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Expression of the Temperature-Inducible Outer Membrane Proteins of Yersiniae

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The expression of the temperature-inducible plasmid-coded outer membrane proteins (YOPs) of *Yersinia pseudotuberculosis* was studied. These proteins were not recovered in the outer membrane fraction when the strain was grown in minimal medium at 37°C, but they were expressed under these conditions. A strict correlation was found between Ca^{2+} dependency in the virulent strain, YPIII(pIB1), and ability to express YOPs. Ca^{2+}-independent plasmid mutants or RNA-polymerase mutants harboring the virulence plasmid were unable to express YOPs, in contrast to the wild-type strain. These strains were also found to be avirulent. Sera recovered from patients or animals undergoing infection with either *Y. pseudotuberculosis*, *Y. pestis*, or *Y. enterocolitica* possessed antibodies directed against YOPs, indicating that they were expressed in all three pathogenic *Yersinia* species during infection. The YOPs of the three different species showed high immunological relatedness.

Pathogenic yersiniae give rise to invasive disease in humans and animals with a variety of symptoms, from diarrhea to fatal disease. However, very little is known about how these invaders exert their pathogenic effects and evade host defense. The three virulent species, *Yersinia pseudotuberculosis*, *Y. pestis*, and *Y. enterocolitica* carry plasmids necessary for virulence (2, 13–15, 22, 29). In animal models, plasmid-bearing strains are virulent, whereas plasmid-cured strains are avirulent (2, 3, 14, 15, 23). Virulence can be restored by introducing the plasmid into a plasmid-cured avirulent strain, as has been shown for *Y. pestis* (21). These plasmids are associated with a number of temperature-inducible features of the bacteria, i.e., production of V and W antigens (7–12, 20), autoagglutination (6, 18, 25), and the expression of certain outer membrane proteins (3, 4, 10, 19, 23, 24, 26).

Another temperature-dependent property of plasmid-containing *Yersinia* strains is that at 26°C they can form colonies on agar medium lacking Ca^{2+}, whereas at 37°C they are unable to grow. However, when Ca^{2+} is added to the agar, colonies can be formed at 37°C (Ca^{2+} dependency). Only bacteria devoid of the plasmid or having a mutation within the plasmid can grow at 37°C in the absence of Ca^{2+} (Ca^{2+} independency) (2, 13–15, 29).

The region of the plasmid containing the Ca^{2+} dependence locus has been shown to be necessary for virulence in *Y. pestis* (21), emphasizing earlier observations of an important correlation between the unknown mechanisms giving rise to Ca^{2+} dependency and virulence of the pathogen (5–8). This region of the plasmid is conserved (24), although the plasmids from *Y. enterocolitica* show about a 50% difference at the DNA level when compared with plasmids from *Y. pestis* and *Y. pseudotuberculosis* (22, 24). The latter plasmids are almost identical (24) and are interchangeable (27a).

All three plasmids have coding capacity for at least some of the temperature-inducible outer membrane proteins, as demonstrated by minicell experiments (24). However, these proteins could be expressed in vitro only by *Y. pseudotuberculosis* and *Y. enterocolitica*, but not by *Y. pestis*, after growth at 37°C (24, 26). When the plasmid of *Y. pestis* pYV019 was transferred to the *Y. pseudotuberculosis* strain YPIII, expression of the proteins could be demonstrated (27a), clearly showing that this plasmid has coding capacity for these proteins.

We have previously shown that one of the plasmid-encoded temperature-inducible outer membrane proteins (YOP1) (molecular weight, 150,000) is not a major virulence determinant (4). The possible role of the other temperature-inducible outer membrane proteins in the infection process remains to be determined.

In a recent study (19), Martinez showed that convalescent sera from patients suffering from infection with *Y. enterocolitica* contained antibodies directed against the plasmid-encoded outer membrane proteins (YOPs). Thus, these proteins appear to be expressed in vivo by *Y. enterocolitica* (19).

In the present study, we show that the YOPs are expressed in vivo by *Y. pestis* and *Y. pseudotuberculosis*, in addition to *Y. enterocolitica*. We also show that the expression of these proteins is correlated to Ca^{2+} dependence and virulence.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used were *Y. pestis* EV76, carrying virulence plasmid pYV019, and its plasmid-free derivative EV76-6 (22); *Y. enterocolitica* 8081, carrying the virulence plasmid pYV8081, and its plasmid-free derivative strain 8081-c (23); and *Y. pseudotuberculosis* YPIII(pIB1), its plasmid-free derivative YPIII (3), and the Tn*5* insertion mutants YPIII(pIB71) and YPIII(pIB101) derived from YPIII(pIB1). Transposon Tn*5* of these mutants is inserted at two different loci about 20 kilobases apart (4). The rifampin-resistant strain YPIII(pIB1)Rif^r was obtained as a spontaneous mutant of the wild-type strain YPIII(pIB1) by selection on plates containing 200 μg of rifampin per ml.

Bacteria were grown in minimal salt medium lacking Ca^{2+} as described elsewhere (3) or in rich medium consisting of

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salt medium as described by Higuchi and Smith (16) supplemented with 1% tryptone (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract (Difco), and 0.2% glucose (19).

Pulse-labeling was performed with strain YPIII(pIB1). The strain was grown in minimal medium under steady-state conditions at 26°C and shifted to growth at 37°C. After 1 h of incubation at 37°C, the bacteria were pulse-labeled with [^{35}S]methionine for 2 min. The bacteria were harvested and boiled in sample buffer, and the total cell content was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Test of Ca^{2+} dependency. Single colonies were suspended in 0.9% sodium chloride, diluted, and plated on blood agar base or magnesium oxalate agar, which consisted of blood agar base (Oxoid, London, United Kingdom), 20 mM $MgCl_2$, and 20 mM sodium oxalate (14). Ca^{2+}-independent strains gave 100% viable counts on magnesium oxalate agar plates at 37°C as compared with the same dilution on blood agar base plates at 26°C. Ca^{2+}-dependent strains gave less than 0.1% viable counts on magnesium oxalate agar plates at 37°C.

Bacterial membrane isolation and SDS-PAGE. Cells (50 ml) were grown at 26 and 37°C to stationary phase. Whole membranes and Triton X-100-insoluble outer membranes were isolated as described by Achtman et al. (1). Outer membranes were suspended in electrophoresis sample buffer (2× buffer is 0.0625 M Tris [pH 6.8], 10% glycerol, 0.001% bromophenol blue, 5% β-mercaptoethanol, 2% SDS), and SDS-PAGE was performed in a discontinuous buffer system as described by Laemmli (17), using gradient gels of 10 to 17 or 5 to 17% acrylamide as the running gel and 3% acrylamide as the stacking gel. Samples were boiled for 5 min immediately before being run, and electrophoresis was performed overnight at a constant current of 8 mA. Gels were stained for 1 h in 0.05% Coomassie brilliant blue–25% isopropanol–10% acetic acid and destained in 5% acetic acid. Molecular weight standards were purchased from Pharmacia AB, Uppsala, Sweden.

Immunoblotting (Western). Western blotting after SDS-PAGE of outer membrane proteins was performed as described by Swanson et al. (27). Mouse antisera against *Y. pseudotuberculosis* were obtained by orally feeding Swiss albino mice with sublethal concentrations of strain YPIII(pIB1) (10^8 bacteria per ml), and blood samples were taken before and in sequence after the challenge with the pathogen. The sera were analyzed by Western blotting, using the homologous strain as the YOP antigen source. Mouse antisera from *Y. pestis*-infected animals were obtained in the following way. A number of Swiss albino mice were infected by intraperitoneal injection of strain EV76 at a dose of about 10^2 organisms in combination with peanut oil and iron as described elsewhere (2). One mouse survived this challenge, and this mouse was repeatedly infected once a week for 3 weeks with an increasing number of bacteria. Immune as well as preimmune sera were analyzed with respect to antibodies directed against the YOPs by using strain YPIII(pIB1) as the antigen source. Rabbit antiserum against virulent *Y. enterocolitica*, prepared as described elsewhere (19), was kindly provided by R. Martinez. The human sera from clinically confirmed cases of infectious *Y. enterocolitica* were kindly supplied by S. Lange, University of Gothenburg, Sweden. Plague convalescence sera were obtained from the Plague Branch, Centers for Disease Control, Ft. Collins, Colo. Membrane protein profiles obtained from plasmid-containing and plasmid-free strains of *Y. enterocolitica* as well as *Y. pseudotuberculosis* grown under conditions allowing expression of the YOPs were used in the immunoblotting experiments with human sera.

Infection of Swiss albino mice. Oral infection of Swiss albino mice with *Y. pseudotuberculosis* strains was carried out essentially as described by Gemski et al. (15). 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 of each strain containing 10^9 bacteria per ml. Interperitoneal injection of strain EV76 was carried out as described elsewhere (2).

RESULTS

Nomenclature and in vitro expression of YOPs. When *Y. pseudotuberculosis* YPIII(pIB1) was grown in rich medium at 37°C, the temperature-inducible outer membrane proteins were inserted into the outer membrane (Fig. 1) (24). These proteins were not seen in the plasmid-free derivative strain

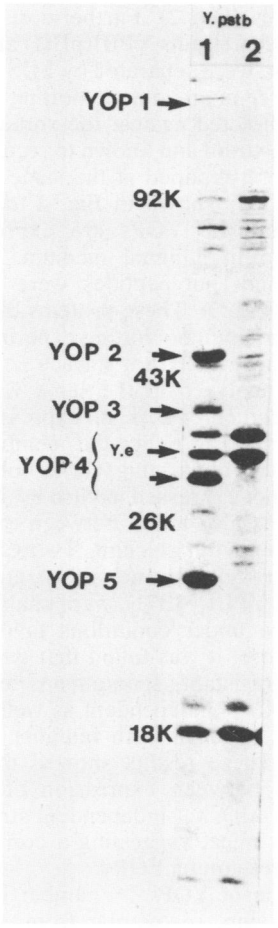

FIG. 1. Outer-membrane protein profile of strains YPIII(pIB1) and YPIII. The strains were grown in rich medium at 37°C and outer membrane was prepared and desolved in SDS sample buffer. The samples were thereafter boiled and subjected to SDS-PAGE. After electrophoresis the gel was stained with Coomassie brilliant blue. Lane 1, strain YPIII(pIB1); lane 2, strain YPIII. The YOPs are denoted YOP1 to YOP5, with the following molecular weights: YOP1, 150,000; YOP2, 44,000; YOP3, 40,000; YOP4, 34,000; and YOP5, 26,000. Y.E. indicates the position of YOP4 (36,000) of *Y. enterocolitica*. Y. pstb, *Y. pseudotuberculosis*.

YPIII (Fig. 1) (24). We suggest that the proteins be named YOP1 through -5 (yersiniae outer membrane proteins) as indicated in Fig. 1 with the corresponding structural genes *yopA* through *yopE*. YOP4 of *Y. enterocolitica* and *Y. pseudotuberculosis* differ somewhat in molecular weight, 36,000 and 34,000, respectively (24). By immunoblotting we learned that these proteins are related (see Fig. 4) and could be given the same designation.

We found earlier that expression of the YOPs is media dependent, i.e., the proteins are clearly produced when the pathogen is grown in rich medium devoid of Ca^{2+} but not in minimal medium lacking Ca^{2+} (24). The question remained, however, whether the proteins were made in minimal medium but not inserted into the outer membrane, or if they were simply not expressed. To address this question, strain YPIII(pIB1) was pulse-labeled in minimal medium, and the total cell content was subjected to SDS-PAGE (Fig. 2). We found that the strain indeed expressed a set of temperature-inducible proteins that had molecular weights corresponding to the YOPs. These proteins were not detected in the appropriate controls (Fig. 2). Furthermore, when the total cell protein profiles of strains YPIII(pIB1) and YPIII, grown in minimal medium, were separated by SDS-PAGE and used as the antigen source in an immunoblotting experiment with rabbit antiserum directed against the surface antigens of a plasmid-containing strain and known to recognize the YOPs, blotting signals were obtained at the same positions as the indicated polypeptides shown in Fig. 2 (data not shown). This indicates that the YOPs are expressed in strain YPIII(pIB1) grown in minimal medium. Two additional high-molecular-weight polypeptides were induced by the temperature shift (Fig. 2). These proteins did not react with the antiserum in the immunoblotting experiment. These two polypeptides are most likely not surface antigens. When *Y. pestis* EV76 was analyzed in the same way as described above for *Y. pseudotuberculosis*, no expression of the YOPs could be demonstrated in either the membrane fraction or the soluble fraction, confirming earlier observations that these proteins are not expressed in vitro by *Y. pestis* (24, 26).

A correlation was also found between expression of the YOPs and dependency on calcium. Two Ca^{2+}-independent Tn*5*-derived insertion mutants of strain YPIII(pIB1), YPIII(pIB101) and YPIII(pIB71), were unable to express the YOPs when grown under conditions favoring expression (Fig. 3). Furthermore, it was found that two independently isolated, rifampin-resistant, spontaneous mutants of strain YPIII(pIB1) were Ca^{2+} independent as well as incapable of expressing YOPs, although both mutants still carried the plasmid (Fig. 3). These results showed that there was a strong correlation between expression of YOPs and response to calcium. All Ca^{2+}-independent strains were found to be avirulent for mice, suggesting a correlation between virulence and expression of YOPs.

In vivo expression of YOPs. A number of different sera obtained from patients recovering from yersiniosis were analyzed by immunoblotting. From one patient, a sequential set of sera obtained early, intermediate late, and late after onset of infection were analyzed with respect to antibodies directed against YOPs. The early sera contained antibodies directed mainly against YOP4 (Fig. 4). The later sera showed an increased serum titer as visualized by an elevated overall intensity of the protein antigen bands of the immunoblot. The serum titer of YOP-specific antibodies was consistently elevated, and antibodies directed against YOP2 through -5 of *Y. pseudotuberculosis* as well as *Y. enterocolitica* could be demonstrated (Fig. 4).

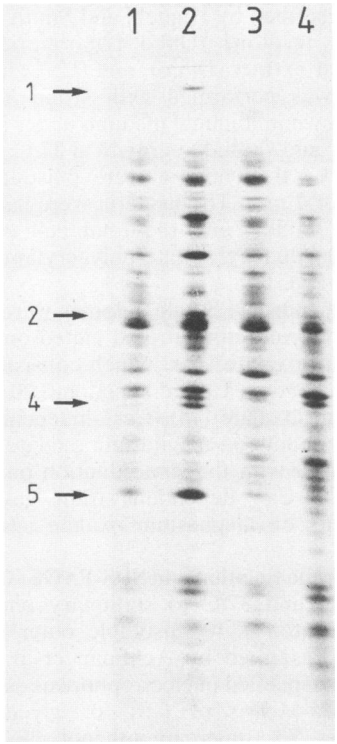

FIG. 2. Total protein profile of the plasmid containing strain YPIII(pIB1) and its cured derivative. Strains YPIII(pIB1) and YPIII were grown under steady-state conditions at 26°C in 10 ml of minimal medium. At an optical density of 550 nm = 0.2, the cultures were divided into two aliquots and one of these was shifted to 37°C. At 1 h after the shift, the cultures were pulse-labeled by the addition of 50 μCi of [^{35}S]methionine. After 2 min nonradioactive methionine was added to give a final concentration of 50 μg/ml, and 3 min later the cultures were chilled on ice and harvested by centrifugation. The cells were washed once in 10 mM Tris buffer (pH 7.0). Total cell pellet was resolved in 100 μl of SDS sample buffer and heated for 5 min at 95°C. A 20-μl amount of each sample was put on an SDS-polyacrylamide gel. Lanes: 1, strain YPIII(pIB1) grown at 26°C; 2, strain YPIII(pIB1) grown in 37°C; 3, strain YPIII grown at 26°C; and 4, strain YPIII grown at 37°C.

Sera obtained from mice infected with sublethal concentrations of YPIII(pIB1) contained antibodies against YOP2 through -5 that could be detected from sera obtained 10 days after the onset of infection (Fig. 5). Preimmune sera, on the other hand, did not show any detectable signal (data not shown).

Convalescent sera obtained from two patients recovering from plague were also analyzed. One of these sera possessed antibodies directed against YOP4 and YOP5 (Fig. 6), and the other sera did not show any specific response towards the YOPs. Because it was impossible to achieve in vitro expression of YOPs of *Y. pestis* strains, strain YPIII(pIB1) was used as the antigen source in these experiments. Although very suggestive, it was not conclusively proven that *Y. pestis* expressed the YOPs in vivo during infection, as no appropriate controls could be executed. Therefore, immune as well as preimmune sera from a mouse infected with strain EV76 were analyzed with respect to antibodies directed against the YOPs, using strain YPIII(pIB1) as the antigen source (Fig. 7). Specific antibodies directed against YOP2, YOP3, and YOP5 could be detected in the immune sera (Fig. 7), whereas no response was obtained from the preimmune

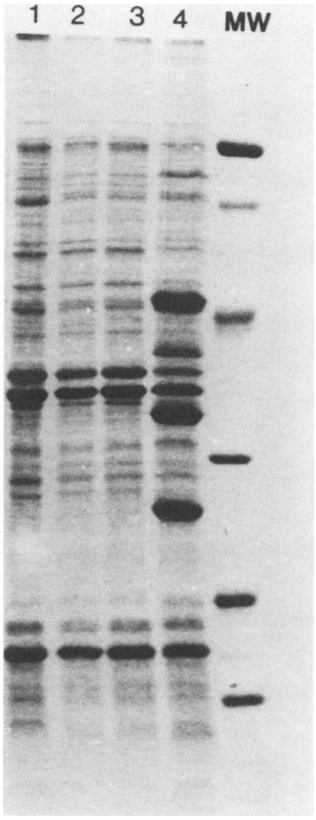

FIG. 3. Outer membrane protein profile of different Ca^{2+}-independent mutants and the wild-type strain YPIII(pIB1). Two different Ca^{2+}-independent Tn*5*-derived insertion mutants YPIII(pIB101) and YPIII(pIB71) and one RNA-polymerase Ca^{2+}-independent mutant YPIII(IB1)Rif^r were analyzed with respect to their outer membrane protein profiles after growth at 37°C in rich medium. Lanes: 1, strain YPIII(pIB71); 2, YPIII(pIB101); 3, YPIII(pIB1)Rif^r, and 4, YPIII(pIB1). MW, Molecular weight markers.

sera (data not shown). These results support the conclusion that YOPs are expressed in vivo during the infection phase of *Y. pestis*.

DISCUSSION

At least five different YOPs (Fig. 1) are induced when cells of *Y. pseudotuberculosis* or *Y. enterocolitica* are shifted from growth at 26 to 37°C (24). Although the virulence plasmid of *Y. pseudotuberculosis* and *Y. pestis* are almost identical, no synthesis of the YOPs can be observed when strains of *Y. pestis* are studied in the laboratory (24). This is puzzling because it is known that the virulence plasmid of *Y. pestis* has the coding capacity for these proteins (24). This raises the important question of whether YOPs are expressed in vivo during infection of all three pathogenic species of yersiniae, or if only *Y. enterocolitica*, as has been shown by Martinez (19), expresses the proteins in vivo.

The appearance of these proteins in the outer membrane fraction is not fully understood. The composition of the growth medium seems to play an essential role in this process (3, 24). From the results presented here, it is obvious that the YOPs were induced at 37°C when *Y. pseudotuberculosis* YPIII(pIB1) was grown in minimal medium, in which YOPs could not be recovered from the outer membrane fraction. This suggests that the proteins are made but degraded rather than inserted into the bacterial outer membrane under these conditions. The reason for this is at present unknown. Moreover, there seems to be a strict correlation between the expression of YOPs and the Ca^{2+} response of strain YPIII(pIB1), because we found that three different classes of Ca^{2+}-independent mutants harboring the virulence plasmid were unable to express YOPs, with the exception of YOP1. This protein, however, seems to be regulated differently than the other YOPs (3, 4). These mutants were also found to be avirulent. Thus, there is a correlation between the expression of YOPs and the virulence of the pathogen. This conclusion was further strengthened by the finding that YOPs were expressed in vivo during infection with pathogenic strains of yersiniae. Although highly likely, in vivo expression of YOPs does not necessarily prove their role in the process of virulence, because as an example, a Tn*5*-induced structural gene mutant of YOP1 does not produce any detectable changes in the virulence behavior of the pathogen (4).

When a sequential set of sera obtained from a patient recovering from yersiniosis was analyzed, we found that early in the infection antibodies directed against YOP2 and YOP4 predominated, whereas later sera contained antibodies directed against the plasmid-coded outer membrane proteins YOP2 through -4. Antibodies reacting with the high-molecular-weight protein YOP1 could also be observed (data not shown).

Swiss albino mice infected with a sublethal concentration of a virulent strain of *Y. pseudotuberculosis* developed an antibody response directed against all the YOPs, clearly indicating that YOPs are expressed during the infection process.

Even though synthesis of YOPs not could be detected when *Y. pestis* EV76 was cultivated in vitro, the strain

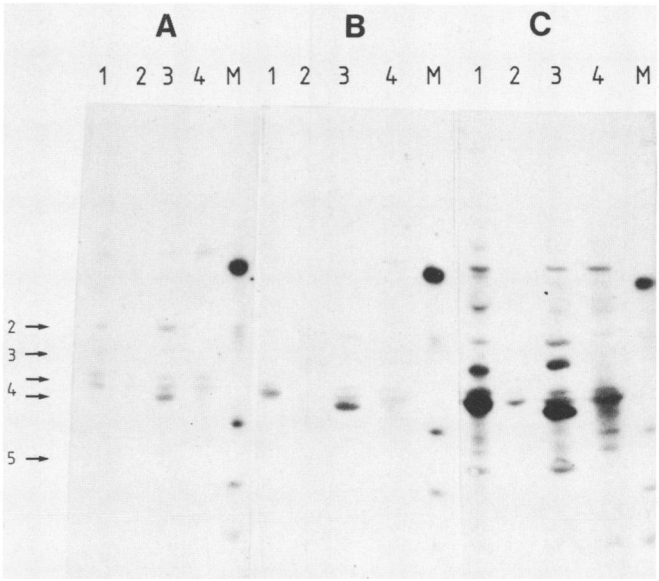

FIG. 4. Immunoblotting of sera recovered from a human case of *Y. enterocolitica* infection. Serum was obtained early (A), intermediate late (B), and late (C) after the onset of infection with *Y. enterocolitica*. These sera were analyzed by immunoblotting, using plasmid-containing and -cured strains of *Y. enterocolitica* and *Y. pseudotuberculosis* as antigen sources. Lanes: 1, *Y. enterocolitica* 8081; 2, *Y. enterocolitica* 8081-C; 3, *Y. pseudotuberculosis* YPIII(pIB1) and 4, *Y. pseudotuberculosis* YPIII. M, Molecular weight markers. Labeled arrows indicate the position of the YOPs.

expressed some of these proteins (YOP2, -3, and -5) during infection in a mouse model system, supporting the earlier finding that plasmid pYV019 has the coding capacity for YOPs (24, 27a). Antibodies directed against YOP4 and YOP5 were also found when convalescent serum from a patient recovering from plague was analyzed. This observation is in accordance with results obtained by Mazza et al. (G. Mazza, H. F. Blank, and D. T. Kingsbury, manuscript in preparation). Thus, *Y. pestis* has the capability to induce and express YOPs in a suitable host. Synthesis of these proteins is never observed when plasmid-containing strains of *Y. pestis* are studied in vitro. Transfer of the plasmid pYV019 of strain EV76 to a plasmid-free strain of *Y. pseudotuberculosis*, YPIII, confers on this strain full virulence and the ability to express YOP2 to -5 in vitro (27a), suggesting that chromosomally encoded regulatory functions are involved in the expression of YOPs. The finding that rifampin-resistant mutants of strain YPIII(pIB1), affected in the RNA polymerase, are unable to express the YOPs supports this conclusion and also indicates that YOPs may be transcriptionally regulated.

It may be suggested that the yersiniae have the ability to sense changes in the environment, turn on the synthesis of YOPs, and express them on the cell surface when needed, for example, when the bacteria are intracellularly located. A similar hypothesis, has been put forward by Brubaker (7), who proposed that *Y. pestis* may recognize an intracellular (low) and extracellular (high) concentration of Ca^{2+}. This low Ca^{2+} concentration would trigger the expression of plasmid-coded determinants (V and W antigens). The same

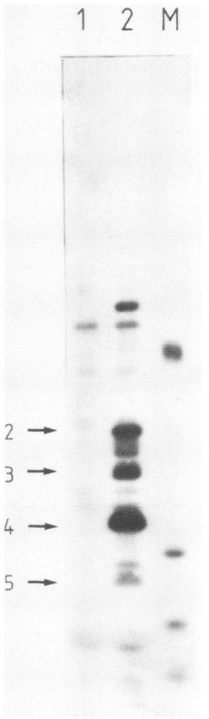

FIG. 5. Immunoblotting of a sera obtained from a mouse infected with *Y. pseudotuberculosis*. Swiss albino mice were orally challenged with *Y. pseudotuberculosis* strain YPIII(pIB1). Sera were taken before and in sequence after the challenge. Shown is the serum obtained at day 10. Strains YPIII(pIB1) and YPIII used were grown under conditions allowing expression of the YOPs. Lanes: 1, YPIII; and 2, YPIII(pIB1). M, Molecular weight markers.

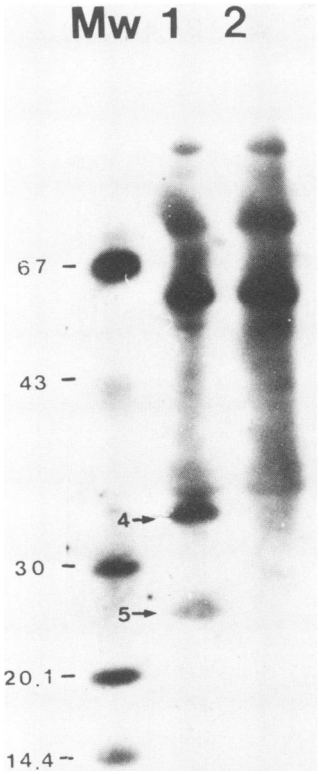

FIG. 6. Immunoblotting of convalescent serum obtained from a patient recovering from plague. *Y. pseudotuberculosis* strains YPIII(pIB1) and YPIII were used as antigen sources. Lanes: 1, YPIII(pIB1) and 2, YPIII. MW, Molecular weight markers.

mechanism might also be applied to the expression of YOPs. Results obtained in this study showing a high correlation between Ca^{2+} dependency and expression of YOPs, together with results obtained by Portnoy et al. (23) showing that YOPs are induced in a low Ca^{2+}-concentration environment, support this hypothesis. By finding out the necessary requirements for the in vitro expression of YOPs, it will perhaps be possible to gain some understanding of the in vivo regulation of these proteins.

Antibody response to the YOPs varies from case to case (compare Fig. 4 and 7). In addition, we analyzed a large number of sera obtained from human cases of yersiniosis. In all cases an antibody response against the YOPs was evident, but sera showed individual differences. In one case, for example, antibodies against YOP4 and -5 could be detected, and in another, antibodies against YOP2, -3, and -5 were present. Whether these results reflect differences in the expression of YOPs during infection or individual variations in the immune response of the host cannot at present be evaluated. It can, however, be concluded that all of the temperature-inducible outer membrane proteins can be expressed during the infection phase in all three pathogenic strains of yersiniae and that the host can induce an immune response directed toward these proteins.

Because the YOPs of *Y. pseudotuberculosis* and *Y. enterocolitica* were used as the antigen source in combination with sera obtained from animals and humans infected with any of the three species, the results point out the interspecies similarities between these proteins. These findings show that the YOPs have been maintained during evolution of the

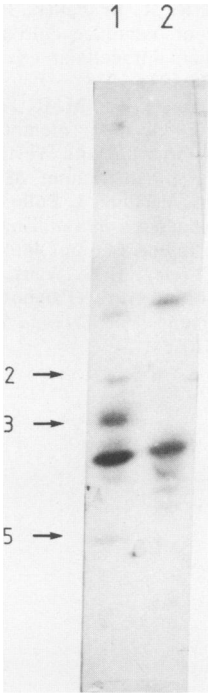

FIG. 7. Analysis of a serum obtained from a mouse infected with *Y. pestis*. Swiss albino mice were infected intraperitoneally with strain EV76 at a dose of 10^2 organisms as described previously (2). One mouse survived this challenge and was repeatedly infected by two additional injections with increasing doses of strain EV76 (10^3 and 10^4). The time interval between the injections was 1 week. After 3 weeks, serum was drawn and analyzed by immunoblotting, with strains YPIII(pIB1) and YPIII as antigen source. Lanes: 1, YPIII(pIB1); and 2, YPIII.

three species of pathogenic yersiniae. The virulence plasmid of *Y. enterocolitica* shows about 50% DNA homology with corresponding plasmids of *Y. pestis* and *Y. pseudotuberculosis* (24). These plasmids have obviously been subjected to evolutionary rearrangements. Thus, there is strong selective pressure for maintenance of YOPs. This evidence can be applied as an additional argument for the importance of YOPs of pathogenic *Yersinia* species in the process of virulence, especially because *Y. pestis* has an ecological niche different than that of *Y. enterocolitica* or *Y. pseudotuberculosis*.

However, more precise information about the YOPs is needed to establish their role in the Ca^{2+} response and virulence. The YOPs seem to be regulated in concert by the same mechanism that gives rise to Ca^{2+} dependency, because three different Ca^{2+}-independent mutants are unable to express YOPs. Furthermore, we have obtained data from molecular cloning experiments showing that the structural genes of the YOPs are scattered around plasmid pIB1 at least 10 kilobases apart and are located outside the Ca^{2+} region. Our interpretation of this is that the Ca^{2+} region codes for regulatory elements affecting the expression of YOPs and that YOPs not are localized within the same operon. The question is whether the YOPs per se are directly involved in the Ca^{2+}-dependent behavior or whether yet unidentified gene products of the Ca^{2+} region are responsible for this effect. The latter suggestion seems more likely as *Y. pestis* shows a Ca^{2+}-dependent behavior in vitro but is unable to express YOPs (24). To elucidate these questions specific mutations must be created in the structural genes of the YOPs. Such studies are in progress in our laboratory.

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