[a]

Strain	Growth temp (°C)	KDO (% [dry weight] of LPS)	Molar ratio of PO_4^{2-} to KDO in LPS
EV76PL$^-$	26	7.3 ± 0.4	12.6 ± 2.5
	37	10.5 ± 0.7	4.0 ± 0.5
EV76	26	7.2 ± 0.3	6.1 ± 1.7
	37	9.6 ± 0.7	3.9 ± 0.2

[a] Similar data were obtained whether calcium was present or absent in the growth medium of cells from which LPS was derived. Results are the means ± standard deviations of at least four assays, using three different LPS preparations.

bilayer membrane, the conductance of the membrane increased severalfold. In control experiments in which no protein was added, the conductance of the membrane did not increase significantly over the time period that the experiments were performed (data not shown).

Similar to other porins, when the amount of protein E added to the aqueous phase was low (1×10^{-9} to 2×10^{-9} M trimers), discrete stepwise increments in conductance could be observed (Fig. 4). These steps were mostly directed upwards, indicating the insertion of single channels into the membrane, although downward events were observed, presumably due to the loss or inactivation of a channel. A similar high incidence of step conductance decreases has been observed for one of three *Salmonella* porins (6), but its physiological relevance, if any, is unknown. In most cases, the size of both the upward and downward steps appeared to be the same (Fig. 4). The sizes of the individual conductance steps were distributed around a mean. A histogram representing the size distribution of the conductance steps observed for protein E in 1 M NaCl is shown in Fig. 5.

A characteristic expected for a large, weakly selective, water-filled channel is that the average conductance increase observed in a given salt solution would correspond to the conductivity of the aqueous phase. Therefore, the average single-channel conductance increase for protein E was determined in three different salt solutions which differed in their bulk conductivity by a factor greater than 3 (Table 3). In each case in which the conductance increase was divided by the bulk conductivity of the salt solution, similar values were obtained (Table 3).

Protein E had a single-channel conductance of 1.1 nS in 1 M KCl. Such a conductance would correspond to a channel diameter of approximately 0.98 nm, using the formula: $\Lambda = \sigma\Pi r^2/l$, where r is the channel radius and l the width (7.5 nm) of the membrane, to estimate the effective channel size (6). Further evidence that protein E formed a water-filled channel was suggested by the fact that the current generated due to the presence of protein E was directly proportional to the voltage being applied across the membrane (Fig. 6). Furthermore, in agreement with published data for other porins (5, 6, 22), no switch-on voltage (i.e., voltage gating) was observed for this pore.

Comparison of cells with and without the 47-megadalton plasmid. Calcium dependence is temperature dependent in that it is expressed when cells are incubated at 37°C but not at 26°C (9). The expression of this virulence determinant is also associated with the presence of a 47-megadalton plasmid (17, 41). Since several changes in the outer membrane protein and LPS composition were shown to occur due to altered growth temperature, we examined outer membranes for differences related to the presence or absence of the 47-megadalton plasmid.

Results similar to those of Straley and Brubaker (46) were obtained, in that there were no new outer membrane polypeptides whose ap-

FIG. 3. SDS-PAGE of purified porin protein E. Protein E was purified as described in the text from *Y. pestis* EV76 cells incubated at 26°C. Protein samples (10 μg) were solubilized at either 30°C (lane 1) or 100°C (lane 2) for 10 min before electrophoresis on a 9% acrylamide gel. Molecular weight standards (Sigma no. MW-SDS-200; lane 3) from top to bottom are: myosin (205,000); β-galactosidase (116,000); phosphorylase B (97,000); bovine albumin (66,000); egg albumin (45,000); carbonic anhydrase (29,000).

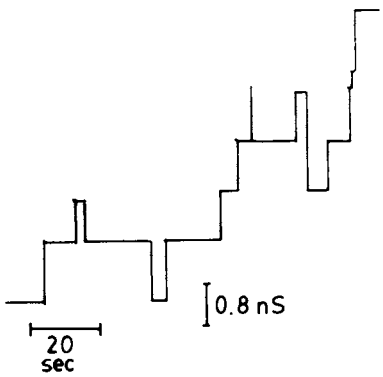

FIG. 4. Stepwise conductance fluctuations of the membrane current in the presence of purified protein E. The aqueous phase contained 1 M NaCl and ca. 1.9×10^{-9} M trimers of protein E. The temperature was kept at 25°C, and 30 mV was applied across a membrane formed from oxidized cholesterol in *n*-decane.

pearance was associated with the 47-megadalton plasmid. The changes in the outer membrane protein composition due to growth temperature or stage of growth (14) were observed for both the calcium-dependent (47-megadalton-plasmid-containing) strain EV76 and the calcium-independent (47-megadalton plasmid-free) derivative EV76PL$^-$. These changes were unaffected by the calcium concentration in the medium, with the exception noted below.

Two changes in the outer membranes of *Y. pestis* associated with the presence of 47-megadalton plasmid were observed. The first difference was observed when calcium-dependent cells were incubated at 37°C under conditions of calcium deficiency before analysis. Spots J, J*, and L,G were observed (Table 1). These major protein staining spots were absent when calcium-dependent cells were grown at 37°C in the presence of calcium. Two-dimensional gels from calcium-independent cells grown at 37°C lacked spots J, J*, and L,G irrespective of the presence of calcium but contained these spots if the bacteria were grown at 26°C.

The second difference involved an alteration of the LPS. When cells were grown at 26°C, there was a significant ($P < 0.01$) decrease in the molar ratio of phosphate to KDO in the 47-megadalton-plasmid-containing, calcium-dependent strain EV76 when compared with strain EV76PL$^-$, which is missing the 47-megadalton plasmid (Table 2). The decrease in the ratio of phosphate to KDO observed for calcium-dependent cells grown at 26°C was also observed in LPS preparations obtained by the method of Darveau and Hancock (15). This effect of the 47-megadalton plasmid was not observed when the cells were grown at 37°C, presumably due to the superimposition of growth temperature related changes in phosphate/KDO ratios. The presence or absence of calcium in the growth medium did not significantly ($P > 0.2$) change either the relative amount of KDO or phosphate/KDO ratios at either growth temperature.

DISCUSSION

We have examined the effects of growth temperature, loss of the 47-megadalton plasmid, and calcium deficiency on the outer membrane protein and LPS composition of *Y. pestis* EV76 and EV76PL$^-$. Variation of growth temperature had strong effects on both the outer membrane protein and LPS composition. For example, raising the growth temperature from 26 to 37°C resulted in a decrease in the amount of a porin protein E. This porin protein appeared to form smaller pores than previously reported porins (6, 7), except for the anion-specific protein P channel of *Pseudomonas aeruginosa* (22). Protein E had a single-channel conductance of 1.1 nS in 1 M KCl, which was about half that observed for *E. coli* porins (7).

The LPS of *Y. pestis* was also different when

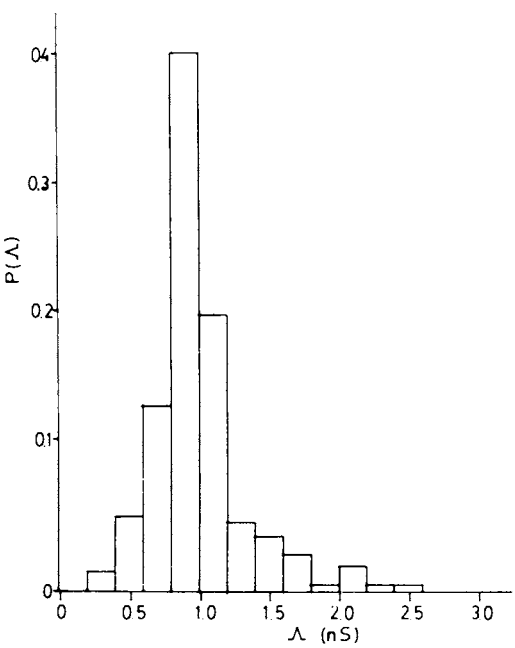

FIG. 5. Histogram of the conductance fluctuations observed with membranes from oxidized cholesterol in *n*-decane in the presence of protein E. The aqueous phase contained ca. 1.9×10^{-9} M trimers of protein E and 1 M NaCl. P(Λ) is the probability of a given conductance increment (Λ) taken from recorder traces such as those shown in Fig. 4. Both upward and downward conductance events were included. An average conductance increment ($\bar{\Lambda}$) of 0.95 nS was obtained by counting 220 events with 50 mV applied across the membrane.

TABLE 3. Average conductance increment $\bar{\Lambda}$ measured on membranes from oxidized cholesterol–*n*-decane in the presence of protein E[a]

Salt	Salt concn	$\bar{\Lambda}$ (nS)	σ (mS/cm)	$\bar{\Lambda}/\sigma$ (10^8 cm) ratio	*n*
NaCl	1.0	0.95	84	1.1	220
KCl	1.0	1.12	112	1.0	76
Tris-chloride	0.5	0.33	30	1.1	137

[a] The pH of the salt solutions was between 6 and 7; temperature was 25°C; applied voltage was 50 mV. $\bar{\Lambda}$ was determined by recording a large number (*n*) of conductance steps and averaging. σ indicates the specific conductance of the aqueous salt solution.

preparations from cells grown at 26 and 37°C were examined. At both growth temperatures, the LPS appeared rough, in agreement with the conclusions of Hartley et al., who used cells grown at 37°C (23). Growth at 37°C appeared to result in a smaller molecule, as shown by the relatively higher mobility upon SDS-PAGE and the increase in the relative amount of KDO in the LPS. However, it should be noted that mobility on SDS-PAGE may be influenced by factors other than size, and the KDO assay is subject to error depending upon the substituent groups present on the KDO (11). Despite this, our data favor a growth temperature-dependent alteration in the LPS of *Y. pestis*. The change in LPS composition observed for *Y. pestis* at higher growth temperatures may represent one of several (9, 50) adaptations necessary for the continued growth and virulence of *Y. pestis*. Growth temperature can affect the LPS compositions of other bacteria (31, 35, 48), including *Y. enterocolitica* (1; R. Darveau and D. Portnoy, unpublished data). The ability of *Y. pestis* and other organisms to alter their LPS compositions at different growth temperatures provides further evidence that LPS synthesis is regulated by environmental factors (12, 36).

Another effect of growth temperature was observed for three major outer membrane proteins. Each of these proteins (L, G, and J) was present as two forms having different apparent molecular weights (proteins L and G also displayed an altered isoelectric point) after two-dimensional analysis of the outer membranes of cells grown at 26°C (Fig. 1). The appearance of two separate spots representing the two different apparent molecular weights of so-called "heat-modifiable" outer membrane proteins has been observed before (3, 24, 30). When cells were grown at 37°C, J,J* and L,G were absent and L* and G* were present. The single exception to this pattern was observed for the 47-megadalton-plasmid-containing strain EV76 incubated at 37°C in the absence of calcium. Under these circumstances, the two-dimensional analysis revealed a protein pattern for these proteins closely resembling that of cells grown at 26°C.

A major effect of variation of the growth temperature of *Y. pestis* is that cells incubated at 37°C, but not those incubated at 26°C, show a dependence on exogenously added calcium medium for continued growth, virulence (8, 9), the production of two virulence antigens (9), and pleiomorphism (10, 20). This effect is commonly referred to as calcium dependence and is associated with the presence of the 47-megadalton plasmid (17, 41). As discussed above, we observed a corresponding calcium-dependent difference in the pattern of outer membrane protein spots seen in two-dimensional analyses of cells grown at 37°C. This calcium effect on the outer membrane could be due to the fact that calcium-deprived *Y. pestis* EV76 cells do not maintain growth long enough at 37°C for this temperature-dependent change to occur. Although this explanation cannot be eliminated with the present data, these cells are similar to stationary phase *Y. pestis* with respect to their outer membrane proteins (14, 46), the decrease in porin protein E, and the parameters of the LPS composition that we measured (Table 2). It is possible, therefore, that this calcium effect on the outer membrane of *Y. pestis* reflects a specific alteration in the outer membrane associated with this unique stage brought about by calcium deprivation.

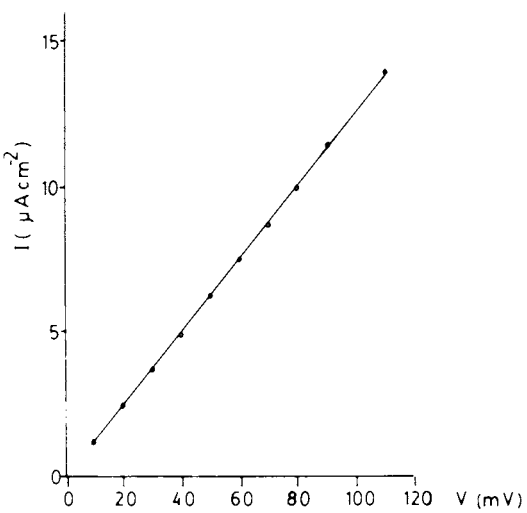

FIG. 6. Current [I(μA/cm^2)] versus voltage [V(mV)] characteristic of a membrane formed from oxidized cholesterol in *n*-decane in the presence of protein E. The aqueous phase contained ca. 3.8×10^{-10} M trimers of purified protein E and 1 M NaCl. The temperature was kept at 25°C. After a stationary level of membrane conductance was obtained, the voltage applied across the membrane was increased, and the resulting current was recorded.

The simplest hypothesis to explain our data is that the failure of a portion of proteins L and G to alter their migration after being heated in SDS and of protein J to enter the IEF gel is related to their interaction with LPS or Ca^{2+} or both. In agreement with this, both divalent cations (21, 37) and LPS (21, 24) have been shown to influence the modification, by solubilization temperature, of the mobility of outer membrane proteins on SDS-PAGE. We were able to obtain some evidence for LPS alterations, although this should be confirmed by more exhaustive analysis of the LPS composition. Overall, this paper provides preliminary evidence that the 47-megadalton plasmid of *Y. pestis* may influence LPS composition. Since LPS binds divalent cations, including Ca^{2+} (13, 44), and changes in LPS composition can influence growth (43) and virulence (33), we feel that a plasmid-related LPS alteration in addition to the observed growth temperature-related alterations in LPS could adequately explain the phenotypic alterations caused by the 47-megadalton plasmid. It is interesting to note that the intracellular survival of another facultative intracellular parasite, *Brucella abortus*, appears to be influenced by the LPS composition (31).

ACKNOWLEDGMENTS

A portion of this work was supported by the Natural Sciences and Engineering Research Council of Canada and by the State of Washington as provided by initiative 171.

We thank Roland Benz for setting up the planar bilayer apparatus in the laboratory of R.E.W.H.

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