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ABSTRACT

Pathogenic Yersinia enterocolitica cells do not induce the chemiluminescence response of human polymorphonuclear leukocytes (PMNs). We tested the chemiluminescence response to Y. enterocolitica mutants affected in the known pYV-encoded factors. We did not detect any influence of the Yops in this phenomenon. By contrast, the presence of YadA correlated with a lack of chemiluminescence. The expression of YadA at the bacterial surface also reduced the phagocytosis by PMNs. Finally, we measured the survival of Y. enterocolitica cells confronted with PMNs by the classical plating method and by a new luminometry assay. We observed that YadA+ bacteria were not killed, while YadA- bacteria were killed. We conclude that the presence of YadA at the surface of Y. enterocolitica cells prevents phagocytosis and killing by PMNs. This conclusion is in good agreement with our recent observation that YadA protects Y. enterocolitica from opsonization by C3b.

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Role of YadA in Resistance of Yersinia enterocolitica to Phagocytosis by Human Polymorphonuclear Leukocytes

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Pathogenic Yersinia enterocolitica cells do not induce the chemiluminescence response of human polymorphonuclear leukocytes (PMNs). We tested the chemiluminescence response to Y. enterocolitica mutants affected in the known pYV-encoded factors. We did not detect any influence of the Yops in this phenomenon. By contrast, the presence of YadA correlated with a lack of chemiluminescence. The expression of YadA at the bacterial surface also reduced the phagocytosis by PMNs. Finally, we measured the survival of Y. enterocolitica cells confronted with PMNs by the classical plating method and by a new luminometry assay. We observed that YadA− bacteria were not killed, while YadA+ bacteria were killed. We conclude that the presence of YadA at the surface of Y. enterocolitica cells prevents phagocytosis and killing by PMNs. This conclusion is in good agreement with our recent observation that YadA protects Y. enterocolitica from opsonization by C3b.

Yersinia enterocolitica cells are enterobacteria frequently involved in human enterocolitis. They have a remarkable capacity to invade the Peyer's patches and to resist the nonspecific defense of the host, i.e., killing by complement (2) and phagocytosis by polymorphonuclear leukocytes (PMNs) (27) or by macrophages (39). This capacity essentially depends on the presence of a 70-kb virulence plasmid called pYV. The pYV plasmid specifies several thermodependent properties, including the secretion of 11 plasmid-encoded proteins, called Yops, and the production of at least two outer membrane proteins, called YadA and YlpA (for reviews, see references 5 and 15). The secretion of Yops involves a machinery of a new type encoded by (at least) loci virA (lcrA) and virC (lcrC) (13, 14, 30–32, 38). A transcriptional activator encoded by gene virF is required for the production not only of the Yops but also of YadA and YlpA (10, 12, 14, 25).

The Yop proteins are important pathogenicity determinants (for a review, see reference 45). For instance, YopH is a phosphotyrosine phosphatase which confers on Yersinia pseudotuberculosis an antiphagocytic activity against macrophages (4, 18, 39). YadA is a polymer composed of four to five subunits with a molecular mass of 45 to 52.5 kDa (41, 43, 48) and forming a tiny fibrillar structure at the surface of Y. enterocolitica (23, 24, 48). In the experimental oral infection of the mouse, YadA contributes to the long-term fecal excretion of bacteria (23). In vitro, YadA plays various roles in relation to its surface localization. It increases the surface hydrophobicity (29) and promotes autoagglutination (41). It confers adherence to epithelial cells (19), to ileal mucus (35), and to the extracellular matrix by binding collagen fibers (17, 40) and fibronectin (47). YadA also inhibits the anti-inflammatory effect of interferon (6). Finally, YadA confers resistance to the bactericidal activity of human serum (2) by promoting the fixation of factor H (9), which leads to the degradation of C3b deposited at the bacterial surface (37). As a consequence, the formation of the membrane attack complex is prevented (37). Although other factors, such as the Ail protein (3, 36) and lipopolysaccharide (42), also play a role in this phenotype, YadA seems to be the major determinant of resistance to complement killing, since YadA-deficient mutants become serum sensitive (2).

When facing human PMNs, Y. enterocolitica strains harboring the pYV plasmid and grown at 37°C induce an unusually low chemiluminescence (CL) response. This lack of burst correlates with an inhibition of phagocytosis (27, 28). In the present study, we analyzed the response of PMNs to several well-characterized pYV mutants (2, 13, 14, 33) to identify the pYV gene(s) involved in this inhibition.

MATERIALS AND METHODS

Bacterial strains and plasmids. Y. enterocolitica W22703 (nalidixic acid resistant) and W22708 (streptomycin resistant) are two restriction mutants (Res− Mod+) of the serotype O:9 strain W227 (11). Y. enterocolitica KNG22703 is a derivative of W22703 which produces luciferase as a result of the insertion of genes luxAB in the chromosomal gene blad (21, 22). The plasmids used in this study are listed in Table 1.

Bacterial growth conditions. For CL and killing experiments, Y. enterocolitica was inoculated at an optical density at 600 nm (OD600) of 0.1 in conical flasks containing 5 ml of brain heart infusion (Difco, Detroit, Mich.) supplemented with 0.4% glucose, 20 mM MgCl2, and 20 mM sodium oxalate (BH-OX) and containing the appropriate antibiotics. The cultures were shaken either for 5 h at room temperature or for 2 h at room temperature and then shifted for 3 h at 37°C.

For phagocytosis experiments, bacteria were inoculated at an OD600 of 0.5 in a conical flask containing 5 ml of BH-OX supplemented with appropriate antibiotics and shaken for 3 h at 37°C. The antibiotics, purchased from Boehringer (Mannheim, Germany), were supplemented at the following final concentrations: nalidixic acid, 35 µg ml−1; gentamicin, 20 µg ml−1; kanamycin, 50 µg ml−1; and tetracycline, 10 µg ml−1.

Serum. Normal human serum (NHS) was purchased from Sigma (St. Louis, Mo.). The absence of anti-Yop antibodies was monitored by immunoblotting as previously described (9).

Preparation of human PMNs. Human PMNs were prepared from peripheral blood obtained from healthy individuals. A 10-ml volume of human venous blood containing heparin (Organon Teknika, Boxtel, Holland) (5 U/ml) was mixed with
TABLE 1. List of plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Characteristic(s)</th>
<th>Reference(s)</th>
</tr>
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<tbody>
<tr>
<td>pBC7</td>
<td>pVYE227/npA::pBC9</td>
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</tr>
<tr>
<td>pBM15</td>
<td>pVYE227/npYopB-MBM15::Tn2507</td>
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<td>33</td>
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<td>pVYE227/npA::pYLA::Tn3</td>
<td>2 and 10</td>
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<tr>
<td>pYE227</td>
<td>Virulence plasmid of Y. enterocolitica W22703</td>
<td>11</td>
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</table>

3 ml of 6% (wt/vol) dextan (Sigma) in saline, and the mixture was allowed to sediment at 37°C during 30 min. The supernatant, consisting of leukocyte-rich plasma, was applied to the upper surface of 5 ml of cold (4°C) lymphocyte separation medium (d, 1.077 ± 0.001) (International Medical, Brussels, Belgium) and centrifuged (1,000 × g at 4°C for 15 min). The pellet was resuspended in 10 ml of hemolysis balanced solution (0.84% NH₂Cl 0.084%, NaHCO₃ 0.043% EDTA), and the solution was centrifuged for 7 min at 150 × g. This second pellet was resuspended in 5 ml of Hanks balanced salt solution (HBSS) (GIBCO, Paisley, Scotland). Cell viability, monitored by trypan blue dye (Sigma) (final concentration, 0.25% exclusion), was greater than 95%. PMNs were counted with a hemocytometer and adjusted to a final concentration of 10⁷ cells per ml of HBSS.

CL assay. Luminol-enhanced CL was measured with a Pico-Lite luminometer (Packard Instrument Co., Downers Grove, Ill.) by using a modification of the procedure described by Lian and Pai (28). A 50-µl volume of a bacterial suspension in HBSS at an OD₅₅₀ of 0.6, 50 µl of luminol (5-amino-2,3-dihydro-1,4-phenathalazine dioxide; Sigma; 10⁻³ M), 50 µl of NHS, and 100 µl of PMNs (10⁸ cells) was mixed in a tube and placed immediately in the luminometer cell, which was preheated at 37°C. The number of counts per minute during about 20 min was recorded (see Fig. 1).

Phagocytosis assays. We slightly modified the microscope assay described by Lian et al. (27). Briefly, 3 × 10⁵ PMNs in 30 µl of HBSS were placed in each well of a 10-well microscope slide (Flow Laboratories, McLean, Va.) and incubated for 1 h at 37°C in 6% CO₂. Bacteria from 1 ml of culture were harvested, washed with phosphate-buffered saline (PBS) (13 mM sodium hydrogenophosphate, 2.8 mM potassium dihydrogenophosphate, 135 mM sodium chloride, [pH 7.4]), and resuspended (OD₅₅₀ of 0.6) in PBS supplemented with 10 mM ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) and 5 mM MgCl₂. The bacterial suspension was first incubated at 37°C for 10 min with 5% (final concentration) NHS and then washed with PBS and resuspended in 1 ml of HBSS. This preosensitive step did not kill serum-sensitive bacteria (survival, more than 90%). The microscope slides containing PMNs were washed with PBS to withdraw the nonadhering cells. A 30-µl volume of the preosensitized bacterial suspension was added to each well, and the wells were incubated for 90 min in a 6% CO₂ atmosphere. Slides were washed with ice-cold PBS, stained for 1 min with acridine orange (0.1% [wt/vol] in PBS), counterstained for 3 min with crystal violet (1% [wt/vol] in PBS), and washed with PBS.

STAINED slides were examined under an epifluorescence microscope (Diapan; Leitz, Wetzlar, Germany) with an oil immersion objective lens. In this assay, intracellular bacteria fluoresce green when they are viable and red when they are nonviable (44). Extracellular bacteria were not visible because of the counterstain. A total of 100 PMNs were counted, and phagocytosis was expressed as the percentage of PMNs containing intracellular bacteria (green or red).

We also measured phagocytosis by a second method based on the flow cytometry of fluorescent bacteria (7). Bacteria were harvested and adjusted to an OD₅₅₀ of 0.6 in carbonate buffer (0.016 M Na₂CO₃, 0.034 M NaHCO₃, [pH 9.5]). Live bacteria were then labeled by the addition of fluorescein isothiocyanate (FITC; Sigma) to a final concentration of 100 µg ml⁻¹ and incubated at 37°C for 30 min. The bacteria were then washed three times with PBS. According to a plating assay, this labeling did not affect the viability of the bacteria. It is also known not to disturb the bacterial surface (34). Labeled bacteria were preopsonized with 5% NHS at 37°C in PBS-E GTA-MgCl₂ as described above. Then, 100 µl of preopsonized bacteria and 200 µl of PMNs suspension (10⁷ cells ml⁻¹ in HBSS) were mixed in a final volume of 1 ml. This mixture was shaken (75 rpm) during 1 h at 37°C and washed twice with ice-cold PBS. The cells were resuspended in 1 ml of PBS, and trypan blue (0.2% final concentration; Sigma) was added in order to extinguish the fluorescence associated with extracellular bacteria (8). The suspension of the PMNs was then applied to a fluorescence-activated cell sorter (FACS) (Epics Elite; Coulter, Hialeah, Fla.). The proportion of fluorescent PMNs was calculated and considered a measure of the percentage of phagocytosis.

Killing assay. We mixed 100 µl of suspension of preopsonized bacteria (OD₅₅₀ of 0.6 in HBSS) in a universal flask with 200 µl of a suspension of PMNs (2 × 10⁷ cells) in a final volume of 1 ml of HBSS. After 1 h of incubation and shaking at 37°C, 200 µl of mixture was harvested and added to 800 µl of ice-cold distilled water in order to stop phagocytosis and to lyse the PMNs. A 100-µl volume of this dilution was plated on tryptic soy agar (GIBCO) supplemented with appropriate antibiotics, and the plates were incubated at 28°C for 48 h. A sample of bacteria was treated in parallel under the same conditions but in the absence of PMNs. The ratio between the number of CFUs counted from samples incubated with PMNs and from samples incubated without PMNs gave the percentage of survival.

The use of strain KNG22703 allowed monitoring of survival also by measurement of the light emitted as a result of the luciferase activity of this strain (22, 26). The assay was performed as described by Kaniga et al. (22). A 250-µl volume of bacterial suspension was placed in a tube containing 50 µl of 0.1% n-decanol (Sigma), and the emitted light was scored for 10 s in the luminometer. The ratio between the number of counts per minute emitted by bacteria that have been exposed to PMNs and by the bacteria that were not exposed to PMNs gave the percentage of survival.

RESULTS

CL of PMNs induced by Y. enterocolitica W22703. We first analyzed the oxidative burst induced by W22703, our wild-type reference strain of Y. enterocolitica serotype O:9. pYV⁺ and pYV⁻ variants of Y. enterocolitica W22703 were grown in BHI-OX either for 5 h at 25°C or for 2 h at 25°C and for 3 h.
at 37°C. They were subsequently engaged in a CL test (1), with PMNs in a ratio of 50 bacteria per PMN in the presence of 20% NHS. The photons emitted in the presence of luminal were counted in a luminometer. Bacteria grown at 25°C induced a strong CL response, irrespective of the presence or absence of the pYV plasmid (Fig. 1A). By contrast, the CL response induced by bacteria grown at 37°C was different according to the presence or the absence of the pYV plasmid: Y. enterocolitica W22703 pYV- induced a significant CL response, while the pYV+ bacteria induced a five- to sevenfold-lower CL response, indicating a lack of oxidative burst.

These results are in agreement with those obtained previously (28, 46) with a Y. enterocolitica strain of serotype O:3 and confirmed that the lack of induction of CL by wild-type Y. enterocolitica correlates with the expression of pYV plasmid-encoded functions at 37°C.

**Role of the Yops in CL inhibition.** Since there was a coincidence between the inhibition of the oxidative burst and the secretion of Yops, both occurring at 37°C and in the absence of Ca²⁺, we monitored the CL response induced by mutants affected in the production of one or several individual Yops. We tested Y. enterocolitica W22703(pGC1256), mutated in yopE; W22703(pGC1152), affected in yopH; W22703 (pGC559), deficient in yopO and yopP; W22703(pBM15), mutated in yopM; W22703(pBM33), deficient in yopQ; and W22703(pBM79), affected in yopB, yopD, and lerV. All of these yop mutants induced the same level of CL as the wild pYV+ strain (Fig. 1B), indicating that none of these Yops was responsible for the inhibition of stimulation of PMNs. Because we could not test mutants that were deficient in YopN and YopR (15), we also tested the pleiotropic virA, virB, virC, and virF mutants. Only the virF mutant induced a significant CL response (Fig. 1C). Since the virA, virB, and virC mutants do not secrete any of the Yops but nevertheless inhibit the CL response, we inferred that neither a particular Yop nor a combination of Yops was involved in the reduction in the oxidative burst.

**Role of YadA in CL inhibition.** We then focused on the YadA protein, because it is produced by virA, virB, and virC mutants but not by virF mutants. We tested the yadA ylpA double mutants W22708(pGB08) and W22708(pGB910), as well as the parental ylpA mutant W22708(pYL4) (2, 11). We also tested W22703(pBC7), a yadA mutant constructed by recombinational integration of a suicide plasmid (9). The yadA mutant and yadA ylpA double mutants induced a strong CL response, while the ylpA mutant behaved like the wild type (Fig. 1D), indicating that YadA was responsible for the inhibition of the oxidative burst.

To confirm the role of YadA, we complemented the three
yadA mutants with plasmid pMS153, which contains an amplified yadA gene cloned under the control of the lac promoter (9). As expected, strains carrying pMS153 produced YadA (data not shown) and no longer induced a CL response (Fig. 1D). YadA, thus, is involved in the CL inhibition phenomenon.

**YadA and phagocytosis.** To determine whether the inhibition of oxidative burst was due to a reduction of phagocytosis, as already suggested by Lian et al. (27), we first used the microscopic method described by these authors. Preopsonized Y. enterocolitica W22703 pYV⁺, W22703 pYV⁻, W22703 (pBC7), and W22703(pBC7)(pMS153) were added to microscope slide wells containing adherent PMNs, and the wells were incubated for 90 min at 37°C. The slides were then stained with acridine orange and counterstained with crystal violet. Acridine orange diffuses in PMNs and accumulates in acidic cellular compartments such as phagolysosomes (16). It intercalates in DNA in the same way as ethidium bromide and stains bacteria and the nuclei of PMNs (Fig. 2). Crystal violet does not enter PMNs, but it stains the bacterial wall and quenches the fluorescence of extracellular bacteria. We examined 100 PMNs and counted those containing bacteria. As shown in Table 2, we observed a clear correlation between the presence of YadA and the reduction of phagocytosis.

This method was quite difficult to apply, because the distinction between intracellular bacteria and acidic granules stained by acridine orange was sometimes difficult. To confirm our result, we thus turned to another approach, based on the use of fluorescent bacteria and flow cytometry. We labeled live bacteria with FITC and checked that the labeling did not affect their viability (data not shown). The FITC-labeled bacteria were then opsonized and mixed with PMNs (ratio, 50:1) for 1 h at 37°C. The mixture of PMNs and bacteria was then counterstained with trypan blue to quench the fluorescence of

![Image](https://example.com/image1)

**FIG. 2.** Light microscopy analysis of phagocytosis of *Y. enterocolitica* by PMNs. (A) *Y. enterocolitica*(pYVe227) incubated at 37°C in BHI-OX in contact with PMNs; (B) fluorescence examination of cells in panel A; (C) *Y. enterocolitica*(pBC7) incubated at 37°C in BHI-OX in contact with PMNs; (D) fluorescence examination of cells in panel C. The arrows indicate individual bacteria; the green fluorescence of the nucleus (n) of an individual PMN is shown. Magnification, ×1,250.

<table>
<thead>
<tr>
<th>Y. enterocolitica strain</th>
<th>Presence of YadA</th>
<th>% Phagocytosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>W22703(pYVe227)</td>
<td>+</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>W22703</td>
<td>−</td>
<td>91 ± 8</td>
</tr>
<tr>
<td>W22703(pBC7)</td>
<td>−</td>
<td>75 ± 12</td>
</tr>
<tr>
<td>W22703(pBC7)(pMS153)</td>
<td>+</td>
<td>35 ± 11</td>
</tr>
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</table>

* PMN monolayers were incubated for 90 min with preopsonized bacteria. Percentages of PMNs having engulfed bacteria and standard deviations for three independent experiments are given. According to a hierarchical F test, the difference between the YadA⁺ and the YadA⁻ bacteria is significant (*P* < 0.01).
extracellular bacteria and was analyzed in the FACS. The FACS monitored all of the PMNs and calculated the percentage of cells that were fluorescent as a result of engulfment of bacteria. The experiment was carried out with \textit{Y. enterocolitica} W22703(pYVe227), W22703 pYV⁻, W22703(pBC7), and W22703(pBC7)(pMS153). As shown in Fig. 3, bacteria expressing YadA were less phagocytosed than the bacteria that did not express YadA. This second approach thus confirmed our microscopic observations. There was a correlation between the lack of oxidative burst and the lack of phagocytosis.

**YadA and killing.** The CL experiments did not resolve the fate of the bacteria ingested by PMNs. We thus measured the killing of \textit{Y. enterocolitica}. For this assay, we used our strain KNG22703, a derivative of W22703 which produces luciferase as a result of the insertion of genes \textit{lucAB} in the chromosomal \textit{bla}A gene (21). Preopsonized bacteria were suspended either in HBSS containing PMNs or in HBSS without PMNs. Bacteria and PMNs were mixed in a ratio of 50 bacteria per PMN. After 60 min of incubation, the PMNs were lysed with ice-cold distilled water, and bacteria were plated on tryptic soy agar supplemented with nalidixic acid for strain KNG22703, with nalidixic acid and gentamicin for strain KNG22703(pBC7), and with nalidixic acid, gentamicin, and tetracycline for strain KNG22703(pBC7)(pMS153). As shown in Table 3, pYV⁺ bacteria were not killed by PMNs, while most of the pYV⁻ bacteria did not survive. The pYV⁺ \textit{yadA} mutant was also killed; however, it survived when the mutation was complemented by the introduction of a cloned \textit{yadA} gene.

Since KNG22703 emits photons in the presence of n-decanal and since there is a linear relation between the number of counts per minute emitted and the number of CFUs (22), we also measured the survival rate by luminometry as described by Kaniga et al. (22). The experiment was realized with \textit{Y. enterocolitica} KNG22703(pYVe227), KNG22703 pYV⁻, KNG22703(pBC7), and KNG22703(pBC7)(pMS153). For each culture, the number of surviving bacteria was estimated both by plating and by luminometry. The results obtained were in very good agreement with those obtained by plating, indicating that the activation of PMNs did not interfere with the light emission resulting from luciferase activity (Table 3). We conclude from these experiments that there is a correlation between the expression of YadA on the cell surface, the inhibition of the CL response of the PMNs, the reduction of phagocytosis by PMNs, and the survival against killing by PMNs.

**TABLE 3. Killing of \textit{Y. enterocolitica} by human PMNs.**

<table>
<thead>
<tr>
<th>\textit{Y. enterocolitica} strain</th>
<th>Presence of YadA</th>
<th>% Survival</th>
<th>% Luminescence</th>
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<tr>
<td>KNG22703(pYVe227)</td>
<td>+</td>
<td>112 ± 18</td>
<td>96 ± 11</td>
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<td>KNG22703</td>
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<td>+</td>
<td>95 ± 22</td>
<td>92 ± 9</td>
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</table>

* Preopsonized bacteria were incubated in HBSS or in HBSS containing PMNs for 60 min at 37°C. After lysis of PMNs, the numbers of viable bacteria were determined by plating (percent survival) or by bioluminescence (percent luminescence). The values given are the percentages of CFU (percent survival) or of counts per minute (percent luminescence) obtained for bacterial suspensions incubated with PMNs, compared with those for control bacterial suspensions incubated in HBSS. According to a analysis of variance 2 analysis, the two methods were not significantly different. Orthogonal-contrast analysis of results obtained by the two methods showed that KNG22703(pYVe227) was significantly different from KNG22703(pBC7) (\(P < 0.01\)) but that KNG22703(pYVe227) was not significantly different from KNG22703(pBC7)(pMS153) and that KNG22703 was not significantly different from KNG22703(pBC7).
DISCUSSION

Lian and Pai (28), showed that Y. enterocolitica cells harboring the virulence plasmid and grown at 37°C do not induce the oxidative burst of PMNs. They showed that a protein present in outer membrane fragment preparations was responsible for this property, and they hypothesized that this protein could be the outer membrane protein now known as YadA. Tertti et al. (46) confirmed the lack of burst, and they hypothesized that it was due to Yop proteins. Moreover, they showed that there was a correlation between the lack of oxidative burst and the reduction of opsonization by complement molecule C3b.

In this study, we tested several well-characterized pYV mutants for their abilities to prevent the oxidative burst of PMNs. The yadA mutants did not prevent the oxidative burst, and this effect of the mutation could be complemented by a cloned yadA gene supplied in trans. Thus, YadA is involved in this phenomenon, as was suggested by Lian et al. (27). We then investigated phagocytosis of Y. enterocolitica and observed that YadA+ Y. enterocolitica cells were less phagocytosed than the YadA- variant. According to acridine orange staining, the intracellular YadA+ bacteria that were phagocytosed were localized in an acidic compartment, an acidified phagosome or a phagolysosome. This acid compartment seemed to be lethal for the bacteria. This conclusion was confirmed by the killing experiments, which showed that the bacteria harboring YadA survive in the presence of PMNs. In conclusion, YadA+ bacteria are phagocytosed and killed, while YadA- bacteria resist phagocytosis and killing. We do not know the fate of the few YadA+ bacteria that are phagocytosed. The fact that YadA inhibits phagocytosis by PMNs could be related to the capacity of YadA to reduce the opsonization by C3b molecules (9). We hypothesize that the presence of YadA prevents the opsonization by C3b molecules, which leads to a reduction of opsonophagocytosis by PMNs as well as to resistance to the bactericidal activity of complement. Y. enterocolitica cells also resist phagocytosis by macrophages, the other professional phagocytes (39). This second resistance involves YopH (39), a phosphotyrosine-phosphatase dephosphorylating macrophage protein (4). We do not see any contradiction between our observation that the inhibition of opsonophagocytosis by PMNs is mediated by YadA and the known role of YopH in the inhibition of phagocytosis by macrophages in the absence of serum opsonins. Indeed, the experiments of phagocytosis by macrophages were carried out in the absence of autologous complement. The systems are thus different. We wondered whether YopH would also prevent phagocytosis by PMNs in the absence of serum. However, in the absence of serum, we could not detect any significant CL response to pYV+ Y. enterocolitica, and most pYV- Y. enterocolitica remained extracellular (10a). Hence, in contrast to macrophages, PMNs require opsonization of the target, to carry out phagocytosis. It is thus not too surprising that Y. enterocolitica developed different protection strategies to face different situations. Since opsonization is required, preventing opsonization seems to be a first-choice strategy to protect from PMNs. This strategy is classical in bacterial pathogenesis. Among others, it is exemplified by group A Streptococcus pyogenes. The M protein of group A. pyogenes prevents phagocytosis by a reduction of complement opsonization (20). We would like to point out the functional similarity between protein M and YadA. Like YadA (9), protein M was indeed reported to enhance the fixation of factor H (20). It is difficult to say whether this functional parallelism results from a similar structure, because the structure of YadA remains unclear.

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