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Plasmid-Mediated Surface Fibrillae of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*: Relationship to the Outer Membrane Protein YOP1 and Possible Importance for Pathogenesis

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The cell surface properties of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* mutants, constructed by insertional inactivation of genes located on the 40- to 50-megadalton virulence plasmid, were examined. Electron microscopy revealed an absolute correlation between expression of four plasmid-dependent, temperature-inducible properties related to the bacterial surface: (i) a fibrillar matrix covering the outer membrane, (ii) outer membrane protein YOP1, (iii) spontaneous autoagglutination, and (iv) mannose-resistant hemagglutination of guinea pig erythrocytes. Immunoelectron microscopy indicated that YOP1 is a structural component of the fibrillae. Experiments demonstrating inhibition of hemagglutination by anti-YOP1 monoclonal antibody suggested a potential role for YOP1 in adhesion. Insertional inactivation of the gene coding for YOP1, with resultant loss of the ability to express fibrillae, led to a significant reduction in the capacity of *Y. enterocolitica*, but not *Y. pseudotuberculosis*, to colonize the ileum of orogastrically infected mice. In both *Y. enterocolitica* and *Y. pseudotuberculosis*, inactivation of the genes coding for Ca^{2+} dependency reduced the ability to maintain intestinal colonization, regardless of the ability to express fibrillae. Both surface fibrillae and Ca^{2+} dependency seem to reflect pathogenic determinants which are required for the establishment of *Y. enterocolitica* infection. In *Y. pseudotuberculosis*, however, no clinical significance of the fibrillae has so far been defined.

A plasmid approximately 40 to 50 megadaltons in size is required for expression of virulence in *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, and *Yersinia pestis* (12–14, 26). The virulence plasmid from all three *Yersinia* species has coding capacity for a series of ancillary outer membrane proteins (YOPs) (3, 27, 31, 33). Another property encoded by the virulence plasmid is a nutritional requirement for Ca^{2+}, which leads to decreased cell division in Ca^{2+}-deficient media (12–14, 23). This Ca^{2+}-dependent growth inhibition is accompanied by synthesis of two virulence-associated proteins known as the V and W antigens (6–9, 23). The phenotypic expression of both YOPs and Ca^{2+} dependency is temperature regulated, these properties being expressed maximally at elevated temperatures of 35 to 37°C.

The virulence plasmid contains a large, highly conserved region which is necessary for expression of Ca^{2+} dependency and V-antigen production (10, 15, 25, 27). The conserved maintenance of this region in virulence plasmids from all three pathogenic *Yersinia* species suggests that the gene products concerned are important in pathogenesis (26). Indeed, a close correlation between Ca^{2+} dependency and virulence has been established for *Y. pseudotuberculosis* and *Y. pestis* (4, 24). It has also been shown that the Ca^{2+} dependency region encodes regulatory components that affect expression of several low-molecular-weight YOPs (3, 10, 31, 34). Recent data indicate that some of these YOPs represent virulence attributes (31). However, one high-molecular-weight protein, YOP1, is regulated by a mechanism different from that responsible for the other YOPs (3, 4, 10). Whereas *Y. pseudotuberculosis* and *Y. enterocolitica* are able to express YOP1, *Y. pestis* lacks this property (3, 31–33). The clinical significance of YOP1 is a matter of debate (1, 4, 30).

Plasmid-bearing strains of *Y. enterocolitica* (17, 18) and *Y. pseudotuberculosis* (17), unlike their plasmid-cured mutants, produce a matrix of fibrillae covering the bacterial surface. These surface structures are distinct from the fimbriae which are elaborated by certain *Yersinia* strains (20). Like the YOPs, the fibrillae are expressed at 35 to 37°C. Kapperud et al. (17) found a close correlation between the presence of fibrillae, the ability to undergo spontaneous autoagglutination (19, 23), and expression of a high-molecular-weight outer membrane protein, probably identical to YOP1. Furthermore, a possible relationship between the fibrillae and a novel mannose-resistant hemagglutinin was indicated (16–18). Though a causal relationship between YOP1 and autoagglutination has previously been indicated (1, 29), no surface appendages were detected in these studies. Likewise, Lachica et al. (18) demonstrated a relationship between fibrillae and autoagglutination. No attempt was made, however, to correlate these factors with outer membrane proteins.

In this paper, we present results obtained with a series of *Y. pseudotuberculosis* and *Y. enterocolitica* mutants constructed by insertional inactivation of distinct genes on the virulence plasmid. The examination of these insertion mutants permitted us to obtain further insight into the interrelationship between surface fibrillae, YOPs, autoagglutination, hemagglutination, and Ca^{2+} dependency. Furthermore, we used an adult mouse model to ascertain whether the ability to express surface fibrillae has any significance in pathogenesis.

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MATERIALS AND METHODS

Bacterial strains. Five derivatives of *Y. pseudotuberculosis* YPIII (serogroup O:III) were kindly provided by I. Bölin and H. Wolf-Watz ((Department of Microbiology, National Defence Research Institute, Umeå, Sweden): the wild-type strain YPIII(pIB1), its plasmid-cured derivative YPIII, and the Tn*5* insertion mutants YPIII(pIB71), YPIII(pIB101), and YPIII(pIB102) derived from YPIII(pIB1) (4). Five derivatives of *Y. enterocolitica* W22708 (serogroup O:9) were kindly donated by G. Cornelis (Université Catholique de Louvain, Unité de Microbiologie, Brussels, Belgium): W22708(pYL4), its plasmid-cured derivative W22708, and the insertion mutants W22708(pGB08), W22708(pGB51), and W22708(pGB910), constructed from W22708(pYL4) by transposon mutagenesis (1). The strains have previously been characterized with respect to genetic details, production of outer membrane proteins, Ca^{2+} dependency, and autoagglutination (1, 3, 4). All strains were stored at −70°C in heat-inactivated horse serum with 17% glycerol.

Media and growth conditions. The strains were cultivated under conditions previously shown to promote expression of YOP1 and surface fibrillae (17). The strains were grown under aerobic and static conditions at 22 or 37°C for 20 h in culture tubes (15 by 150 mm) containing 5 ml of Eagle basal medium with 10% fetal calf serum (Gibco Europe Ltd., Paisley, Scotland). The bacteria were then inoculated into tubes containing 5 ml of the same medium at an optical density at 620 nm of 0.05 (Hitachi 101 spectrophotometer). The tubes were subsequently incubated at 22 or 37°C for 20 h, and the bacterial concentration was finally adjusted by turbidimetric adjustment.

Autoagglutination. The strains were examined for their ability to undergo spontaneous autoagglutination at 37°C, using the methods and criteria of Laird and Cavanaugh (19), except that the medium was changed to Eagle basal medium with 10% fetal calf serum (see above).

Hemagglutination and hemagglutination inhibition. The strains were tested for their ability to agglutinate guinea pig erythrocytes in vitro, using a small-scale hemagglutination procedure. Blood from a single guinea pig was citrated by addition of 0.75 ml of citrate glucose phosphate buffer (0.15 M glucose, 0.09 M sodium citrate, 0.015 M citric acid, 0.016 M NaH_2PO_4) per 5 ml of blood. The blood cells were washed three times with phosphate-buffered saline (PBS) (0.1 M; pH 7.4) containing 10 mM NaN_3, and the washed and packed erythrocytes were diluted to 3% in PBS. The bacteria were cultivated at 37°C, harvested by low-speed centrifugation (2,000 × *g* for 5 min), and suspended in PBS to approximately 10^{10} bacteria per ml by turbidimetric adjustment. Serial twofold dilutions in PBS were performed in microtiter plates with U-shaped wells, and equal volumes (25 μl) of a suspension containing 3% guinea pig erythrocytes and 2% D-(+)-mannose in PBS were added to each well. The microtiter plates were kept at 4°C and examined after 1 and 20 h of incubation.

The ability of anti-YOP1 monoclonal antibody (described below) to inhibit hemagglutination was examined as follows. Bacterial suspension was mixed 1:1 with a 100-fold dilution of monoclonal antibody in PBS and incubated at room temperature for 2 h before testing for hemagglutination as described above. Controls included (i) bacterial suspension mixed 1:1 with 10-fold and 100-fold dilutions of specific serotype O:9 antiserum prepared by intravenous inoculation of rabbits, using boiled bacterial antigen as described by Wauters (G. Wauters, thesis, University of Louvain, Brussels, Belgium, 1970); (ii) bacterial suspension mixed 1:1 with PBS without antibody; and (iii) antibody suspensions without bacteria.

Electron microscopy. Expression of surface fibrillae was evaluated by electron microscopic examination of 37°C-grown bacteria as described previously (17). Before preparation, carbon-filmed grids were conditioned by glow discharge in air to facilitate spreading of both specimens and stain (22). The specimens were negatively stained with 0.25% sodium phosphotungstic acid (pH 7.0) and examined in a JEM 100B electron microscope operated at 80 keV.

Monoclonal antibody. A monoclonal antibody (immunoglobulin M) to YOP1 was produced in a hybridoma cell line prepared by the semisolid-agar method (11) using spleen cells from mice immunized with purified YOP1 from a *Y. enterocolitica* O:3 strain (2, 29). The culture supernatants of hybridoma clones were screened for specific antibodies by using an immunoblotting technique (28). The monoclonal antibody was precipitated from the culture supernatant with a 50% saturation of $(NH_4)_2SO_4$, solubilized in a minimal amount of distilled water, dialyzed against several changes of PBS (0.1 M; pH 7.2), and stored at −20°C. The specificity of the antibody was demonstrated by immunoblotting in a previous work (30).

Immunoelectron microscopy. For immunoelectron microscopy, the bacteria were cultivated at 37°C, washed, and suspended in distilled water as outlined previously (17). Carbon-filmed grids were coated with bacterial suspension and incubated face down for 3 min on drops of a 1,000-fold dilution of the monoclonal antibody. The grids were then washed on several drops of PBS (0.01 M phosphate; pH 7.4) before being incubated face down for 3 min with a 10-fold dilution of goat anti-mouse immunoglobulin M conjugated to 5-nm colloidal gold particles (Janssen Pharmaceutica, Beerse, Belgium). After a series of washings on drops of PBS, followed by distilled water, the specimens were finally stained with 0.25% sodium phosphotungstic acid (pH 7.0) for 30 s. All dilutions of antibody and gold probe were performed in PBS (0.01 M; pH 7.4). The specimens were examined by electron microscopy as described above. *Y. enterocolitica* 8265 P^+ (serogroup O:3) was used as a positive control. This strain has previously been shown to produce both YOP1 and fibrillae (17). Its plasmid-cured derivative 8265 P^- served as a negative control. The specificity of the gold conjugate was evaluated by incubating the bacteria with gold particles conjugated to goat anti-mouse immunoglobulin M in the absence of anti-YOP1.

Mouse virulence assay. Test strains were grown at 22°C, and the bacterial concentration was finally adjusted to approximately 10^9 bacteria per ml (optical density at 620 nm of 0.8) in the growth medium. A single dose of 0.5 ml of bacterial suspension was administered orogastrically with a feeding tube into the stomach of adult female Bom:NMRI (specific-pathogen-free) mice weighing about 25 g. The mice were observed daily for signs of illness or death. Groups of four mice were killed by cervical dislocation at 1, 2, 3, and 4 days after challenge. The spleen, approximately 0.1 g of the left liver lobe, and a 5-cm segment of the distal ileum were removed aseptically. The ileal segment was cut open longitudinally and placed in 0.5 ml of sterile saline. Ileal contents

TABLE 1. Correlation among plasmid-dependent, temperature-regulated properties of *Y. pseudotuberculosis* and *Y. enterocolitica*

Strain	Plasmid-dependent properties[a]					
	CAD[b]	YOP1[b]	Anti-YOP1	FIB	MRHA	AA
Y. pseudotuberculosis						
YPIII(pIB1)	+	+	+	+	+	+
YPIII(pIB71)	−	+	+	+	+	+
YPIII(pIB102)	+	−	−	−	−	−
YPIII(pIB101)	−	−	NT[c]	−	−	−
YPIII	−	−	−	−	−	−
Y. enterocolitica						
W22708(pYL4)	+	+	+	+	+	+
W22708(pGB51)	−	+	+	+	+	+
W22708(pGB910)	+	−	NT	−	−	−
W22708(pGB08)	+	−	−	−	−	−
W22708	−	−	−	−	−	−

[a] CAD, Ca^{2+} dependency; YOP1, outer membrane protein YOP1; Anti-YOP1, binding of monoclonal antibodies against YOP1 (see Fig. 2); FIB, surface fibrillae (see Fig. 1); MRHA, mannose-resistant hemagglutination; AA, autoagglutination.

[b] The ability to express Ca^{2+} dependency and YOP1 has previously been documented by Bölin and Wolf-Watz (4) and Balligand et al. (1).

[c] NT, Not tested.

were washed out by blending with a sterile glass rod. The number of yersiniae in the ileal contents was determined by plating 0.1-ml portions of decimal saline dilutions onto bromothymol blue-lactose agar in duplicate. The plates were examined for presence of the challenge strain after incubation for 48 h at 22°C. The number of organisms recovered was expressed as $\log_{10}$ CFU per 5-cm segment of the distal ileum. Spleen and liver samples were placed separately in 0.5 ml of sterile saline and homogenized by maceration with a sterile glass rod. Presence of the challenge strain was determined by plating decimal saline dilutions onto blood agar as described for the ileal contents.

RESULTS

Expression of surface fibrillae. The insertion mutants of *Y. pseudotuberculosis* and *Y. enterocolitica* were initially tested for their ability to express surface fibrillae when cultivated at 37°C under conditions previously shown to promote production of the outer membrane protein YOP1 (17). Electron microscopic examination demonstrated a close relationship between the presence of a fibrillar matrix on the bacterial surface and the ability to express YOP1 (Table 1, Fig. 1). No fibrillae could be detected on the surface of the mutants YPIII(pIB102) (Fig. 1b), W22708 (pGB08) (Fig. 1d), or W22708(pGB910), all of which have putative transposon insertions within the plasmid gene coding for YOP1. In contrast, numerous fibrillae were expressed by the wild-type parent strains YPIII(pIB1) (Fig. 1a) and W22708(pYL4) (Fig. 1c), as well as by their insertion mutants YPIII(pIB71) and W22708(pGB51), all of which are able to produce YOP1 (Table 1). The diameter of the individual fibrillae measured 1.5 to 2.0 nm, which is in accordance with our previous findings. The fibril length, however, was found to vary from 50 to 125 nm, in contrast to the fairly uniform dimension of 50 to 70 nm documented for a clinical isolate of *Y. enterocolitica* O:9 in our previous work (17).

Autoagglutination and hemagglutination. The ability to undergo spontaneous autoagglutination and to agglutinate guinea pig erythrocytes in vitro was displayed only by bacteria having the fibrillae exposed on their surface; nonfibrillated strains were invariably negative (Table 1). An absolute correlation between four temperature-inducible and plasmid-dependent properties of *Y. pseudotuberculosis* and *Y. enterocolitica* was thus demonstrated: (i) surface fibrillae, (ii) YOP1, (iii) autoagglutination, and (iv) a narrow-spectrum, mannose-resistant hemagglutinin.

Immunoelectron microscopy. The results presented above provide strong evidence that YOP1 and the surface fibrillae both reflect the same determinant, and these results encourage speculations as to whether YOP1 constitutes a structural part of the fibrillae. To test this hypothesis, we used an immunogold labeling technique. All bacteria having the fibrillae exposed on their surface were specifically labeled with gold particles, whereas with nonfibrillated bacteria no binding of gold was seen (Table 1, Fig. 2). Neither were any gold particles observed on specimens incubated with the gold probe in the absence of anti-YOP1. It proved difficult to determine the exact localization of the gold particles due to the minute dimensions of the fibrillae, which were masked by stabilizing proteins present in the antibody preparation. Nevertheless, the electron micrograph shown in Fig. 2a shows labeling of the fibrillar matrix. The results thus provide strong evidence that the fibrillae contain YOP1.

Role of YOP1 in hemagglutination. The observation that YOP1 production was correlated with agglutination of guinea pig erythrocytes prompted us to study whether anti-YOP1 monoclonal antibody could inhibit hemagglutination. Strains W22708(pYL4) and W22708(pGB51), both of which produce YOP1, were incubated with the anti-YOP1 monoclonal antibody and subsequently tested for hemagglutination. Both strains showed significantly reduced reaction (three twofold-dilution steps) as compared with bacteria tested in the absence of anti-YOP1, indicating that YOP1 is involved in the hemagglutination process. In contrast, hemagglutination was not inhibited by the addition of antibody not specific for YOP1 (i.e., O:9 antiserum).

Mouse virulence. To test whether the surface fibrillae have any significance in the pathogenesis of *Y. pseudotuberculosis* or *Y. enterocolitica* infections, we compared the pathogenic potential of the strains listed in Table 1, using an adult mouse model. The number of bacteria recovered from the ileal contents of mice after orogastric challenge is shown in Fig. 3 and 4.

(i) *Y. pseudotuberculosis*. Insertional inactivation of the genes coding for YOP1 did not significantly reduce the capability of *Y. pseudotuberculosis* to colonize the mouse ileum. The Ca^{2+}-dependent (CAD^+), nonfibrillated (FIB^-) mutant YPIII(pIB102) and its wild-type parent strain YPIII(pIB1) (CAD^+ FIB^+) colonized with about equal efficiency. On the other hand, inactivation of the genes responsible for expression of Ca^{2+} dependency led to an appreciable decline in the number of bacteria recovered: the ability of the mutant YPIII(pIB71) (CAD^- FIB^+) to maintain colonization was significantly reduced as compared with that of YPIII(pIB1). Although YPIII(pIB71) was still able to express surface fibrillae, its capacity for colonization was comparable with that of the avirulent plasmid-cured derivative YPIII.

(ii) *Y. enterocolitica*. In accordance with the results obtained for *Y. pseudotuberculosis*, insertional inactivation of the Ca^{2+} dependency genes consistently reduced the ability of *Y. enterocolitica* to colonize the ileal lumen, regardless of the ability to express fibrillae. Although all strains were

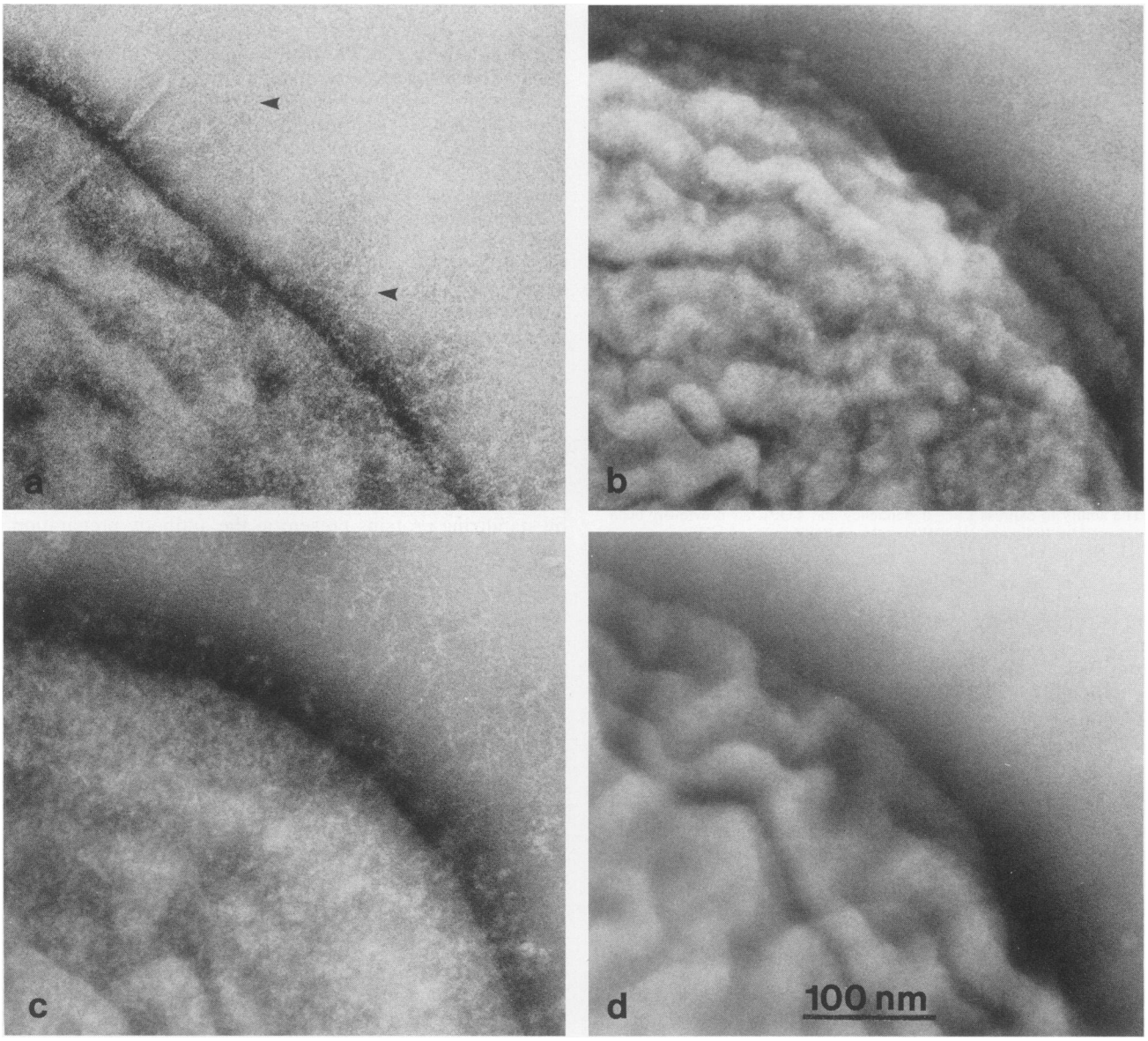

FIG. 1. Electron micrographs showing (a) *Y. pseudotuberculosis* YPIII(pIB1), (b) its insertion mutant YPIII(pIB102), (c) *Y. enterocolitica* W22708(pYL4), and (d) its insertion mutant W22708(pGB08). Micrographs a and c show the fibrillated surface of the parent strains, which express YOP1. Note the total absence of fibrillae on the surface of bacteria having transposon insertions within the gene for YOP1 (b and d). The specimens are negatively stained with 0.25% phosphotungstic acid (pH 7.0). Magnification, ×200,000.

present in the ileal contents in large numbers at 1 day after inoculation, the viable counts of the mutant W22708(pGB51) (CAD^- FIB^+) and the avirulent plasmid-cured derivative W22708 (CAD^- FIB^-) both decreased to an undetectable level within 2 to 3 days. In contrast, the parent strain W22708(pYL4) increased by more than 2 logs during the same period of time. However, unlike *Y. pseudotuberculosis*, insertional inactivation of the gene coding for YOP1 led to a significant reduction in the ability of *Y. enterocolitica* to colonize the mouse ileum, irrespective of Ca^{2+} dependency. The mutants W22708(pGB08) (CAD^+ FIB^-) and W22708(pGB910) (CAD^+ FIB^-), both of which have putative transposon insertions within the structural gene encoding YOP1, decreased to about 0 to 10^2 CFU after 3 days, at which time their parent strain reached about 10^6 CFU. It is notable, though, that the Ca^{2+}-dependent mutants W22708 (pGB08) and W22708(pGB910) were cleared more slowly from the ileal lumen than their Ca^{2+}-independent counterparts W22708 and W22708(pGB51). All mice inoculated with W22708(pGB08) and W22708(pGB910) were still heavily infected after the first 2 days, whereas the viable count of W22708(pGB51) fell precipitously over the same period of time and reached an undetectable level in three of the four mice examined.

Surprisingly, YPIII(pIB102) was recovered from the spleen in a much greater number (10^5 to 10^7 CFU per spleen at day 3) than its parent strain YPIII(pIB1) (0 to 10^2 CFU at day 3) (data not shown). Likewise, YPIII(pIB102) reached high counts in the liver (10^4 to 10^5 CFU/g at day 3), whereas YPIII(pIB1) was not detected at all throughout the investi-

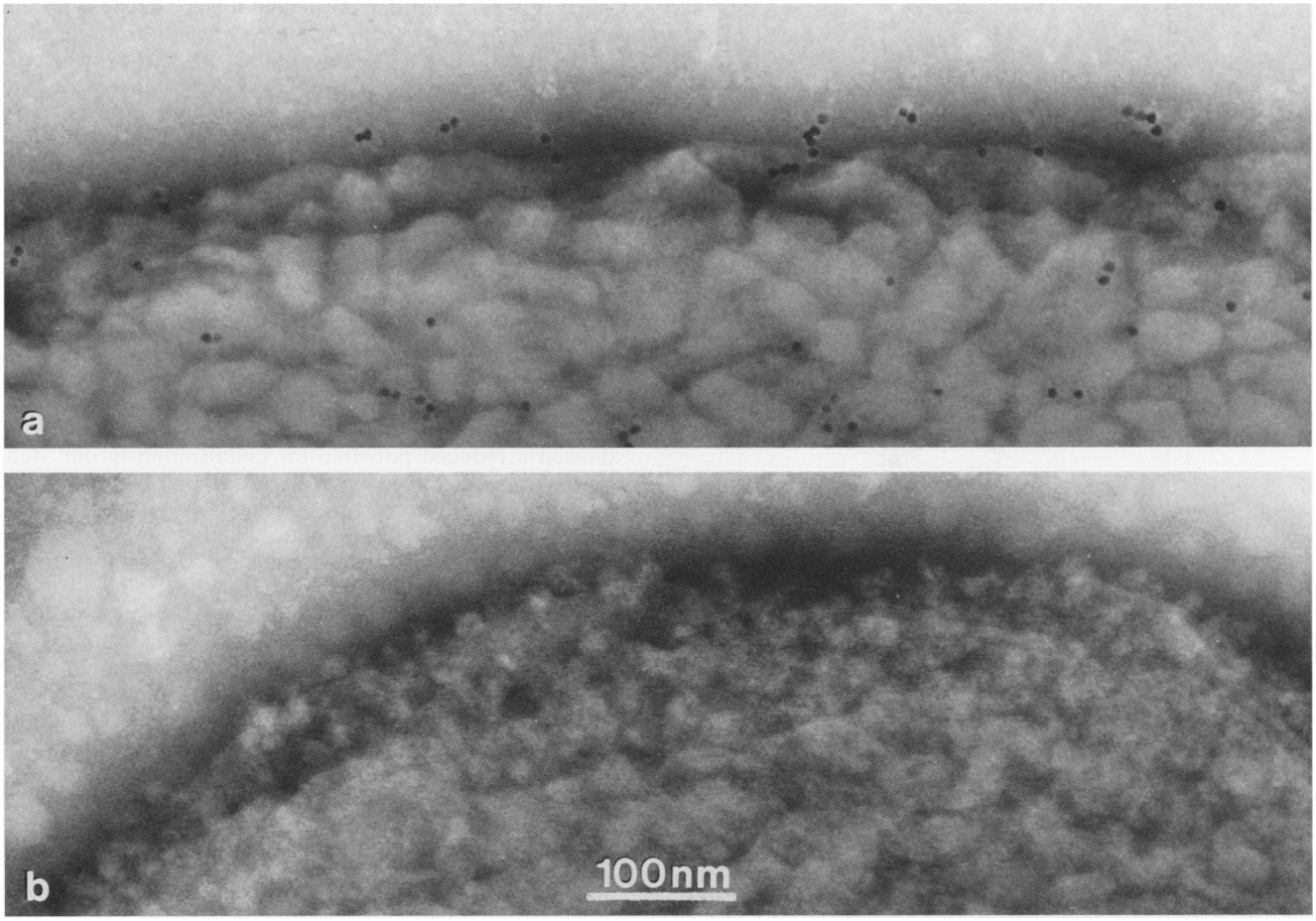

FIG. 2. Electron micrographs showing two insertion mutants of *Y. pseudotuberculosis* incubated with a monclonal antibody to YOP1 and a 5-nm gold probe. The specimens are negatively stained with 0.25% phosphotungstic acid (pH 7.0). (a) Labeling of the fibrillated strain YPIII(pIB71). (b) No labeling of strain YPIII(pIB102), which is unable to express fibrillae. Magnification, ×200,000.

gation. The remaining strains of both *Y. pseudotuberculosis* and *Y. entrerocolitica* were only sporadically recovered in low numbers from liver or spleen. No deaths were recorded during the 4 days encompassed by this study.

DISCUSSION

This work completes a cycle of correlations. Previous studies have demonstrated a correlation between YOP1 and autoagglutination (1, 29), between presence of fibrillae and autoagglutination (18), between presence of fibrillae and expression of both autoagglutination and an outer membrane protein probably identical to YOP1, and between expression of fibrillae and mannose-resistant hemagglutination (17). The present study employed six insertion mutants of *Y. pseudotuberculosis* and *Y. enterocolitica*, previously constructed and described in the literature (1, 4), to demonstrate an absolute correlation between the ability to express YOP1 and the phenomena of autoagglutination, hemagglutination, and presence of surface fibrillae (Table 1). Although it cannot completely be ruled out that the insertions concerned have a polar effect on adjacent genes, our results provide strong evidence that these attributes all reflect the same determinant(s). The phenotypic expression of these properties seems to be regulated in concert by a mechanism distinct from that responsible for Ca^{2+}-dependent growth and production of the low-molecular-weight YOPs.

In this study, the presence of YOP1 in the fibrillar matrix was established by immunoelectron microscopy (Table 1, Fig. 2). This finding is further supported by the results of Martinez (21) and Zaleska et al. (35). Martinez showed that the YOPs are, at least partly, exposed on the bacterial surface, as indicated by radioiodination and susceptibility to exogenously added proteases. The presence of correlated surface structures might thus be expected. Zaleska et al. (35) reported that a plasmid-containing variant of *Y. enterocolitica* serogroup O:3 expressed "tacklike" projections covering the cell surface when cultivated at 37°C. It seems likely that these surface projections represent condensations of the fibrillar matrix to form occasional electron-dense tacks as a result of the preparative procedure. In accordance with our results, the tacklike projections were associated with autoagglutination and expression of one major outer membrane protein of approximately 200 kilodaltons. Like YOP1, the 200-kilodalton protein was a polymer composed of subunits of about 50 kilodaltons (29), indicating that these proteins are identical. Furthermore, the tacks were specifically labeled with antibodies to the protein subunits, strongly indicating that these surface projections contained YOP1 (35).

It has been shown that sera from patients and animals

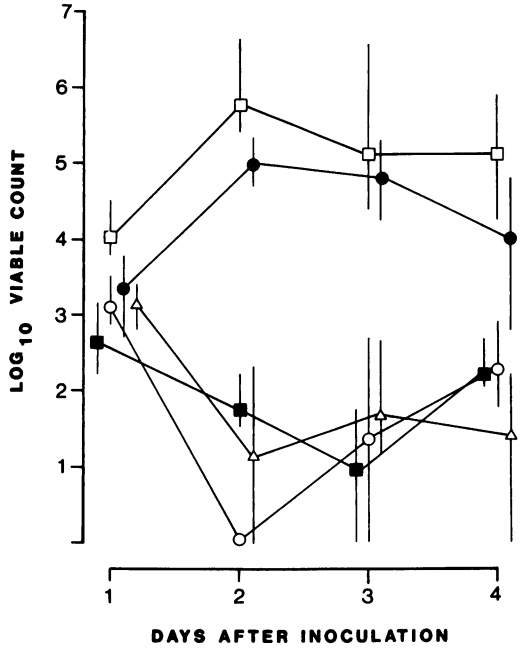

FIG. 3. Isolation of five *Y. pseudotuberculosis* strains from the ileal contents of mice after orogastric inoculation. Symbols: □, YPIII(pIB1); ■, YPIII; ○, YPIII(pIB71); ●, YPIII(pIB102); △, YPIII(pIB101). Mean values for four mice are indicated. Vertical bars represent total ranges.

undergoing infection with either *Y. pseudotuberculosis* or *Y. enterocolitica* possess antibodies directed against YOPs, including YOP1 (3, 21). This observation indicates that the surface fibrillae are expressed during infection, and it encourages speculations as to whether they are involved in the pathogenic process. The present results suggest that the fibrillae impart some rather striking properties to the bacteria which significantly influence their adhesive potential. The fibrillae seem to represent an adhesin which enables the bacteria to attach to each other (autoagglutination) and to guinea pig erythrocytes (hemagglutination). The correlations established in this work are not sufficient to prove these functions definitely. However, our finding that anti-YOP1 monoclonal antibody caused inhibition of hemagglutination provides more direct evidence for a potential role of YOP1 in adhesion. Lachica et al. (18) demonstrated that the formation of fibrillae was associated with increased cell surface charge and hydrophobicity. The combination of hydrophobic and electrostatic forces is known to play a decisive role in the adherence of bacteria to host cell surfaces. If the fibrillae are expressed in the intestinal lumen before penetration of the epithelial lining, it seems likely that they may contribute to the pathogenic process by promoting adherence to the intestinal mucosa. Indeed, Skurnik and Poikonen (30) found that *Y. enterocolitica* serogroup O:3, cultivated at 22°C, rapidly expressed YOP1 in the intestinal lumen of orogastrically infected rats before penetration of the epithelium. Our present investigation demonstrated that insertional inactivation of the gene coding for YOP1, with resultant loss of the ability to express fibrillae, led to a reduced capability of *Y. enterocolitica* to maintain intestinal colonization of mice challenged orogastrically with 22°C-grown bacteria. Analogous results have been obtained by G. Balligand and G. Cornelis (G. Cornelis, personal communication). However, further investigations are needed to determine whether these findings are attributable to reduced adherence to epithelial cells or whether they reflect an impaired function of other virulence determinants. It is not known whether the yersiniae isolated from intestinal contents are progeny of bacteria adhering to epithelial cells or are derived from those that have invaded the lamina propria (26). If the latter is the case, intestinal colonization, as determined in our study, may reflect survival in the lamina propria rather than adherence to the epithelial lining.

To survive and multiply in the lamina propria, the yersiniae must tolerate the potential bactericidal action of serum components bathing the interstitial spaces (26). Balligand et al. (1) presented genetic evidence which strongly suggested that YOP1 is necessary, though not sufficient, for serum resistance in *Y. enterocolitica*. They reported that W22708(pGB08) and W22708(pGB910), both of which showed a reduced potential for colonization in our study, were serum sensitive, whereas W22708(pYL4), which was capable of effective colonization, was serum resistant. It is, therefore, tempting to speculate whether serum resistance may, at least partly, have contributed to the intestinal colonization observed in the present work.

The mechanism of virulence is known to vary within the genus *Yersinia* (5). For example, the serum resistance of *Y. pseudotuberculosis* and *Y. pestis*, unlike that of *Y. enterocolitica*, is unrelated to expression of YOPs (23). If serum resistance contributes to the intestinal colonization observed, one would expect that insertional inactivation of the YOP1 gene should not drastically reduce the capability of *Y. pseudotuberculosis* to maintain colonization. This was, indeed, found to be the case. In contrast to the results obtained with *Y. enterocolitica*, we failed to establish a correlation between intestinal colonization and the ability to express

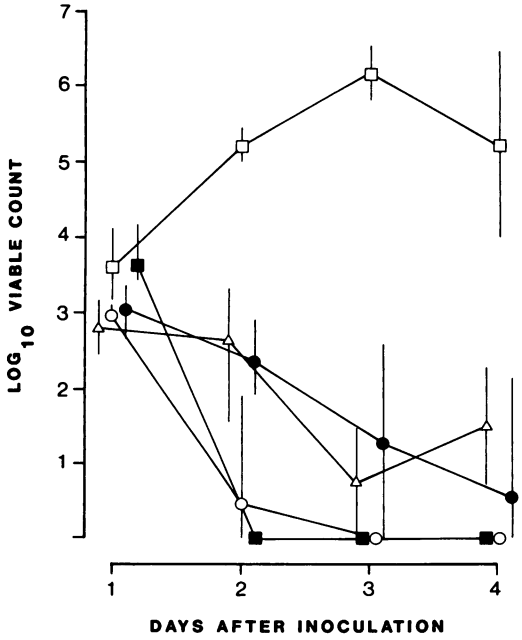

FIG. 4. Isolation of five *Y. enterocolitica* strains from the ileal contents of mice after orogastric inoculation. Symbols: □, W22708 (pYL4); ■, W22708; ○, W22708(pGB51); ●, W22708(pGB910); △, W22708(pGB08). Mean values for four mice are indicated. Vertical bars represent total ranges.

fibrillae and YOP1 in *Y. pseudotuberculosis*. It should be emphasized, however, that the *Y. enterocolitica* and *Y. pseudotuberculosis* mutants might not be completely comparable, since the operons disrupted by the insertions concerned have not been exactly delineated. The insertion mutant YPIII(pIB102), which is unable to express fibrillae and YOP1, colonized with about the same efficiency as its wild-type parent. In accordance with this observation, Bölin and Wolf-Watz (4) found no detectable reduction in the ability of YPIII(pIB102) to kill orally infected mice, thus indicating that the fibrillae are not a major virulence determinant in *Y. pseudotuberculosis*. Our finding that YPIII(pIB102) was isolated from the spleen and liver in greater numbers than YPIII(pIB1) may be looked at in connection with the inability of *Y. pestis* to express YOP1 (3, 31–33). It might be speculated that expression of YOP1 is disadvantageous for bacteria causing a systemic infection.

In both *Y. enterocolitica* and *Y. pseudotuberculosis*, insertional inactivation of the genes coding for Ca^{2+} dependency reduced the number of bacteria recovered from the ileal lumen, regardless of the ability to express fibrillae. This observation is consistent with previous studies indicating that there is a genetic link between Ca^{2+} dependency and virulence in *Y. pestis* and *Y. pseudotuberculosis* (4, 24). In conclusion, the ability to express both Ca^{2+} dependency and surface fibrillae seems to be required for optimal expression of virulence in *Y. enterocolitica*, whereas in *Y. pseudotuberculosis* no significant role of the fibrillae has so far been established.

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LITERATURE CITED

1. **Balligand, G., Y. Laroche, and G. Cornelis.** 1985. Genetic analysis of virulence plasmid from a serogroup 9 *Yersinia enterocolitica* strain: role of outer membrane protein P1 in resistance to human serum and autoagglutination. Infect. Immun. **48**:782–786.
2. **Bölin, I., L. Norlander, and H. Wolf-Watz.** 1982. Temperature-inducible outer membrane protein of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* is associated with the virulence plasmid. Infect. Immun. **37**:506–512.
3. **Bölin, I., D. A. Portnoy, and H. Wolf-Watz.** 1985. Expression of the temperature-inducible outer membrane proteins of yersiniae. Infect. Immun. **48**:234–240.
4. **Bölin, I., and H. Wolf-Watz.** 1984. Molecular cloning of the temperature-inducible outer membrane protein 1 of *Yersinia pseudotuberculosis*. Infect. Immun. **43**:72–78.
5. **Brubaker, R. R.** 1979. Expression of virulence in yersiniae, p. 168–171. *In* D. Schlessinger (ed.), Microbiology—1979. American Society for Microbiology, Washington, D.C.
6. **Brubaker, R. R., and M. J. Surgalla.** 1964. The effect of Ca^{++} and Mg^{++} on lysis, growth, and production of virulence antigens by *Pasteurella pestis*. J. Infect. Dis. **114**:13–25.
7. **Burrows, T. W., and G. A. Bacon.** 1956. The basis of virulence in *Pasteurella pestis*: an antigen determining virulence. Br. J. Exp. Pathol. **37**:481–493.
8. **Burrows, T. W., and G. A. Bacon.** 1960. V and W antigens in strains of *Pasteurella pseudotuberculosis*. Br. J. Exp. Pathol. **39**:278–291.
9. **Carter, P. B., R. J. Zahorchak, and R. R. Brubaker.** 1980. Plague virulence antigens from *Yersinia enterocolitica*. Infect. Immun. **28**:638–640.
10. **Cornelis, G., M. P. Sory, Y. Laroche, and I. Derclaye.** 1986. Genetic analysis of the plasmid region controlling virulence in *Yersinia enterocolitica* O:9 by mini-Mu insertions and *lac* gene fusions. Microbiol. Pathogen. **1**:349–359.
11. **Davis, J. M., J. E. Pennington, A.-M. Kublern, and J.-F. Conscience.** 1982. A simple, single-step technique for selecting and cloning hybridomas for the production of monoclonal antibodies. J. Immunol. Methods **50**:161–171.
12. **Ferber, D. M., and R. R. Brubaker.** 1981. Plasmids in *Yersinia pestis*. Infect. Immun. **31**:839–841.
13. **Gemski, P., J. R. Lazere, and T. Casey.** 1980. Plasmid associated with pathogenicity and calcium dependency of *Yersinia enterocolitica*. Infect. Immun. **27**:682–685.
14. **Gemski, P., J. R. Lazere, T. Casey, and J. A. Wohlhieter.** 1980. Presence of a virulence-associated plasmid in *Yersinia pseudotuberculosis*. Infect. Immun. **28**:1044–1047.
15. **Goguen, J. D., J. Yother, and S. C. Straley.** 1984. Genetic analysis of the low calcium response in *Yersinia pestis* Mu d1(Ap *lac*) insertion mutants. J. Bacteriol. **160**:842–848.
16. **Kapperud, G., and J. Lassen.** 1983. Relationship of virulence-associated autoagglutination to hemagglutinin production in *Yersinia enterocolitica* and *Yersinia enterocolitica*-like bacteria. Infect. Immun. **42**:163–169.
17. **Kapperud, G., E. Namork, and H.-J. Skarpeid.** 1985. Temperature-inducible surface fibrillae associated with the virulence plasmid of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. Infect. Immun. **47**:561–566.
18. **Lachica, R. V., D. L. Zink, and W. R. Ferris.** 1984. Association of fibril structure formation with cell surface properties of *Yersinia enterocolitica*. Infect. Immun. **46**:272–275.
19. **Laird, W. J., and D. C. Cavanaugh.** 1980. Correlation of autoagglutination and virulence of yersiniae. J. Clin. Microbiol. **11**:430–432.
20. **MacLagan, R. M., and D. C. Old.** 1980. Haemagglutinins and fimbriae in different serotypes and biotypes of *Yersinia enterocolitica*. J. Appl. Bacteriol. **49**:353–360.
21. **Martinez, R. J.** 1983. Plasmid-mediated and temperature-regulated surface proprties of *Yersinia enterocolitica*. Infect. Immun. **41**:921–930.
22. **Namork, E., and B. V. Johansen.** 1982. Surface activation of carbon film supports for biological electron microscopy. Ultramicroscopy **7**:321–330.
23. **Perry, R. D., and R. R. Brubaker.** 1983. Vwa^+ phenotype of *Yersinia enterocolitica*. Infect. Immun. **40**:166–171.
24. **Portnoy, D. A., H. F. Blank, D. T. Kingsbury, and S. Falkow.** 1983. Genetic analysis of essential plasmid determinants of pathogenicity in *Yersinia pestis*. J. Infect. Dis. **148**:297–304.
25. **Portnoy, D. A., and S. Falkow.** 1981. Virulence-associated plasmids from *Yersinia enterocolitica* and *Yersinia pestis*. J. Bacteriol. **148**:877–883.
26. **Portnoy, D. A., and R. J. Martinez.** 1985. Role of a plasmid in the pathogenicity of *Yersinia* species. Curr. Top. Microbiol. Immunol. **118**:29–51.
27. **Portnoy, D. A., H. Wolf-Watz, I. Bolin, A. B. Beeder, and S. Falkow.** 1984. Characterization of common virulence plasmids in *Yersinia* species and their role in the expression of outer membrane proteins. Infect. Immun. **43**:108–114.
28. **Skurnik, M.** 1985. Expression of antigens encoded by the virulence plasmid of *Yersinia enterocolitica* under different growth conditions. Infect. Immun. **47**:183–190.
29. **Skurnik, M., I. Bölin, H. Heikkinen, S. Piha, and H. Wolf-Watz.** 1984. Virulence plasmid-associated autoagglutination in *Yersinia* spp. J. Bacteriol. **158**:1033–1036.
30. **Skurnik, M., and K. Poikonen.** 1986. Experimental intestinal infection of rats by *Yersinia enterocolitica* O:3. A follow-up study with specific antibodies to the virulence plasmid specified antigens. Scand. J. Infect. Dis. **18**:355–364.
31. **Straley, S. C., and W. S. Bowmer.** 1986. Virulence genes regulated at the transcriptional level by Ca^{2+} in *Yersinia pestis* include structural genes for outer membrane proteins. Infect. Immun. **51**:445–454.
32. **Wolf-Watz, H., I. Bölin, Å. Forsberg, and L. Norlander.** 1986. Possible determinants of virulence of yersiniae, p. 329–334. *In* D. L. Lark (ed.), Protein-carbohydrate interactions in biological

systems. Molecular biology of pathogenic microorganisms. Academic Press Inc. (London), Ltd., London.
33. **Wolf-Watz, H., D. A. Portnoy, I. Bölin, and S. Falkow.** 1985. Transfer of the virulence plasmid of *Yersinia pestis* to *Yersinia pseudotuberculosis*. Infect. Immun. **48:**241–243.
34. **Yother, J., and J. D. Goguen.** 1985. Isolation and characterization of Ca^{2+}-blind mutants of *Yersinia pestis*. J. Bacteriol. **164:**704–711.
35. **Zaleska, M., K. Lounatmaa, M. Nurminen, E. Wahlström, and P. H. Mäkelä.** 1985. A novel virulence-associated cell surface structure composed of 47-kd protein subunits in *Yersinia enterocolitica*. EMBO J. **4:**1013–1018.