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Inhibition of Phagocytosis in *Yersinia pseudotuberculosis*: a Virulence Plasmid-Encoded Ability Involving the Yop2b Protein

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Virulence plasmid-containing cells of *Yersinia pseudotuberculosis* had the ability to inhibit phagocytosis by mouse peritoneal macrophages cultured in vitro, but cells of its plasmid-cured derivative did not. Inhibition was most pronounced when the pathogen was incubated under Ca^{2+}-deficient conditions, which allowed a high level of expression of outer membrane proteins (Yops). The addition of 2.5 mM Ca^{2+} to the growth medium reduced the degree of inhibition by the pathogen, but it was still significantly higher than that of the plasmid-cured strain. An avirulent mutant strain, from which the entire *yopH* gene was deleted, was impaired in its phagocytosis inhibition ability. This mutant could be *trans*-complemented by the *yopH*$^+$ gene back to the wild-type phenotype with respect to virulence, as well as the ability to inhibit phagocytosis, demonstrating that the ability to inhibit phagocytosis is an important virulence function. The mutant strain was still cytotoxic for HeLa cells, indicating that inhibition of phagocytosis can be genetically separated from the ability to cause a cytotoxic effect.

Virulent strains of *Yersinia pseudotuberculosis* possess a virulence plasmid (2, 4) which is closely related to similar plasmids found in *Yersinia pestis* and *Yersinia enterocolitica* (1, 20, 22). These plasmids are involved in Ca^{2+}-dependent behavior and expression of seven temperature-inducible outer membrane proteins (Yops) (3, 9, 20, 22). The expression of Yops is regulated at the transcriptional level by temperature and the concentration of calcium in the growth medium (3, 5, 22, 23). At 37°C, in the absence of calcium, Yops are expressed at high levels, and they can be recovered both from the outer membrane fraction and from the culture supernatant (1, 9, 12, 19). When the bacteria are incubated at the same temperature in a medium exceeding 2.5 mM calcium, the synthesis of Yops is lowered, and they are only recovered in the outer membrane fraction (1, 9). At 26°C no expression has been observed (1, 9, 19). The structural genes of Yops have been cloned from plasmid pIB1 of *Y. pseudotuberculosis* (9). The DNA sequences of the structural genes of the Yop2b (*yopH*) and Yop5 (*yopE*) proteins were recently determined. By directed mutagenesis it has been shown that they encode essential virulence determinants (5, 10).

These two genes are also conserved in the three species of *Yersinia* given above, although the virulence plasmid of *Y. enterocolitica* is rearranged (1, 20, 22). This fact supports a common role for the Yop2b and Yop5 proteins in the virulence of yersiniae.

Y. pestis and *Y. enterocolitica* have the ability to inhibit phagocytosis and induce a cytotoxic effect on cultured cells (6, 7, 11, 16, 19, 21). These properties have been correlated with the presence of a common virulence plasmid. However, the significance of phagocytosis inhibition and the actual role of the common gene products in the process of virulence have not been revealed. Here we show that a virulent strain of *Y. pseudotuberculosis* exhibits a property that allows it to inhibit phagocytosis by mouse macrophages. This property is also encoded by the *Y. pestis* plasmid pYV019. The inhibition was most pronounced when the bacteria were preincubated under conditions that allowed high-level expression of Yops, i.e., without Ca^{2+}. One specific mutant strain, YPIII (pIB29), that was unable to express the Yop2b protein showed a reduced ability to inhibit phagocytosis and was avirulent. The ability to inhibit phagocytosis could be complemented in *trans* by the introduction of plasmid pYOP21 carrying the *yopH*$^+$ gene. This latter construct was also virulent.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. These strains were derivatives of wild-type YPIII(pIB1) of *Y. pseudotuberculosis* serotype III. The *Y. pseudotuberculosis* strains were grown in a rich medium, as described by Martinez (17), that was either supplemented with 2.5 mM $CaCl_2$ or deprived of Ca^{2+} by the addition of 20 mM sodium oxalate. The cultures were grown overnight at 26°C on a rotary shaker. The overnight cultures were diluted 1/20 in the same medium, and incubation was continued for about 1 h at 26°C. The cell cultures were divided into two parts, and incubation was continued at 26 or 37°C.

After 2 h of incubation the exponentially growing cells were put on ice and the bacterial density was determined by measuring the optical density at 550 nm in a spectrophotometer (DU-7; Beckman Instruments, Inc., Fullerton, Calif.). The cells were diluted in sterile 0.9% NaCl to a cell concentration of about 10^7 bacteria per ml.

Cultivation of resident mouse peritoneal macrophages. Resident mouse peritoneal macrophages were obtained from male Swiss albino mice. The mice were killed by carbon dioxide suffocation, and the peritoneal cells were harvested by lavage with 2 ml of cold Dulbeccos modified Eagle medium (DMEM; Flow Laboratories, Inc., McLean, Va.) containing 100 U of penicillin per ml. The cells were washed twice and finally diluted to a concentration of 0.8×10^6 cells per ml in DMEM containing 10% heat-inactivated (56°C for 30 min) fetal bovine serum and 100 U of penicillin per ml. A total of 1 ml of this cell suspension was seeded per well in a tissue culture multiwell plate (24 wells per plate; 2.0 cm^2/well; Linbro; Flow Laboratories) containing sterile cover

TABLE 1. Genotypes of the different *Y. pseudotuberculosis* strains used in this study

Strain	Relevant genotype	Reference
YPIII(pIB1)	Wild type	2
YPIII	Plasmid$^-$	2
YPIII-P4	pYV019::Tn*5*(d)	23
YPIII(pIB29)	*yopA yopH*	5
YPIII(pIB29, pYOP21)	*yopA yopH*$^+$	5
YPIII(pIB102)	*yopA yopH*$^+$	4

slips (diameter, 13 mm). After incubation for about 2 h at 37°C in a humidified atmosphere of 5% CO_2 in air, the cell cultures were washed with warm DMEM to remove nonadherent cells. Adherent cells were incubated for 18 h in DMEM containing 10% heat-inactivated fetal bovine serum and penicillin. The cell cultures were washed again, and the incubation was continued for another 24 h in DMEM containing 10% heat-inactivated fetal bovine serum and 100 U of penicillin per ml. On day 3 the cells were again washed, covered with prewarmed culture medium without penicillin, and incubated for 1 to 2 h before infection of macrophages with bacteria.

Infection of macrophages with bacteria. The macrophage cell cultures were infected with 0.1 ml of the diluted bacterial suspension (10^7/ml). To facilitate contact between bacteria and cells, the infected cells were centrifuged for 5 min at 400 × *g*. This resulted in the infection of about 80% of the macrophages with about 5 bacteria per macrophage. Infected cell cultures were incubated at 37°C; 30 min after infection the cell cultures were washed twice with 1 ml of DMEM and then prepared for the double-immunofluorescent antibody test.

Discrimination between intra- and extracellularly located bacteria. To distinguish between intra- and extracellularly located bacteria, the double-immunofluorescent antibody test described by Heesemann and Laufs (14) was used. Briefly, 30 min after infection the cell cultures were washed and the cover slips were overlaid with anti-*Yersinia* rabbit antiserum diluted in phosphate-buffered saline (PBS) and incubated for 30 min on ice. The antiserum was prepared as described by Doyle et al. (8) by using strain YPIII(pIB1) as the antigen. The antiserum reacted to the same extent with strains grown at 26°C as it did with those grown at 37°C. Excess antiserum was washed off by dipping the cover slips 4 times in PBS. After the cover slips were air dried, the monolayers were fixed in methanol at −15°C for 1 to 2 min. The cover slips were again air dried and subsequently overlaid with trirhodamin isothiocyanate-labeled goat anti-rabbit immunoglobulin G (IgG) serum (Sigma Chemical Co., St. Louis, Mo.) diluted 1:25 in PBS and incubated for 20 min at 37°C to stain extracellularly located bacteria. After the monolayers were washed by dipping them 3 times in PBS, they were treated a second time with anti-*Yersinia* rabbit antiserum for 1 h at 37°C. During this step, antibodies were able to react with intracellular bacteria. After the monolayers were washed, fluorescein isothiocyanate-labeled sheep anti-rabbit IgG serum (The National Bacteriological Laboratory, Stockholm, Sweden) diluted 1:20 in PBS was added to the monolayers and incubated for 30 min at 37°C. The cells were again rinsed with PBS and then mounted in glycerol-PBS (9:1; vol/vol) under a glass cover slip. The specimens were examined with a fluorescent microscope (Orthoplan; Leitz/Opto-Metric Div. of E. Leitz Inc., Rockleigh, N.J.) with epifluorescence illumination and a ×63 oil immersion lens (final magnification, ×630). In each experiment duplicate samples were analyzed and at least 100 macrophages were counted from randomly selected fields without preview. The number of extracellular bacteria was counted with the trirhodamin isothiocyanate-filter system, and the total number of cell-associated (intracellular plus extracellular) bacteria was determined with the fluorescein isothiocyanate-filter system.

RESULTS

When the virulent strain of *Y. pseudotuberculosis*, YPIII (pIB1), and its plasmidfree isogenic derivative, YPIII, grown at 26°C were added to cultured mouse peritoneal macrophages, both strains were phagocytosed at the same level; i.e., at least 90% of the cell-associated bacteria were found to be intracellular (Fig. 1 and Table 2). However, when the virulent strain was shifted from growth at 26°C to growth at 37°C for 2 h in a Ca^{2+}-deficient medium, about 34% of the cell-associated bacteria were found to be intracellular, while the remaining 66% were found at the surface of the macrophages (Fig. 1 and Table 2). The addition of 2.5 mM Ca^{2+} to the growth medium reduced the frequency of extracellular bacteria to about 40% (Table 2). Strain YPIII containing the virulence plasmid pYV019 of *Y. pestis* EV76 was also tested. Identical results to those described above were obtained. When the avirulent strain YPIII was tested, we found that above 90% of the cell-associated bacteria were phagocytosed, irrespective of the preincubation conditions (Table 2). In addition, in all experiments described above the level of total macrophage-associated bacteria was about the same. About 80% of the macrophages were infected with a mean value of 5 bacteria per cell.

Previously, mutant YPIII (pIB29) was constructed from which the entire *yopH* gene was deleted (5). This mutant was found to be avirulent. Introduction of the hybrid plasmid pYOP21 into this strain restored the virulence property. Analysis of the gene products of these two strains revealed that YPIII(pIB29) was unable to express the Yop2b polypeptide, in contrast to YPIII(pIB29, pYOP21), which was able to do so (5). When these strains were analyzed, it was observed that YPIII(pIB29) showed a reduced ability to inhibit phagocytosis, in contrast to YPIII(pIB29, pYOP21), which showed the wild-type level (Table 3). One additional mutant, YPIII(pIB102), which was impaired in the expression of the Yop1 protein, was also tested. This strain showed the same level of inhibition as its corresponding wild-type strain (Table 3).

We have recently shown that the virulent strain YPIII(pIB1) also has the ability to induce a cytotoxic response on cultured HeLa cells (21). In this study we noticed a similar effect on cultured macrophages. Therefore, the cytotoxic effect and the ability to inhibit phagocytosis could be a result of the same molecular mechanism. When the cytotoxic effect on cultured macrophages and HeLa cells was recorded, it was observed that strain YPIII(pIB29) induced a cytotoxic response on cultured cells (Table 3), indicating that the ability to resist phagocytosis can be genetically separated from the ability to induce a cytotoxic effect.

DISCUSSION

We showed here that the ability to inhibit phagocytosis is caused by a plasmid-encoded entity and that the Yop2b protein, which is encoded by the *yopH* gene, is involved in a

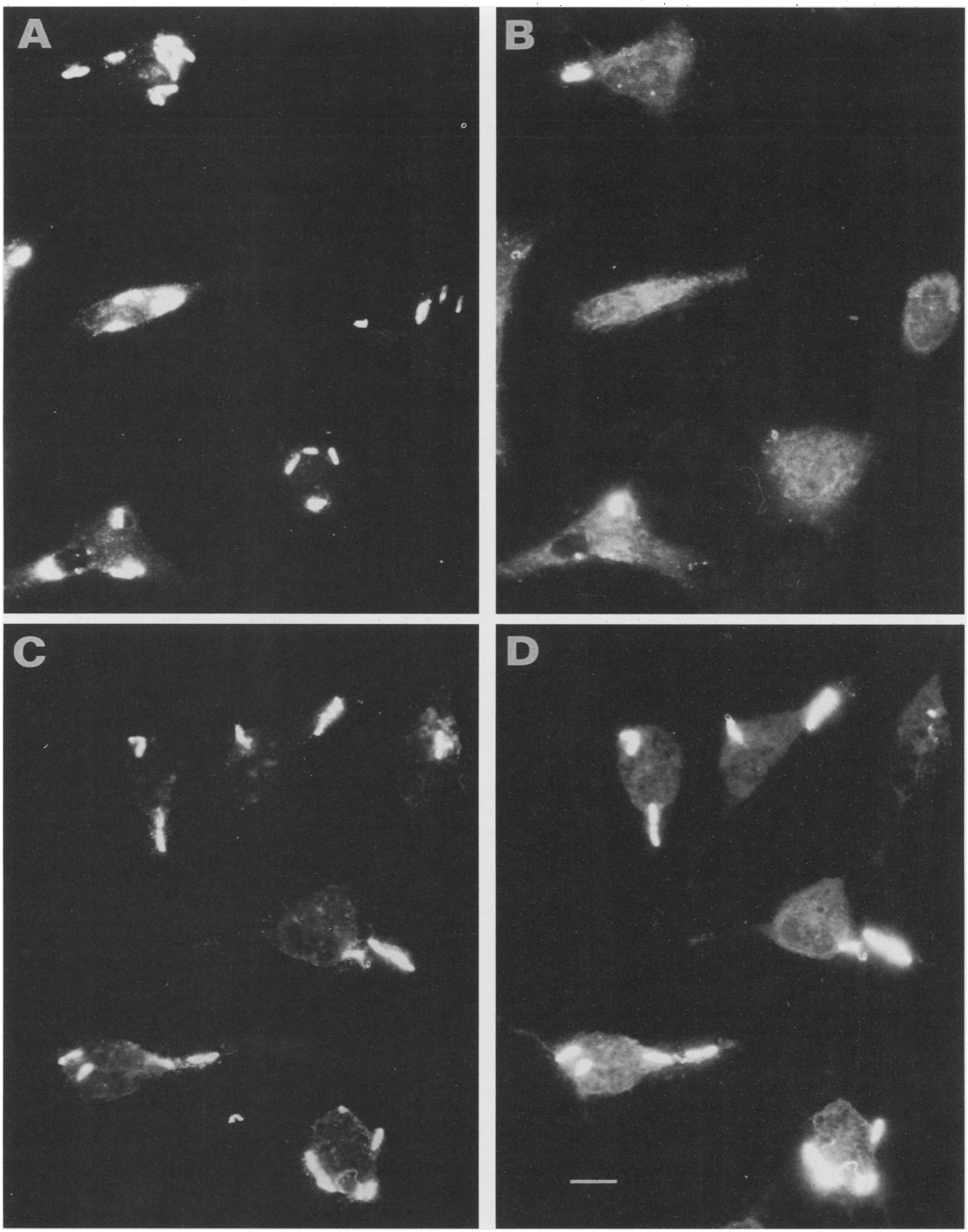

FIG. 1. Inhibition of phagocytosis of the virulent strain YPIII(pIB1). Strain YPIII(pIB1) was grown at 26 or 37°C in a rich medium that was depleted of Ca^{2+}. The bacteria were diluted in sterile saline and added to cultured mouse peritoneal macrophages to give a final concentration of about 10 bacteria per macrophage. The infected cultures were incubated for 30 min at 37°C. Thereafter, the cultures were prepared for the immunofluorescence assay. Total cell-associated bacteria (intracellular and extracellular) were visualized by the use of the green immunofluorescence dye fluorescein isothiocyanate, while the red dye trirhodamin isothiocyanate only visualized extracellular bacteria. (A) Total macrophage-associated bacteria pregrown at 26°C. (B) Extracellular macrophage-associated bacteria pregrown at 26°C. (C) Total macrophage-associated bacteria pregrown at 37°C. (D) Extracellular macrophage-associated bacteria pregrown at 37°C. Phagocytosis is illustrated in panels A and B; inhibition of phagocytosis is illustrated in panels C and D. Bar, 7 μm.

TABLE 2. Inhibition of phagocytosis by strains YPIII(pIB1) and YPIII

Strain	Ca^{2+} concn (mM)	% Extracellular bacteria of total cell-associated bacteria at[a]:	
		26°C	37°C
YPIII(pIB1)	<0.1	5 ± 1 (5)	66 ± 5* (6)
YPIII(pIB1)	2.5	5 ± 2 (3)	40 ± 7** (4)
YPIII	<0.1	6 ± 1 (2)	6 ± 2 (5)
YPIII	2.5	6 ± 1 (2)	5 ± 2 (3)

[a] The different strains were preincubated at 26 or 37°C for 2 h in a rich medium, with or without calcium. The bacteria were added to cultured macrophages, and the number of extracellular macrophage-associated bacteria after 30 min of incubation was determined by the method described in the legend to Fig. 1. The data presented are mean values ± standard error of the mean for the number of experiments indicated in parentheses. *, $P < 0.001$; **, $P < 0.01$; significant differences were tested by Student's t test.

molecular process that leads to the inhibition of phagocytosis. Results of our earlier studies (5, 10) indicated that two of the plasmid pIB1-encoded, temperature-inducible, Ca^{2+}-regulated proteins of *Y. pseudotuberculosis*, Yop2b and Yop5, are indispensable virulence determinants. Thus, the ability to resist phagocytosis is a virulence attribute.

This was demonstrated by showing that the *yopH* deletion mutant YPIII(pIB29) had a reduced ability to inhibit phagocytosis, while the *trans*-complemented strain YPIII(pIB29, pYOP21) regained the ability to inhibit phagocytosis to the same extent as the wild-type strain. However, the level of inhibition of the mutant strain was about 20%, irrespective of the incubation conditions, while the corresponding value was 5% for the plasmid-cured strain YPIII, indicating that the intermediate level of inhibition is dependent on the presence of the plasmid. Since Yops are coregulated by a common activator and repressor system (5, 10), the deletion of the *yopH* gene could cause a change in the expression of the other plasmid-encoded gene products. This could indicate that there are components other than Yop2b that are involved in phagocytosis inhibition, for example, the cytotoxin (21).

Although the cytotoxic effect was genetically separated from the full inhibition of phagocytosis, we cannot exclude the possibility that the Yop2b protein is one component in a complex system that involves the cytotoxin or other plasmid-encoded products which act in concert to give an optimal inhibition of macrophage activity.

It has been shown that the presence of the Yop1 polypeptide mediates adherence to but blocks the invasion of *Y. enterocolitica* into HEp-2 cells, and the question of whether the Yop1 protein could inhibit phagocytosis of *Y. enterocolitica* by "professional phagocytes" was raised (13). We showed here that the absence of Yop1 does not affect the ability of strain YPIII(pIB102) to inhibit phagocytosis of macrophages. Thus, the molecular mechanism leading to phagocytosis inhibition by mouse macrophages can be discriminated from the Yop1-promoted inhibition of invasion of HEp-2 cells. It has been suggested that the entry process of HEp-2 cells is triggered by a receptor-mediated endocytotic pathway (15). It is therefore possible that the Yop1 polypeptide promotes binding to another receptor, which does not trigger endocytosis.

Y. pestis and *Y. enterocolitica* exhibited the capability of inhibiting phagocytosis, and this function can be coupled to the common virulence plasmid (6, 7, 16). Thus, all three species of pathogenic *Yersinia* discussed here have in common the plasmid-encoded ability to prevent phagocytosis. Because the *yopH* gene is conserved among all virulent yersiniae, it is reasonable to suggest that the Yop2b protein is of fundamental importance in the virulence, as well as in the ability to prevent phagocytosis, of all three species. It remains to be established, however, whether it is the membrane-bound form, the secreted form, or both that are involved in this process. The surface location and the basic nature of the Yop2b protein (1, 9) could possibly generate a charged surface which per se could be sufficient to explain the mechanism of phagocytosis inhibition, in accordance with the suggestions made by Öhman et al. (18), who presented data that indicate that there is a correlation between a charged bacterial surface and the property of phagocytosis inhibition. Although the amount of the Yop2b protein was substantially reduced when the pathogen was grown in the presence of Ca^{2+}, the inhibition of phagocytosis was still at a high level. Moreover, under these conditions the protein is mainly recovered in the membrane-bound form (1, 9). Since the ability to resist phagocytosis is an extracellular event, the pathogen most likely must exert this property in a Ca^{2+}-containing milieu. Therefore, we favor the possibility that during infection the Yop2b protein is expressed in an extracellular Ca^{2+}-containing environment in which the protein is expressed at a low level in a membrane-bound form.

The question remains as to whether the Yop2b protein is an integral component of a complex system that mediates inhibition of phagocytosis. To answer this question, new mutants that carry mutations in two or more plasmid-encoded genes must be constructed. Such studies are in progress in our laboratory.

TABLE 3. Inhibition of phagocytosis by strains YPIII(pIB29), YPIII(pIB29, pYOP21), and YPIII(pIB102)

Strain	% Extracellular bacteria of total cell-associated bacteria at[a]:		Virulence[b]
	26°C	37°C	
YPIII(pIB29)	17 ± 2 (5)	23 ± 3 (5)	Avirulent
YPIII(pIB29, pYOP21)	10 ± 1 (2)	63 ± 4 (5)	Virulent
YPIII(pIB102)	8 ± 3 (3)	58 ± 7 (3)	Virulent

[a] The strains were grown in a medium that lacked calcium (see legend to Fig. 1 and footnote *a* of Table 2 for experimental details). Cytotoxicity was determined as described previously (21). A cytotoxic effect was observed in all three strains.

[b] Virulence data were from previous reports (4, 5).

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