Temperature-Inducible Outer Membrane Protein of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* Is Associated with the Virulence Plasmid

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A strain of *Yersinia pseudotuberculosis* which harbors a 63-kilobase plasmid was found to cause a lethal infection in Swiss albino mice. The rate of infection paralleled the ability of the pathogenic organism to attach to a monolayer of HeLa cells. One novel outer membrane protein (protein 1) with a molecular weight of 140,000 was found to be associated with the possession of the 63-kilobase plasmid of *Y. pseudotuberculosis*. This protein was expressed during growth at 37°C but not at 26°C, and expression was moderately affected by the concentration of calcium in the growth medium. Moreover, it was found that synthesis of protein 1 is induced within 2 min after a temperature shift from 26 to 37°C. A plasmid-associated outer membrane protein showing similar properties was also found to be expressed in plasmid-containing strains of *Yersinia enterocolitica*. The properties of protein 1 indicate that it could be identical to the previously described virulence W antigen.

The three recognized species of the facultative intracellular parasite yersinia, *Y. pestis, Y. enterocolitica*, and *Y. pseudotuberculosis*, are known to be virulent for humans and animals (10, 25, 28). In all three species, the virulence has been associated with the presence of plasmids ranging in molecular weight from 60 to 75 kilobases (15, 17, 18, 34, 41). These virulence plasmids are correlated with production of V and W antigens, which are released from bacterial cells during prolonged growth at 37°C (4, 7, 8, 9, 22). The synthesis of these virulence antigens is repressed at room temperature or at 37°C in the presence of 2.5 mM Ca²⁺ (3).

No information is available regarding the function of these two antigens. It has been suggested that they may provide protection against phagocytosis or serve to permit intracellular growth within host cells (3, 6). Straley and Brubaker recently showed that V antigen is a protein (molecular weight, 38,000) which could be recovered in the cytoplasmic fraction of the cell (37). Less information is available concerning W antigen. Lawton et al. (22), who have purified this antigen 1,000-fold, estimated the molecular weight of the W antigen to 145,000 and claimed that 38% of its dry weight was lipid, indicating a possible membrane localization.

All three species of yersinia show a calcium dependency when incubated at 37°C on magnesium oxalate agar, a medium virtually free of calcium (6, 17–19). The fraction (0.1 to 1%) of bacterial cells, which has lost the plasmid, can grow on such medium at 37°C (17, 18). Such plasmid-free bacteria are no longer virulent and do not produce V and W antigens. In addition, growth of *Y. pestis* on magnesium oxalate agar at 37°C can occasionally give rise to mutants which have no requirement for calcium. One class of mutants retains the ability to produce V and W antigens but are no longer virulent (5, 33).

It is known that *Y. pseudotuberculosis* and *Y. enterocolitica* have the ability to interact with a monolayer of HeLa cells (2, 23, 32). A positive correlation between penetration of HeLa cells and the ability to produce disease has been reported (27, 39). It is, however, unclear whether the HeLa cell invasivity reflects the fact that the pathogen is a facultative intracellular parasite or if it is a measure of the ability of the bacteria to attach to cell surfaces (11).

In this study, we report results which indicate that there is a correlation between the adherence of *Y. pseudotuberculosis* to HeLa cells and the ability of the bacteria to cause a fatal infection in mice. However, this adherence is not correlated with the presence of a plasmid in the pathogen, supporting results obtained by others (34). This study also provides information concerning a novel temperature-inducible plasmid-associated outer membrane protein (protein 1) having a molecular weight of about 140,000.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The bacterial strains used in this study were: *Y. pseudotuberculo-
sis serotype III (denoted YP III(p⁺)), a strain of Y. enterocolitica Wa (YE (Wa⁺)) both obtained from P. Gemski (17, 18), and a plasmid-containing strain of Y. enterocolitica (YE 1) obtained from D. A. Portnoy (34).

The minimal medium used was medium E as described by Vogel and Bonner (40), which was supplemented with 0.2% glucose and the essential L-amino acids (25 µmol/ml) except for methionine.

The rich media were either nutrient broth (Oxoid Ltd., London, England) or brain heart infusion broth (Oxoid Ltd.), prepared according to the instructions of the manufacturer. Bacteria were grown either at 26 or 37°C in a rotary shaker water bath. Growth was recorded by following the increase in optical density at a wavelength of 550 nm.

Analysis of plasmid content. The presence of plasmids in the yersiniae strains was analyzed by a modification of the method by Holmes and Quigley (20). Bacterial colonies (one loop) were scraped off plates and suspended in 1 ml of a buffer consisting of 8% sucrose, 5% Triton X-100, 50 mM EDTA, and 50 mM Tris (pH 8.0). The cells were lysed by the addition of 10 µl of lysozyme (20 mg/ml) and incubated for 5 min at 95°C. After centrifugation for 10 min (Eppendorf centrifuge), the supernatant was treated with 1 µl of RNase (10 mg/ml) for 15 min at 37°C. Diethylylcarboxylate, 10 µl of a 10% solution in ethanol, was added. The mixture was incubated for 10 min at 65°C and finally precipitated by the addition of ammonium acetate and isopropanol. The washed and dried pellet was suspended in 50 µl of TE buffer (10 mM Tris-hydrochloride [pH 8.0], 1 mM EDTA) (H. Lerach, personal communication). The samples were electrophoresed on 0.7% agarose slabs gels with a buffer containing 89 mM Tris-borate buffer, and 2.5 mM EDTA.

Plasmid-containing strains were cured by selecting for colonies growing on magnesium oxide agar plates at 37°C (17-19).

Preparation of the outer membrane by the Sarkosyl method. For the preparation of membranes, 50 ml of culture was centrifuged at 4°C. The bacterial pellet was suspended in 5 ml of a solution containing 10 mM Tris-hydrochloride (pH 7.8), 5 mM EDTA (pH 7.8), and 1 mM β-mercaptoethanol. The cells were then disrupted by four 15-s bursts at the full power of a Branson ultrasonic disintegrator. Cell debris was removed by low-speed centrifugation, and membranes were pelleted from the supernatant fraction by centrifugation at 100,000 × g for 1 h. The membrane pellet was suspended in 5 ml of a solution containing 0.5% Sarkosyl and 1 mM β-mercaptoethanol in distilled water (16). The total membrane fraction was incubated for at least 30 min at 4°C (in most cases it was convenient to incubate overnight), and then the suspension was centrifuged at 100,000 × g for 1 h. The final outer membrane-containing pellet was suspended in 100 µl of sample buffer (62.5 mM Tris-hydrochloride [pH 6.8], 1% SDS, 0.5% β-mercaptoethanol, and 10% glycerol).

Other methods used to prepare outer membrane was performed as described by Actman et al. (1) and Osborn et al. (30).

SDS-polyacrylamide gel electrophoresis. The proteins were analyzed in SDS-polyacrylamide gels essentially by the procedure by Laemmli (21). To separate the outer membrane proteins, we used gels containing 16% acrylamide and 0.094% bisacrylamide or gradient gels (17.5 to 10% acrylamide and 0.46 to 0.20% bis-acrylamide). Proteins were fixed by immersing the gel for 10 min in a mixture of 45% methanol and 9% acetic acid. The proteins were then stained for 30 min in 0.25% Coomassie brilliant blue in 7% methanol and 5% acetic acid and destained in 7% methanol and 5% acetic acid with several changes. All steps were carried out at 37°C. When proteins were visualized by autoradiography, the gel was dried in an LKB Instruments slab gel dryer and then put in direct contact with Cronex-4 X-ray film. After exposure for 48 h at ~70°C, the film was developed.

Oral infection of Swiss albino mice. Oral infection of Swiss albino mice with Y. pseudotuberculosis was essentially carried out as described by Gemski et al. (18). Groups of five Swiss albino mice weighing 17 to 20 g were deprived of water for 18 h and then allowed to drink freely from a 50-ml water suspension containing 10¹⁰, 10⁹, or 10⁸ bacteria of each strain per ml. After incubation at 4°C for 90 min, a 0.1-ml suspension containing 3 × 10⁴ bacteria per ml of phosphate-buffered saline was added. The cultures were further incubated for 90 min at 4°C. After the cells were washed three times with ice-cold phosphate-buffered saline, they were fixed by the use of 1% formaldehyde (usually overnight). Bacteria attached to the surface of the HeLa cells were demonstrated by the immunofluorescent technique, using rabbit antisera directed against whole formaldehyde-killed Y. pseudotuberculosis and fluorescein-conjugated anti-rabbit globulin (National Bacteriological Laboratory, Stockholm, Sweden).

Usually, 100 HeLa cells were counted, and the fraction of HeLa cells having one or more bacteria attached to the cell surface was estimated (32).

RESULTS

Correlation between adhesive properties and virulence of Y. pseudotuberculosis. It is well documented that yersiniae show several different physiological changes when the cells are shifted from growth at 26°C to growth at 37°C (4, 7, 8, 22). This was also found to be true for the ability of Y. pseudotuberculosis to adhere to a monolayer of HeLa cells. When the plasmid-containing strain YP III(p⁺) was grown at 26°C, the bacteria adhered to HeLa cells to a high degree. In contrast, when this strain was incubated at 37°C in the same Ca²⁺-containing medium, it attached to the HeLa cells at a reduced level (Table 1). This observed decrease in adherence
TABLE 1. Attachment of different strains of *Y. pseudotuberculosis* to a monolayer of HeLa cells.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Temperature (°C)</th>
<th>Attachment to HeLa cells (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>YP III(p&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>26</td>
<td>100</td>
</tr>
<tr>
<td>YP III(p&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>37</td>
<td>21</td>
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<tr>
<td>YP III(p&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>26</td>
<td>100</td>
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<td>YP III(p&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>37</td>
<td>19</td>
</tr>
</tbody>
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<sup>a</sup> The bacteria were grown in nutrient broth medium supplemented with 5 mM Ca<sup>2+</sup> at the indicated temperature overnight.

<sup>b</sup> The fraction of bacteria which adhered to HeLa cells was estimated as described in the text.

was not due to a loss of the plasmid, since more than 99% of the bacterial cells grown at 37°C still contained plasmid. To test whether any function of the plasmid is involved in the mechanism of attachment to HeLa cells, we constructed a plasmid-free derivative, YP III(p<sup>-</sup>) (Fig. 1). The attachment behavior of YP III(p<sup>-</sup>) was found to be identical to that of YP III(p<sup>+</sup>) (Table 1). Taken together, these results indicate that the plasmid is not correlated to the HeLa cell attachment of *Y. pseudotuberculosis*.

When YP III(p<sup>-</sup>) grown at 26°C was given orally to Swiss albino mice, an infection was rapidly established and the mice died within 3 to 7 days after challenge (Fig. 2). The rate of the fatal infection showed a dose response, and an average of 10<sup>8</sup> bacteria per mouse was required to establish a lethal infection, i.e., the mice survived a dose of 10<sup>7</sup> bacteria per mouse. The plasmid-free derivative YP III(p<sup>-</sup>) did not cause a fatal infection when given at a dose of 10<sup>10</sup> bacteria per mouse (data not shown). This result strongly indicates a significant role of the plasmid in bacterial virulence. However, when YP III(p<sup>+</sup>) was grown at 37°C under conditions which maintained the plasmid, the bacteria were found to be less infective (Fig. 2). Thus, a dose of 10<sup>9</sup> bacteria per mouse caused a fatal infection in only two of five mice within 18 days, whereas all five mice were dead within 7 days when the corresponding dose of bacteria grown at 26°C was administered (Fig. 2). There is apparently a correlation between the power of YP III(p<sup>+</sup>) to cause a lethal infection in mice and the ability of the pathogen to adhere to a monolayer of HeLa cells. These results suggest that the adherence properties of the bacteria may be of importance in the process of infection.

**Outer membrane protein profiles of *Y. pseudotuberculosis***. Since the ability to cause infection seems to be correlated to the adhesive properties of the pathogen, one might suspect that cell surface structures are involved in the mechanism of virulence. To investigate this possibility, we studied the outer membrane protein profile.
of the YP III(p⁺) and its isogenic plasmid-free derivative YP III(p⁻).

YP III(p⁺) was subjected to a temperature shift to 37°C after growth in calcium-free minimal medium containing 40 mM Mg²⁺ at 26°C. In contrast to its plasmid-free derivative, this strain ceased to grow two generations after the shift. However, the addition of 5 mM Ca²⁺ to the growth medium reverted the temperature effect on the growth of the bacteria. Thus, the plasmid-containing strain YP III(p⁺) showed the characteristic response of yersiniae with respect to Ca²⁺, Mg²⁺, and growth temperature (4, 9).

For [³⁵S]methionine labeling and analysis of outer membrane proteins, YP III(p⁺) was grown in minimal medium (40 mM Mg²⁺) at 26°C. At an optical density of 0.15 at 550 nm, the culture was split and put into three separate flasks. One culture was maintained at 26°C. The other two cultures were transferred to 37°C. At an indicated time (70°C), 50 μCi of [³⁵S]methionine was added to each flask, and 5 min later, the cultures were harvested. Outer membrane was isolated by using the Sarkosyl method as described in Material and Methods. The respective outer membrane protein profile was analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Three outer membrane polypeptides, showing molecular weights of 140,000 (protein 1), 17,000 (protein 2), and 15,000 (protein 3), were found to be induced by the temperature shift (Fig. 3). A minor decrease in the amount of protein 1 was observed when Ca²⁺ had been added to the growth medium, whereas proteins 2 and 3 were unaffected by the addition of Ca²⁺ (Fig. 3). A parallel experiment with the plasmid-free strain YP III(p⁻) revealed that only proteins 2 and 3 were induced by the temperature shift. No detectable synthesis of protein 1 was observed in this strain (Fig. 3).

In an attempt to measure how soon after a temperature shift from 26 to 37°C the synthesis of protein 1 is turned on, we pulse-labeled a
FIG. 5. Outer membrane protein profiles of YP III(p⁺) incubated in different growth media containing 5 mM Ca²⁺ at 37°C. After incubation overnight, each culture was harvested, and outer membrane was prepared by either the method of Osborn et al. or the Sarkosyl method as described in Material and Methods. Outer membrane protein profiles were then analyzed by SDS-polyacrylamide gel electrophoresis. Outer membrane protein profile of cells grown in the following medium: lane 1, nutrient broth medium (Osborn et al.); lane 2, brain heart infusion broth (Sarkosyl method); lane 3, nutrient broth (Sarkosyl method); and lane 4, minimal medium (Sarkosyl method).

logarithmically growing culture of YP III(p⁺) for 1 min with [³⁵S]methionine at different times before and after the temperature shift. Analysis of the outer membrane protein profiles showed that the amount of protein 1 was increased less than 2 min after the shift, indicating that the synthesis of protein 1 very rapidly responded to the changed temperature (Fig. 4).

In previous studies describing temperature-inducible proteins of yersiniae no high-molecular-weight outer membrane protein was reported (34, 37). We therefore compared three different methods to isolate the outer membrane: the Sarkosyl method, the method described by Osborn et al. (30) (Fig. 5, lane 1), and the method described by Achtman et al. (1; data not shown). All three methods gave the same result, and regardless of the method used, we were able to detect protein 1 (34, 37).

The synthesis of protein 1 was, however, found to be affected by the medium in which the bacteria were incubated. Cells grown in rich nutrient broth medium was synthesizing this protein in smaller amounts compared with cells grown in minimal medium. After growth in brain heart infusion broth, protein 1 was hardly detectable (Fig. 5).

**Outer membrane protein profiles of Y. enterocolitica.** A comparative study between outer membrane protein profiles of *Y. pseudotuberculosis* and *Y. enterocolitica* revealed differences (Fig. 6). Two different plasmid-containing isolates of *Y. enterocolitica* possessed a temperature-inducible outer membrane protein with the same molecular weight as protein 1 of YP III(p⁺) (Fig. 6). This outer membrane protein of *Y. enterocolitica* was found to be associated with the virulence plasmid, since an oxalate-cured plasmid-free strain of *Y. enterocolitica* lacked this protein (Fig. 6). This result indicate that *Y. pseudotuberculosis* and *Y. enterocolitica* may have a plasmid-coded temperature-inducible outer membrane protein in common.

**DISCUSSION**

In agreement with results obtained by others (24), we have been unable to detect pili on the surface of strains of *Y. pseudotuberculosis* adhering to HeLa cells (data not shown). It must therefore be concluded that the ability of YP III(p⁺) to adhere to HeLa cells is not mediated by pili. Furthermore, the gene(s) involved in the attachment function is most likely chromosomally located, since a plasmid-free derivative, YP III(p⁻), retained the capacity to adhere to HeLa cells. This conclusion is in agreement with recent results obtained from similar studies with *Y. enterocolitica* (11, 34).

It is now accepted that colonization of the surface of the interior of the intestine by enteropathogenic bacteria is an essential step in the process of infection (26, 35). Many of these bacteria produce proteinaceous surface antigens, pili, which promote the colonization of the intestine in vivo and promote the attachment of the bacteria to different types of erythrocytes in vitro (12, 31). These colonization factors (CFA), which are a prerequisite for the organism to establish an infection, also show host specificity and are in many cases encoded by plasmids, e.g., the colonization factors of enterotoxigenic *Escherichia coli*: K99 (calf), K88 (pig), and CFAI and CFAII (human) (13, 14, 29, 35, 38). The power of these different enteropathogenic bacteria to agglutinate and bind different erythrocytes has been correlated to and used as a measure of the capacity of the pathogens to adhere in vivo (16, 35). The fact that *Y. pseudotuberculosis* shows a positive correlation between attachment to HeLa cells and oral infectivity may reflect the ability of the strain to colonize the intestine in vivo as a primary step in the process of infection rather than as a measure of the invasiveness of the pathogen (Table 1;
Fig. 6. Outer membrane protein profiles of two different strains of *Y. enterocolitica*. Each strain was incubated at 26°C in minimal medium supplemented with 0.2% Casamino Acids. At an optical density of 0.4 at 550 nm, the culture was divided into two portions. One sample was maintained at 26°C, and the other was transferred to 37°C. After incubation overnight, the cultures were harvested, and outer membrane was isolated and subjected to SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel was fixed and stained with Coomassie blue. Lane 1, YP III(p⁺) incubated at 37°C; lane 2, YP III(p⁺) incubated at 26°C; lane 3, YE(Wa⁺) incubated at 26°C; lane 4, YE(Wa⁺) incubated at 37°C; lane 5, YE(Wa⁺) incubated at 37°C; lane 6, YE I incubated at 28°C; and Lane 7, YE I incubated at 37°C.

Fig. 2). Thus, the adherence properties of the pathogen may be an additional virulence factor aside from the plasmid-encoded virulence factors.

The plasmid of YP III(p⁺) is apparently not involved in the bacterial attachment to HeLa cells, but seems to be of fundamental importance for the ability of the strain to cause a fatal infection in Swiss albino mice. This study of the outer membrane protein profiles of YP III(p⁺) revealed that expression of a protein (protein 1) with a molecular weight of approximately 140,000 was associated with the virulence plasmid (Fig. 3). The protein was found to be induced immediately after a temperature shift from 26 to 37°C, and its rate of synthesis after such a shift was found to be affected by the presence of 5 mM Ca²⁺ in the growth medium. Plasmid-containing strains of *Y. enterocolitica* were also found to express a similar outer membrane protein. It might therefore be hypothesized that protein 1 is a common virulence factor of yersiniae. Virulent strains of yersiniae have two antigens in common, the virulence antigens V and W, respectively (4). The W antigen shows similar properties compared with protein 1 with respect to temperature inducibility, plasmid association, molecular weight, and calcium response (4). Furthermore, protein 1 is an outer membrane protein, and as such it might be exposed as a surface antigen. Since there are obvious similarities between W antigen and protein 1, it is tempting to speculate that these two proteins are one and the same. However, the ultimate answer to that question must await until these proteins have been fully characterized.

The function of protein 1 is presently unclear, but one possibility is that it could work as a porin, which gives the bacteria new properties of advantage in the process of infection. The fact that synthesis of protein 1 was partially suppressed during growth of bacteria in rich medium compared with minimal medium may support this hypothesis. We have obtained results which indicate that V antigen is released into the growth medium when the bacteria are grown under conditions which are known to induce V and W antigens (unpublished data). Protein 1 may therefore be involved in the passage out from the cell of V antigen, which in turn may be an active molecule involved in bacterial virulence. Alternatively, protein 1 may change the bacterial cell surface in such a way that the pathogen will resist phagocytosis or become resistant to intracellular killing by the phagocytes.

It is likely but not proven that the structural gene of protein 1 is to be found on the virulence plasmid. To find out more about its function and regulation we have cloned plasmid DNA fragments into pHC79. We are now in the process of identifying the different gene products of the 63-kilobase virulence plasmid of *Y. pseudotuberculosis* strain YP III(p⁺). We hope, by using this strategy, to be able to answer some of the questions concerning the possible role of plasmid-coded products in the process of infection by *Y. pseudotuberculosis*.

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**LITERATURE CITED**


