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Identification and Mapping of the Temperature-Inducible, Plasmid-Encoded Proteins of *Yersinia* spp.

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The structural genes of the outer membrane polypeptides of *Yersinia* spp. (YOPs) and the V antigen of plasmid pIB1 of *Yersinia pseudotuberculosis* were recently cloned and mapped (Å. Forsberg, I. Bölin, L. Norlander, and H. Wolf-Watz, Microb. Pathogen. 2:123–137, 1987). The corresponding genes were localized on pYV019 and pYV8081 of *Yersinia pestis* and *Yersinia enterocolitica*, respectively. No obvious differences were observed on comparison of pIB1 and pYV019, whereas pYV8081 showed intragenic as well as extragenic changes. However, one region of plasmid pYV8081, which coded for the V antigen, YOP3, and YOP4a, was essentially conserved among the three plasmids. Since this region is connected with the Ca^{2+} region, we suggest that the conserved region of the virulence plasmids of *Yersinia* spp. be extended to include both of these regions. Low amounts of the YOPs were detected in the membrane fraction at 37°C in the presence of 2.5 mM calcium. Only minor differences were noticed when the individual YOPs of *Y. pestis* and *Y. pseudotuberculosis* were compared. Several differences were observed when the YOPs of *Y. enterocolitica* were included for comparison. All *Y. enterocolitica* proteins, except YOP1, YOP4b, and the V antigen, exhibited changes in their characteristic molecular sizes. Although these differences were within a range of ±2 kilodaltons, the isoelectric point was retained for each protein type.

Three members of the genus *Yersinia* are recognized pathogens that are capable of causing diseases which elicit a variety of symptoms in humans and animals. Each of the three virulent species *Yersinia pseudotuberculosis*, *Yersinia pestis*, and *Yersinia enterocolitica* carry plasmids that are necessary for virulence (3, 7, 13, 14, 25, 40). Plasmids of *Y. enterocolitica* show approximately 50% homology when compared with the corresponding plasmids of *Y. pestis* and *Y. pseudotuberculosis*. The plasmids of *Y. pestis* and *Y. pseudotuberculosis* are almost identical and are functionally interchangeable (37). Animal models have been used to demonstrate that plasmid-bearing derivatives are virulent, whereas plasmid-cured strains are avirulent. The virulence plasmids are associated with a number of temperature-inducible features of the bacteria: Ca^{2+}-dependent growth at 37°C (low calcium response) (3, 7, 13–15, 27), production of V and W antigens (4–6, 10, 33), autoagglutination (30), and the expression of outer membrane proteins (YOPs) (7, 8, 12, 21, 26, 28, 32).

One region of the virulence plasmid has been shown to be involved in the low calcium response. This region consists of about one-third (20 kilobases [kb]) of the plasmid (11, 15, 25). Recent data indicate that the Ca^{2+} region is involved in the regulation of the plasmid-encoded, temperature-inducible proteins (8, 32, 38, 39). Furthermore, this region is highly conserved among the three *Yersinia* species (28).

All three plasmids exhibit a coding capacity for the temperature-inducible YOPs and the V antigen (17, 25, 28, 37), but there is one exception; *Y. pestis* does not express the high-molecular-weight surface protein YOP1 (28, 37). It has also been shown that all three species secrete the YOPs into the growth medium in the absence of Ca^{2+} at 37°C (17, 36). These proteins appear in high amounts within 2 h after a temperature shift from 26 to 37°C. Following prolonged incubation (8 h), the proteins can also be detected in the outer membranes of *Y. pseudotuberculosis* YPIII(pIB1) and *Y. enterocolitica* 8081. After extended incubation, however, various degrees of breakdown products of these proteins can be observed in the culture supernatant fluid of *Y. pestis* EV76 (36). No proteins could be observed in the outer membrane in this case.

The structural genes for the YOPs and the V antigen have recently been cloned and mapped on plasmid pIB1 of *Y. pseudotuberculosis* (12). The striking observation is that these genes are localized outside the Ca^{2+} region with one exception, YOP4b. Furthermore, they do not form part of a common operon. Results of these studies also showed that the YOPs can be separated into the following seven polypeptides (M_r): YOP1 (47,000), YOP2a and YOP2b (45,000), YOP3 (41,000 to 42,000), YOP4a and YOP4b (34,000), and YOP5 (26,000). Similar results have been obtained in studies with the *Y. pestis* plasmid pCD1 (23, 32).

We used Southern blotting analysis and gene cloning to physically map the respective structural genes of the YOPs (except for YOP2a) and the V antigen on plasmids pYV019 and pYV8081. It was found that the localization of the genes was identical when plasmids pIB1 and pYV019 of *Y. pseudotuberculosis* and *Y. pestis* were compared, whereas that of plasmid pYV8081 of *Y. enterocolitica* was somewhat different (Fig. 1). Only minor differences in molecular weights were observed when the proteins expressed by the three *Yersinia* species were compared.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in this study are described in Table 1. The *Yersinia* strains were grown in a medium consisting of 1% tryptone, 0.5% yeast extract, and 0.2% glucose (21) supplemented with the salt components described by Higuchi et al. (18) and 20 mM sodium oxalate. When Ca^{2+} was added to

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Comments	Reference
Strains		
YPIII(pIB1)	Wild-type *Y. pseudotuberculosis* serotype III	7, 14
YPIII	Plasmid-cured derivative of YPIII(pIB1)	7
EV76	Pgm$^-$ strain of *Y. pestis*	25
8081	Wild-type *Y. enterocolitica* serotype O:8	26
P678-54	Minicell-producing strain of *E. coli*	2
C600	*E. coli* K-12 r$^-$ m$^+$	20
Plasmids		
pIB1	Wild-type virulence plasmid from strain YPIII(pIB1)	7
pYV019	*Y. pestis* virulence plasmid identical to the virulence plasmid of EV76	25
pYV8081	Virulence plasmid from 8081	26

the medium, sodium oxalate was omitted. *Escherichia coli* strains were grown in Luria broth or on Luria agar.

Cell fractionation. Total membranes were isolated from 10-ml samples of culture, as described by Achtman et al. (1), and dissolved in electrophoresis sample buffer (0.0625 M Tris [pH 6.8], 10% glycerol, 5% β-mercaptoethanol, 2% sodium dodecyl sulfate [SDS]).

Culture supernatant fluids (10 ml) were filtered on 0.45-μm-pore-size filters, precipitated by the addition of trichloroacetic acid to a final concentration of 10% (0.1 g/ml), and incubated for 1 h at 4°C. Precipitated proteins were collected by centrifugation, washed once in acetone, dried, and finally dissolved in electrophoresis sample buffer.

SDS-PAGE. SDS-polyacrylamide gel electrophoresis (PAGE) was performed in a discontinuous buffer system, as described by Laemmli (19), by using gradient gels of 10 to 17% acrylamide as the separating gel and 3% acrylamide as the stacking gel.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was performed essentially as described by O'Farrell (22) with [^{35}S]methionine-labeled supernatant proteins (12).

Immunoblotting (Western). Western blotting after SDS-PAGE was performed as described by Swanson et al. (35). Rabbit antiserum against the secreted proteins (anti-YOP antiserum) was obtained by immunizing rabbits with the trichloroacetic acid-precipitated proteins from the culture supernatant fluids of strain YPIII(pIB1) grown in Ca^{2+}-deficient medium at 37°C, as described previously (12). A protein molecular weight standard (Pharmacia AB, Uppsala, Sweden) was labeled with ^{125}I as described by Greenwood et al. (16).

The preparation of rabbit antiserum against purified, released proteins corresponding to YOP2a, YOP2b, YOP3, YOP4a, YOP4b, and YOP5 has been described previously (12). The monoclonal antibody against YOP1 has also been described previously (31). Monospecific antiserum against the V antigen was kindly provided by Robert R. Brubaker.

Minicell assay. Minicells were prepared from *E. coli* P678-54, which carries hybrid plasmids (2). The minicell proteins were labeled and precipitated as described previously (12).

Cloning. Plasmid DNA isolation, restriction enzyme digests, ligation, transformation, and Southern blottings were performed as described by Maniatis et al. (20). DNA fragments were purified by electroelution from agarose gels (GTG; Seakem). Construction of the *Bam*HI gene bank of pIB1 has been described previously (12). *Cla*I gene banks of pIB1, pYV019, and pYV8081 were constructed by using pBR322 as the vector and *E. coli* C600 as the host strain. In subcloning experiments, the vectors pBR322 and pSP64 (Promega Biotec, Madison, Wis.) were used. The resulting clones were analyzed by restriction enzyme digestions of mini-clear lysates and then transformed into minicell-producing *E. coli* P678-54 for the analysis of protein expression.

RESULTS

Localization of the YOP structural genes. From results of a previous study (9), it was known that the amino-terminal portion of the YOP1 structural gene is located within the 1.6-kb *Bam*HI fragment 10 of pIB1. When a minicell clone containing *Bam*HI fragment 10 was analyzed by immunoprecipitation with specific YOP1 monoclonal antibodies, no YOP1 was detected. This suggests that only a part of the structural gene of YOP1 is located within fragment 10.

*Bam*HI fragment 10 contained a single *Cla*I site which divided the fragment into 1,100 and 485-base-pair parts. In an attempt to obtain the whole structural gene, *Cla*I fragments of pIB1 were cloned into the single *Cla*I site of pBR322. Parallel *Cla*I clonings were also performed with pYV019 and pYV8081. The resulting hybrid plasmids were transformed into *E. coli* C600. These *Cla*I banks were screened by colony hybridization, with the 485- and 1,100-base-pair *Bam*HI-*Cla*I fragments used as probes. The hybridization-positive clones were analyzed for YOP1 expression by SDS-PAGE analysis. YOP1 was expressed by clones from pIB1 and pYV8081 that hybridized with the 485-base-pair probe. Some of the pIB1 and pYV8081 clones failed to express YOP1 in *E. coli*. They were found to have the cloned fragment in the opposite orientation compared with that of the clones that expressed YOP1. This indicates that the pBR322 promoter P1 is used to express YOP1 (34). No expression was observed from the corresponding pYV019 clones, irrespective of orientation.

These clones were analyzed with restriction endonucleases, and were shown to contain either a 5.2-kb *Cla*I insert (pIB1 and pYV019) or a 6.2-kb *Cla*I fragment (pYV8081) (Fig. 1B). The restriction maps of the 5.2-kb *Cla*I fragments of pIB1 and pYV019 were found to be identical, while the 6.2-kb *Cla*I fragments of pYV8081 differed. Subclones were constructed from the *Cla*I clones, and the structural genes of YOP1 were localized to within 2-kb fragments (Fig. 1B). Although differences were noted, some restriction sites (*Cla*I, *Pvu*II, and *Pst*I) seemed to be conserved in all three species.

We have previously shown (12) the localization of structural genes for the YOPs on pIB1 (Fig. 1A). By the use of different probes derived from pIB1 clones that contained the identified genes, in Southern blotting experiments the corresponding genes were mapped on plasmids pYV019 and pYV8081. Plasmid pYV019 gave the same hybridization pattern as pIB1 with all restriction enzymes tested. In contrast, pYV8081 showed differences in some regions.

The major difference when the two plasmids (pYV8081 and pIB1) were compared was the localization of the YOP5 gene (Fig. 1). DNA regions adjacent to the structural gene showed no restriction enzyme site homology. Intragenic changes were also detected (Fig. 1C).

The YOP2b gene was localized closer to the Ca^{2+} region on pYV8081 compared with that on pIB1. In this case, no common restriction enzyme sites were found (Fig. 1C).

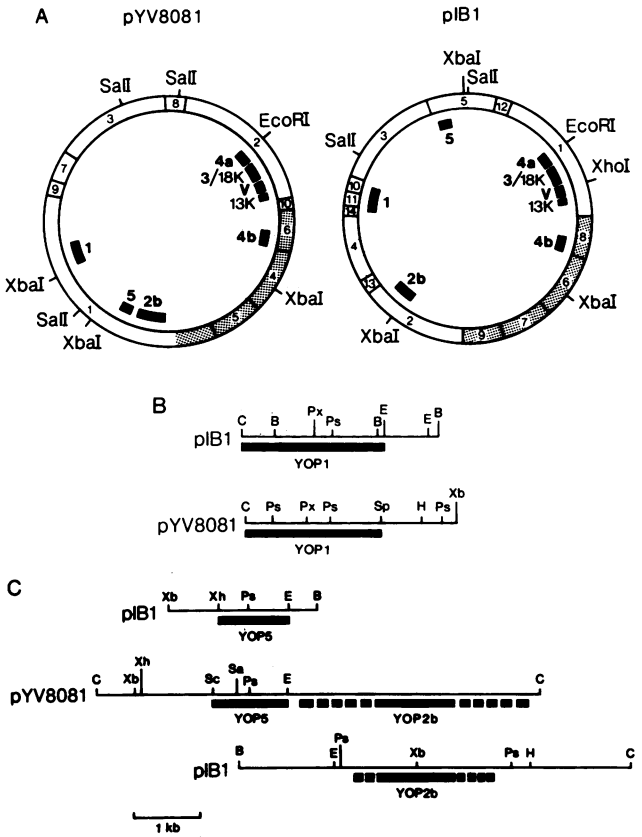

FIG. 1. (A) Genetic localization of the YOPs and the V antigen on the virulence plasmids pYV8081 (*Y. enterocolitica*) and pIB1 (*Y. pseudotuberculosis*). The plasmids are shown as circular *Bam*HI restriction maps, with the fragments numbered according to size. The stippled areas indicate the Ca^{2+} region. The positions of the structural genes of the YOPs and the V antigen are represented by black bars marked with their respective YOP designations. In addition, the positions of the structural genes of the 18- (18K) and 13-kDa (13K) polypeptides known to be localized to the V-antigen region are shown (12, 24). (B) Restriction enzyme maps of DNA fragments of pIB1 and pYV8081 containing the YOP1 structural genes. (C) Structural genes of YOP2b and YOP5. The smallest fragments containing the structural genes are indicated by black bars. Abbreviations used for restriction enzymes are as follows: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Px, *PvuII*; Ps, *Pst*I; Sa, *Sac*I; Sp, *Sph*I; Xb, *Xba*I; Xh, *Xho*I.

These results suggest that the structural genes of YOP2b and YOP5 of pIB8081 are localized as depicted in Fig. 1. To further strengthen the location and identity of the pYV8081 genes, they were cloned and expressed in minicells.

One hybrid plasmid obtained from a *Cla*I bank of pYV8081 in pBR322 contained the 6.7-kb *Cla*I fragment shown in Fig. 1C. This clone expressed in minicells a protein that was slightly larger than YOP2b of pIB1 but that was identical to the protein seen in the wild-type strain 8081 (data not shown). As expected from the Southern blotting data, this clone had no restriction site homology with the pYV019 and pIB1 clones. Subcloning localized the gene for the YOP2b of pYV8081 to within the 3.8-kb *Eco*RI-*Cla*I fragment (Fig. 1C).

The *Eco*RI-*Xba*I fragment (Fig. 1C) containing the YOP5 structural gene of pYV8081 was cloned in pSP64. This clone expressed in minicells a polypeptide of 25.5 kilodaltons (kDa) that could be immunoprecipitated with anti-antiserum against purified YOP5 from YPIII(pIB1) (data not shown).

The other three identified YOP genes and the V-antigen gene showed a conserved localization on pYV8081 compared with that on pIB1. Only minor differences were noted (Fig. 1A).

YOP protein analysis. We have previously shown (12) that strain YPIII(pIB1) expresses two pairs of polypeptides with similar sizes, i.e., YOP2a and YOP2b, and YOP4a and YOP4b. These proteins have never been resolved in *Y. enterocolitica* 8081 or *Y. pestis* EV76. Furthermore, our genetic analysis revealed genetic rearrangements in strain 8081. Therefore, it was of great importance to identify each individual YOP in the respective strain.

The released proteins and membrane proteins were analyzed by immunoblottings with anti-YOP antiserum, as well as with antisera against the individual YOPs (YOP1, YOP2a, YOP2b, YOP3, YOP4a, YOP4b, YOP5) and V-antigen antiserum. In addition, two dimensional gel electrophoresis was performed. Each specific YOP had the same isoelectric point and quantitative pattern of expression, irrespective of variations in molecular size (data not shown). The results from these experiments are summarized in Table 2. The comparison between *Y. pseudotuberculosis* and *Y. pestis* revealed only one difference, i.e., YOP2a. On the other hand, most of the YOPs from *Y. enterocolitica* varied in molecular size (at most, 2 kDa) in comparison with the YOPs from the other two strains.

The YOP3 protein was expressed in lower amounts compared with the Ca^{2+}-regulated YOPs, and was therefore difficult to identify. Using specific YOP3 antiserum, we have shown previously (12) for YPIII(pIB1) that YOP3 can be recovered in two different forms. This was also shown to be the case for YOP3 in *Y. enterocolitica* and *Y. pestis* (Fig. 2). In the membrane fraction the YOP3 polypeptide appeared as a distinct band, whereas the secreted form appeared as a broad band with an increased molecular size.

Expression of the YOPs in cells grown at 37°C in the presence of 2.5 mM Ca^{2+} was also monitored by immunoblotting (Fig. 3). These proteins could not be seen in Coomassie blue-stained gels. In all three species the YOPs were expressed in low amounts under these conditions and could be observed primarily in the membrane fractions. As was the case under Ca^{2+}-deficient conditions, the amount of YOPs recovered from *Y. pestis* was lower when compared with that from the other two species.

TABLE 2. Sizes of the immunologically related YOPs and the V antigen of *Yersinia* spp.[a]

Protein	Sizes (kDa) of proteins in the following strains:		
	Y. pseudotuberculosis YPIII(pIB)	*Y. pestis* EV76	*Y. enterocolitica* 8081
YOP1	47[b]	ND[c]	47[b]
YOP2a	45	44	44
YOP2b	45	45	46
YOP3	41–42[d]	41–42[d]	40–41[d]
V antigen	38	38	38
YOP4a	34	34	36
YOP4b	34	34	34
YOP5	26	26	25.5

[a] Sizes were determined by SDS-PAGE.
[b] Subunit molecular size.
[c] ND, Not detected.
[d] The range in molecular sizes is due to the presence of two different forms of YOP3 (Fig. 2).

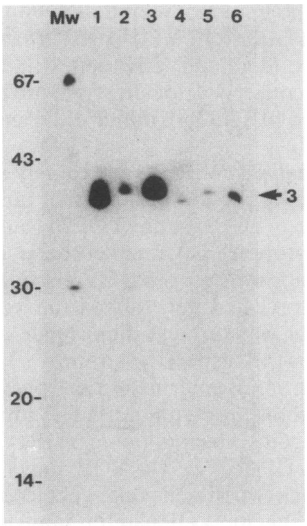

FIG. 2. Immunoblotting of released and membrane-associated proteins with anti-YOP3 antiserum after a temperature shift of *Yersinia* strains grown in a Ca^{2+}-deficient medium. Cultures of 10 ml were grown in Ca^{2+}-deficient medium at 26°C to an optical density at 550 nm of 0.1 and then shifted to 37°C. Two hours after the shift, the cells were harvested by centrifugation. Preparation of whole membranes and precipitation of released proteins from culture supernatant fluids was performed as described in the text. Both whole membranes and supernatant fluid proteins were dissolved in 200 μl of SDS sample buffer, and 10 μl of each sample was used. Lanes: 1, Released proteins from *Y. enterocolitica* 8081; 2, released proteins from *Y. pestis* EV76; 3, released proteins from *Y. pseudotuberculosis* YPIII(pIB1); 4, whole membranes from *Y. enterocolitica* 8081; 5, whole membranes from *Y. pestis* EV76; 6, whole membranes from *Y. pseudotuberculosis* YPIII(pIB1); Mw, ^{125}I-labeled molecular weight standard (indicated to the left, in thousands). The arrow indicates the position of YOP3.

DISCUSSION

In this study we have extended results of our previous study (12), with regard to the localization and expression of the YOPs, to include virulence plasmids pYV8081 and pYV019 of *Y. enterocolitica* and *Y. pestis*, respectively. The similarities between plasmids pIB1 and pYV019 were further strengthened because detailed restriction enzyme analysis of the regions encompassing these genes revealed no obvious differences. Surprisingly, the DNA region of plasmid pYV019 that corresponded to the structural gene of YOP1 was also conserved, even though YOP1 was not expressed from this plasmid. The inability to express YOP1 may be due to minor changes in the structural gene, to defects in its regulation, or both.

In agreement with previously published results (27), rearrangements at the DNA level were observed when plasmid pYV8081 was compared with plasmid pIB1 (Fig. 1). Both intragenic and extragenic changes were established. The region of the plasmids containing the structural genes of the V antigen, YOP3, and YOP4a was found to be highly conserved. This region of the *Y. pestis* plasmid pCD1 was recently characterized (24). Those data are in agreement with the data presented here. We did not, however, observe a 26-kDa polypeptide. This protein might be a truncated form of YOP4a from the cloned 3.6-kb *Hin*dIII fragment, because there is a *Hin*dIII region within the pIB1 structural gene of YOP4a (12). This V-antigen region is adjacent to the Ca^{2+} region. Therefore, we suggest that the conserved region may be expanded to include both regions (Fig. 1).

Because of the observed genetic rearrangement of pYV8081, it was important to establish the identity of the individual YOPs of the respective strains. Only minor differences were observed with respect to the YOPs when strains EV76 and YPIII(pIB1) were compared. The molecular size of the acidic YOP2a protein of EV76 was smaller (44 kDa), while its isoelectric point was similar. In agreement with earlier observations, strain EV76 did not express YOP1 (28, 37). In *Y. enterocolitica* 8081, only three polypeptides, YOP1, V antigen, and YOP4b, showed the same molecular size when compared with that of strain YPIII(pIB1). The other proteins exhibited differences in molecular sizes, which fell within a range of 2 kDa (Table 2).

It has been shown that YOP3 can be recovered in a membrane-bound form and as a secreted form that appears

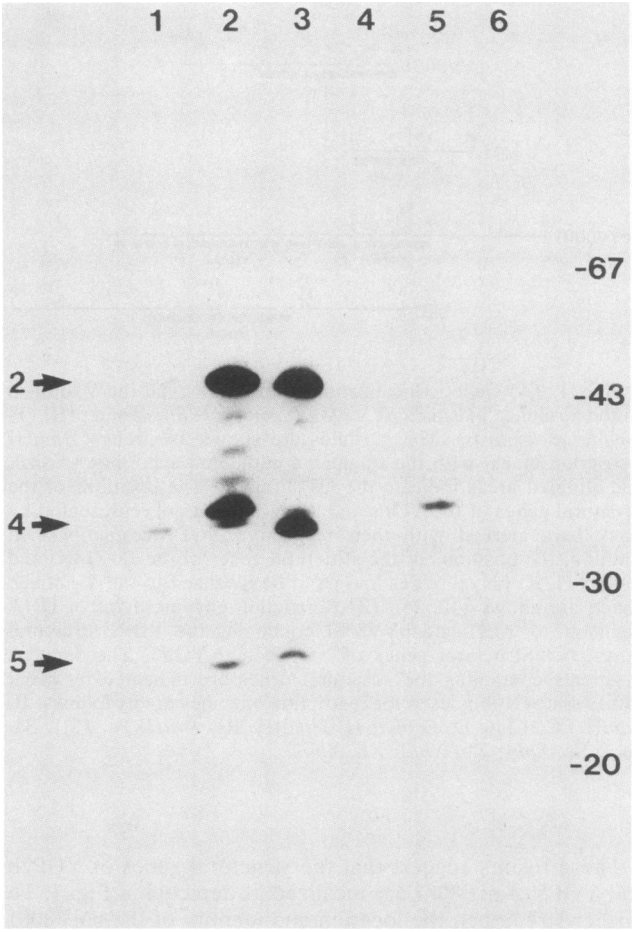

FIG. 3. Released proteins and membrane-associated proteins after a temperature shift of *Yersinia* strains grown in a Ca^{2+}-containing medium. Cultures of 10 ml were grown in a Ca^{2+}-containing (2.5 mM) medium at 26°C, shifted to 37°C, and fractionated as described in the legend to Fig. 2. Membranes and supernatant fluid proteins were dissolved in 50 μl of SDS sample buffer and analyzed by immunoblotting with YOP antiserum. A total of 25 μl of each sample was used. Lanes: 1, Whole membranes from *Y. pestis* EV76; 2, whole membranes from *Y. enterocolitica* 8081; 3, whole membranes from *Y. pseudotuberculosis* YPIII(pIB1); 4, released proteins from *Y. pestis* EV76; 5, released proteins from *Y. enterocolitica* 8081; 6, released proteins from *Y. pseudotuberculosis* YPIII(pIB1). Arrows indicate YOP2, YOP4, and YOP5. Numbers to the right indicate molecular weights (in thousands) of the protein standard.

as a broad band (12). This was also true of the YOP3 from strains EV76 and 8081. The difference in the released and the membrane-bound forms of YOP3 suggests that, although destined for the outer membrane, during conditions of Ca^{2+} starvation it is cosecreted into the medium to a certain extent with the other YOPs. This could also be the case for the V antigen, which was observed in limited amounts in the membrane fraction, as well as in the growth medium.

When the sensitive immunoblotting technique was employed, it was possible to detect small amounts of YOPs in all three strains after incubation at 37°C in a Ca^{2+}-containing medium. The amount of YOPs observed in strain EV76 was lower than that in the other two strains. We favor the idea that this is due to proteolytic activity, as has been observed for this strain when grown in a Ca^{2+}-deficient medium (36). However, the possibility cannot be excluded that the regulation of the YOPs is different. The fact that the YOPs are produced in a Ca^{2+}-containing medium might indicate that they are also expressed in vivo during infection in a Ca^{2+}-containing environment. Support for this idea was obtained when EV76 was grown in vivo in semipermeable capsules in the peritoneal fluid of guinea pigs; in this Ca^{2+}-containing environment, the expression of virulence plasmid-encoded antigens was detected (29). If these proteins play an important role in infection, it is possible that they fulfill their function when expressed at low levels in an extracellular environment. It is important to consider the fact that the high expression of the YOPs during conditions of Ca^{2+} starvation in vitro can be correlated with the decreased cell yield of the bacteria.

The virulence plasmids of the three species have apparently been subjected to rearrangements throughout evolution, but they have retained the coding capacity for the temperature-inducible, Ca^{2+}-regulated proteins, as well as the Ca^{2+} regulon. This reflects the importance of these proteins for pathogenic *Yersinia* spp.

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