

The pH 6 Antigen Is an Antiphagocytic Factor Produced by *Yersinia pestis* Independent of *Yersinia* Outer Proteins and Capsule Antigen

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The pH 6 antigen (pH 6 Ag; PsaA) of *Yersinia pestis* has been shown to be a virulence factor. In this study, we set out to investigate the possible function of *Y. pestis* PsaA in a host cell line, RAW264.7 mouse macrophages, in order to better understand the role it might play in virulence. *Y. pestis* KIM5 derivatives with and without the pCD1 plasmid and their *psaA* isogenic counterparts and *Escherichia coli* HB101 and DH5 α carrying a *psaA* clone or a vector control were used for macrophage infections. Macrophage-related bacteria and gentamicin-resistant intracellular bacteria generated from plate counting and direct microscopic examinations were used to evaluate these RAW264.7 macrophage infections. *Y. pestis* *psaA* isogenic strains did not show any significant difference in their abilities to associate with or bind to mouse macrophage cells. However, expression of *psaA* appeared to significantly reduce phagocytosis of both *Y. pestis* and *E. coli* by mouse macrophages ($P < 0.05$). Furthermore, we found that complementation of *psaA* mutant *Y. pestis* strains could completely restore the ability of the bacteria to resist phagocytosis. Fluorescence microscopy following differential labeling of intracellular and extracellular *Y. pestis* revealed that significantly lower numbers of *psaA*-expressing bacteria were located inside the macrophages. Enhanced phagocytosis resistance was specific for bacteria expressing *psaA* and did not influence the ability of the macrophages to engulf other bacteria. Our data demonstrate that *Y. pestis* pH 6 Ag does not enhance adhesion to mouse macrophages but rather promotes resistance to phagocytosis.

Yersinia pestis, the causative agent of plague, was originally considered a facultative intracellular parasite that can survive and multiply inside host macrophages (7, 8, 37). The cited studies and others have suggested that the interaction of *Y. pestis* with host macrophages might be important in the pathogenesis of plague infection. Paradoxically, many of the characterized virulence factors of *Y. pestis* are involved in maintaining the organism's extracellular location. The best characterized of these virulence determinants is the type III secretion system and the effector proteins, Yops (*Yersinia* outer proteins). Yops are involved in preventing phagocytosis as well as promoting cytotoxicity and modulation of the immune response (9). Specifically, two of these Yops, YopH and YopE, are involved in the ability of *Yersinia* to inhibit phagocytosis (30, 32). Grosdent et al. recently reported that YopT and YopO are also involved in resistance to phagocytosis and demonstrated that YopH, YopT, YopO, and YopE act synergistically to increase the resistance of *Yersinia enterocolitica* to phagocytosis by macrophages (13). Additionally, the *Y. pestis*-specific capsular antigen F1 also protects the organism from phagocytosis (12). Adhesins such as YadA, Inv, and Ail of *Yersinia pseudotuberculosis* and *Y. enterocolitica* have been reported to mediate initial adhesive events between the enteropathogenic *Yersinia* and eukaryotic cells and thus can manipulate host cell signaling

pathways to the advantage of the pathogen (1, 4, 19, 28, 29, 36). However, none of these adhesins appear to be generally functional in *Y. pestis* (11, 26, 32, 34). Accordingly, the adhesin responsible for *Y. pestis* attachment and delivery of Yops to host cells remains unidentified.

Two potential adhesins expressed by *Y. pestis* are Pla and the pH 6 antigen (pH 6 Ag; PsaA). Pla localizes to the outer membrane and has a proteolytic activity that can cleave and activate plasminogen, a property that has been shown to be important for the ability of *Y. pestis* to infect via the peripheral route (35). Pla could also mediate adherence of *Y. pestis* to epithelial cells and the mammalian extracellular matrix (10, 22). PsaA of *Y. pestis* is expressed at 37°C in acidic media, mediates agglutination of erythrocytes of many species, and has been physically recognized as a fimbrial structure (1, 2, 24). Also, *psaA* mutants of a pigmentation-negative *Y. pestis* strain have been shown to be attenuated by the intravenous route of infection (23). PsaA has also been suggested to possibly be involved in the binding of the organism to allow effective delivery of Yops to target cells (36). In *Y. pseudotuberculosis*, PsaA was reported to be a thermoinducible adhesin that allowed binding of the organism to cultured mammalian epithelial cells (18, 20, 39). However, the virulence function of the antigen remains undefined. A recent report demonstrated that purified PsaA selectively binds to apolipoprotein B (apoB)-containing lipoproteins (LDL) in human plasma. LDL at concentrations close to the physiological concentration in human blood (250 μ g of human LDL per ml) almost abolished the interaction of purified PsaA with macrophages. This process

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant comment(s)	Source or reference
Strains		
<i>Y. pestis</i>		
KIM5-3001	Spontaneous Str ^r mutant of KIM5 (Pgm ⁻), <i>psaA</i> ⁺	23
KIM5-4001	pCD1 ⁻ from KIM5-3001, Str ^r	This study
KIM5-3001.1	<i>psaA</i> mutant of KIM5-3001, Chl ^r	
KIM5-4002	pCD1 ⁻ from KIM5-3001.1, Chl ^r	This study
KIM5-4003	KIM5-3001.1 with pDG5, Chl ^r Amp ^r	This study
KIM5-4004	KIM5-3001.1 with pIC20R, Chl ^r Amp ^r	This study
KIM5-4005	KIM5-4002 with pDG5, Chl ^r Amp ^r	This study
KIM5-4006	KIM5-4002 with pIC20R, Chl ^r Amp ^r	This study
<i>E. coli</i>		
DH5 α		Laboratory stock
HB101		Laboratory stock
DH5 α pUT	Kan ^r	Laboratory stock
Plasmids		
pIC20R	pUC-like plasmid with more restriction sites, Amp ^r , 9.4-kb ClaI fragment with <i>psaA</i> cloned into pIC20R	24
pDG5		24

could prevent recognition of the pathogen by host defense systems (25). It was suggested that immune masking might be important for the ability of the pathogen to cause disease in the susceptible host (25).

In the current study, we set out to investigate the possible function of *Y. pestis* PsaA by analyzing the interaction of bacteria with host cells either expressing or not expressing the pH 6 Ag. Given that the type III secretion system encoded on the large virulence plasmid, pCD1, is known to be a major antiphagocytic virulence determinant, we included in the macrophage infection assay isogenic plasmid-bearing and plasmid-cured derivatives of *psaA* mutant and wild-type *Y. pestis* strains. We also included *E. coli* HB101 and DH5 α carrying the *psaA* cloned locus or a vector control in our macrophage infection assay in order to better understand the role PsaA might play in *Y. pestis* virulence. Our results indicate that PsaA is not necessary for adherence of *Y. pestis* to these cells and is not involved in delivery of Yops. However, the ability to express PsaA on the surface of the bacteria is antiphagocytic.

MATERIALS AND METHODS

Strains and culture conditions. The strains and plasmids used in this study are listed in Table 1. *Y. pestis* isogenic pairs of *psaA*⁺ (KIM5-3001) and *psaA* mutant (KIM5-3001.1) derivatives were described previously (23). We generated pCD1-negative strains of KIM5-3001 and KIM5-3001.1 by plating the organisms on magnesium-oxalate (MgOX) medium at 37°C as previously described (15). These strains were designated *Y. pestis* KIM5-4001 and KIM5-4002, respectively (Table 1). The *psaA* clone, pDG5 (24), and its vector pIC20R were transformed into *E. coli* HB101 and *E. coli* DH5 α , KIM5-3001.1, and KIM5-4002. After transformation, KIM5-3001.1 carrying pDG5 or pIC20R were designated KIM5-4003 and KIM5-4004, respectively, and KIM5-4002 carrying pCD5 or pIC20R were designated KIM5-4005 and KIM5-4006, respectively (Table 1). To grow bacteria for infection of RAW264.7 mouse macrophage cells, *Y. pestis* was grown in brain heart infusion (BHI; Invitrogen, Rockville, Md.) broth at 26°C overnight and diluted 1/25 in fresh supplemented BHI (SBHI) broth (23). For induction of *psaA* the medium was adjusted to pH 6 with HCl before sterilization. Some experiments were performed under Yop-inducing conditions by adding MgOX to the medium (15). Different *Y. pestis* derivatives were grown in broth for 2 h at 26°C followed by 6 h of growth at 37°C with shaking (180 rpm; Innova 4300; New Brunswick Scientific) to allow PsaA or Yop proteins to be synthesized and were

used for infection of macrophage monolayers. *E. coli* harboring pDG5 or pIC20R was grown in BHI broth at 37°C overnight before RAW264.7 infection (23, 24). When necessary, antibiotics were added to the culture medium as follows: ampicillin (100 μ g/ml), chloramphenicol (25 μ g/ml), kanamycin (40 μ g/ml), and streptomycin (25 μ g/ml).

Macrophage infection assay. RAW264.7 mouse macrophage cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) high-glucose formulation supplemented with 10% fetal bovine serum, 200 mM L-glutamine, and 10 mM HEPES (Invitrogen) at 37°C in 5% CO₂. Macrophage cells (5 × 10⁵) were seeded in 24-well tissue culture plates (1 ml/well). The infection assays were performed as previously described (5, 17, 33). Briefly, *Y. pestis* derivatives and *E. coli* strains (23) were added to cell monolayers in 24-well tissue culture plates at a multiplicity of infection generally of 10:1 (bacteria to macrophages). After incubation at 37°C for 30 min, duplicate wells of infected cell monolayers were washed three times with Hank's balance salt solution (Invitrogen) and the number of total macrophage cell-associated bacteria was determined. Total cell-associated bacteria were determined by harvesting in 1 ml of 0.1% Triton X-100 in 1 × phosphate-buffered saline (PBS). After 10 min, infected cell lysates were collected and serially diluted 10-fold in PBS, and aliquots were inoculated onto BHI agar plates with suitable antibiotics to assess viable bacterial CFU. This number was considered as the total cell-associated bacteria. A second set of duplicate infected monolayer wells were washed twice with Hank's balance salt solution. Dulbecco's modified Eagle's medium containing 50 μ g of gentamicin/ml (Invitrogen) was added to these wells for 2 h to kill extracellular bacteria. The infected monolayers then were lysed and treated as above to determine the number of intracellular bacteria. The percentage of cell-associated bacteria was determined by calculating the total number of cell-associated bacteria divided by the total CFU in the inoculum and multiplying by 100. The percent phagocytosis was calculated by dividing the number of intracellular bacteria by the number of cell-associated bacteria and multiplying by 100. Each experiment was repeated two to four times on different days, and each sample of bacteria was used to infect duplicate wells of macrophage monolayers. Since the experiments were performed on different days, the Bonferroni *t* test was used for statistical analysis (16).

Western blotting. Protein samples were prepared from bacteria grown under the same conditions as those described above for infection of macrophages. The bacterial suspensions were adjusted to the same optical density at 600 nm (0.5). A 50- μ l volume of bacterial suspension was pelleted, washed, and suspended in protein sample loading buffer (Novex, San Diego, Calif.) and boiled for 15 min. A 15- μ l volume of material was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 4 to 20% Tris-glycine gel (Novex, San Diego, Calif.) and transferred to polyvinylidene difluoride membranes (Novex). The primary antibody was anti-F1 monoclonal antibody (MabF1-04-AG1; provided by Jeffery Adamovicz and Gerald Andrews, Bacteriology Division, U.S. Army Medical Research Institute of Infectious Disease, Ft. Detrick, Md.) or rabbit

anti-pH 6 Ag polyclonal antibody (23) applied for 1 h at room temperature (RT). After being washed (50 mM Tris, 150 mM NaCl [pH 7.4]) the membranes were incubated with alkaline phosphatase-labeled anti-mouse or anti-rabbit antibody (KPL, Gaithersburg, Md.) for 1 h at RT. Blots were developed by the addition of Western Blue Stabilized Substrate (Promega, Madison, Wis.).

Fluorescence microscopy. Intra- and extracellular bacteria were identified by a double immunofluorescence assay as previously described (12). Briefly, macrophage cells ($\sim 1 \times 10^5$) were seeded in eight-well chamber slides (Lab-Tek II Chamber Slide System 154941; Nalge Nunc International, Naperville, Ill.) to make the semiconfluent macrophage monolayers. The infection assay was the same as that described above. After 30 min of infection by KIM5-3001 (*psaA*⁺) or KIM5-3001.1 (*psaA* mutant) the coverslips with the infected monolayers were washed three times with PBS and processed for labeling. To label extracellular bacteria, the infected-monolayer coverslips were incubated with anti-F1 antibody (MabF1-04-AG) for 15 min at RT and rinsed three times with PBS. The coverslips were then fixed in ice-cold methanol for 90 s to permeabilize the macrophage cells and incubated with tetramethyl rhodamine isocyanate-conjugated rabbit anti-mouse immunoglobulin (Jackson ImmunoResearch Laboratories, Inc.) for 30 min at 37°C. To label all macrophage-associated bacteria, the infected-monolayer permeabilized coverslips were again incubated with anti-F1 monoclonal antibody for 30 min at 37°C, washed three times, and finally incubated with fluorescein isothiocyanate-conjugated rabbit anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc.) for 30 min at 37°C. Fluorescent bacteria were viewed with a fluorescence microscope (Nikon E-800) and a confocal system (Bio-Rad Radiance 2100). Under these conditions, intracellular bacteria fluoresce green while extracellular bacteria fluoresce red or orange (12). The overall morphology of macrophage cells was viewed by differential interference contrast microscopy. The infection experiments for fluorescence microscopy were repeated three times. For each sample, five fields from KIM5-derivative-infected monolayers with similar cell densities were observed with the fluorescence microscope. Representative images were photographed with a microscope-mounted camera (Nikon). Extracellular (red-orange) and intracellular (green) bacteria were also counted manually from five fields of one experiment. The Student *t* test was used for statistical analysis.

RESULTS

Effect of PsaA on phagocytosis and adherence of *Y. pestis* RAW264.7 mouse macrophages. To test whether PsaA of *Y. pestis* had any influence on adherence to and phagocytosis by macrophages, different *Y. pestis* KIM5-3001 derivatives were used to infect RAW264.7 mouse macrophage cells. There was no significant difference in the ability of *Y. pestis* KIM5-3001 (*psaA*⁺) and KIM5-3001.1 (*psaA* mutant) to bind to or associate with RAW264.7 macrophages with or without the virulence plasmid pCD1 (Fig. 1A) as determined by the plate counting method ($P > 0.05$) under our growth conditions. Approximately 30% of the input bacteria associated with the macrophages regardless of genotype. In contrast, *Y. pestis* *psaA*⁺ strains KIM5-3001 and -4001 showed approximately five- to eightfold lower levels of phagocytosis by the macrophages than did *psaA* mutant strains KIM5-3001.1 and -4002 ($P < 0.05$; Fig. 1B). When we compared strain KIM5-3001 (*psaA*⁺ pCD1⁺) with KIM5-4001 (*psaA*⁺ pCD1⁻) and KIM5-3001.1 (*psaA* mutant pCD1⁺) with KIM5-4002 (*psaA* mutant pCD1⁻), we did not detect any significant difference in the phagocytosis of the bacteria, although the trend of uptake of pCD1⁺ *Yersinia* was lower than pCD1⁻ *Yersinia* (Fig. 1B; Table 2). The results of experiments whose results are shown in Fig. 1B were performed with *Y. pestis* grown in MgOX SBHI at 37°C and pH 6. However, when we grew these *Y. pestis* derivatives at pH 7 in Yop-inducing MgOX BHI broth instead of pH 6 MgOX SBHI, pCD1-positive strains (KIM5-3001 and -3001.1) showed approximately 24- to 26-fold lower levels of phagocytosis compared to the pCD1⁻ strains (KIM5-4001 and -4002) (Table 2) ($P < 0.05$). These results suggested that a pH

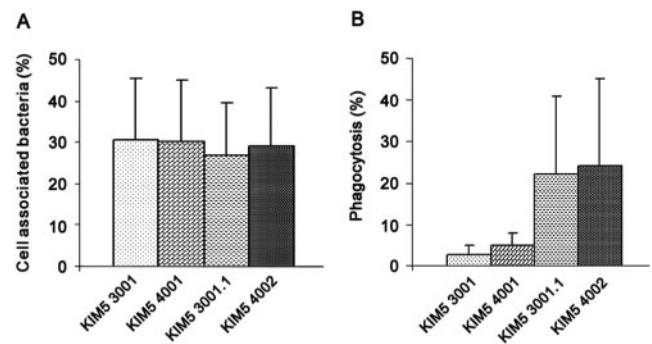


FIG. 1. RAW264.7 mouse macrophage cell-associated *Y. pestis* KIM5 derivatives (percent) (A) and phagocytosis (percent) of *Y. pestis* KIM5 derivatives by the macrophages (B). The infection assay was conducted as described in Materials and Methods. The percent phagocytosis was calculated as the number of intracellular bacteria divided by the macrophage cell-associated bacteria times 100, while the percent cell-associated bacteria was calculated as the number of macrophage cell-associated bacteria divided by the inoculum times 100. Results are shown for KIM5-3001 (*psaA*⁺ pCD1⁺), KIM5-4001 (*psaA*⁺ pCD1⁻), KIM5-3001.1 (*psaA* mutant pCD1⁺), and KIM5-4002 (*psaA* mutant pCD1⁻). Bars represent means plus standard deviations for four experiments, each with duplicate samples. (A) *psaA*⁺ strains KIM5-3001 and KIM5-4001 showed approximately five- to eightfold lower phagocytosis by the macrophages than did *psaA* mutant strains KIM5-3001.1 and KIM5-4002 ($P < 0.05$).

of 7 and a depleted calcium environment are the most efficient conditions for Yop expression and observation of their antiphagocytic effects under our experimental conditions. These results also suggested that the Yops might not be highly expressed under our PsaA-inducing growth conditions. To investigate this possibility, a Western blot experiment was performed. This experiment confirmed that Yops, including YopD, YopN, and YopE, expressed much less or undetectable levels when the KIM5 *Y. pestis* derivatives were grown in pH 6 MgOX SBHI medium compared to growth in pH 7 MgOX BHI medium (data not shown). Although the pCD1⁺ *psaA*⁺ strain KIM5-3001 showed approximately fourfold lower

TABLE 2. Comparison of phagocytosis of *Y. pestis* KIM5 isogenic strains grown in different media by RAW264.7 mouse macrophages

<i>Y. pestis</i> strain	Phagocytosis (%) ^a	
	Growth in pH 6 MgOX SBHI	Growth in pH 7 MgOX BHI
KIM5-3001 (pCD1 ⁺ <i>psaA</i> ⁺)	2.8 ± 2.8*	0.08 ± 0.07
KIM5-4001 (pCD1 ⁻ <i>psaA</i> ⁺)	5.1 ± 3.1	2.1 ± 1.8
KIM5-3001.1 (pCD1 ⁺ <i>psaA</i> mutant)	22.1 ± 17.0	0.37 ± 0.38
KIM5-4002 (pCD1 ⁻ <i>psaA</i> mutant)	24.4 ± 19	9 ± 2.9
KIM5-4003 (pDG5 pCD1 ⁺ <i>psaA</i> mutant)	0.27 ± 0.15	ND ^b
KIM5-4005 (pDG5 pCD1 ⁻ <i>psaA</i> mutant)	0.33 ± 0.34	ND
KIM5-4004 (pIC20R pCD1 ⁺ <i>psaA</i> mutant)	10.3 ± 7.8	ND
KIM5-4006 (pIC20R pCD1 ⁻ <i>psaA</i> mutant)	12.4 ± 3.4	ND

^a Values represent means ± standard deviations for three or four experiments each with duplicate samples.

^b ND, not determined

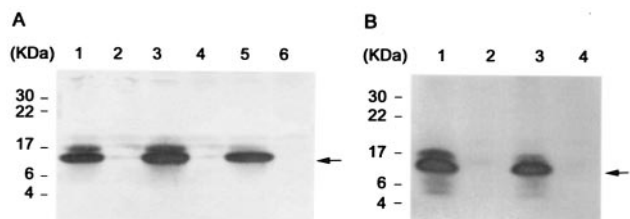


FIG. 2. Western blot analysis of pH 6 Ag expression by KIM5-3001 derivatives, *E. coli* HB101, or *E. coli* DH5 α carrying the pH 6 Ag operon clone, pDG5, or the control vector, pIC20R. The arrows indicate pH 6 Ag. (A) Lanes: 1, KIM5-4003 (KIM5-3001.1 carrying pDG5); 2, KIM5-4004 (KIM5-3001.1 carrying pIC20R); 3, KIM5-4005 (KIM5-4002 carrying pDG5); 4, KIM5-4006 (KIM5-4002 carrying pIC20R); 5, KIM5-3001; and 6, KIM5-3001.1. (B) Lanes: 1, *E. coli* HB101 carrying pDG5; 2, *E. coli* HB101 carrying pIC20R; 3, *E. coli* DH5 α carrying pDG5; and 4, *E. coli* DH5 α carrying pIC20R.

phagocytosis than its *psaA* mutant counterpart KIM5-3001.1 and the pCD1⁻ *psaA*⁺ strain KIM5-4001 showed an approximately fourfold lower rate of uptake than its *psaA* mutant counterpart, KIM5-4002, after growth at pH 7 in MgOX BHI broth, there was no statistically significant difference ($P > 0.05$). Taken together, these data suggested that the pH 6 Ag functions at least in part as a newly defined antiphagocytic factor of *Y. pestis*.

Complementation of *psaA* mutants. In order to confirm the above results, pDG5 carrying the *psaA* operon and the vector pIC20R were transformed into *psaA* mutants KIM5-3001.1 and -4002 (Table 2). Figure 2A shows that *psaA* was expressed well by pDG5 in those *psaA* mutants (KIM5-4003 and KIM5-4005). Complementation of *Y. pestis* *psaA* mutants resulted in significantly less phagocytosis of the mutant strains (Table 2). The phagocytosis of the *psaA* mutant strains KIM5-3001.1 and -4002 harboring pDG5 (KIM5-4003 and -4005) by the macrophages was ~38-fold lower than that of the two *psaA* mutants carrying the pIC20R vector plasmid (KIM5-4004 and -4006) (Table 2). The influence of pCD1 was not significant in these experiments since the bacteria were not grown under maximum Yop-inducing conditions. As further evidence that PsaA is antiphagocytic, *psaA* mutant *Y. pestis* harboring pDG5 (*psaA* operon) was taken up ~10-fold less than the isogenic parent strains (compare KIM5-3001 with KIM5-4003 and KIM5-4001 with KIM5-4005 in Table 2). This may have been caused by the higher copy number of the *psa* operon encoded by pDG5 compared to the chromosomal single copy in the parent strain.

Levels of F1 antigen expressed by *Y. pestis* derivatives. The F1 capsular antigen is well known to be an antiphagocytic factor of *Y. pestis* (6, 12). In order to investigate if F1 antigen was a factor in the observed antiphagocytosis in these experiments (Fig. 1), the bacteria used for infection of macrophages were analyzed by Western blotting for F1 production. Figure 3 shows that there was no obvious difference in F1 antigen expression by the *Y. pestis* derivatives. The *psaA* mutants and the strains with the pDG5 plasmid expressed similar amounts of F1 antigen compared with their parent strains, but the levels of phagocytosis were significantly different (Table 2). These data demonstrated that the observed decrease in phagocytosis of *psaA*-expressing bacteria was not due to alterations in the level of F1 capsule expression.

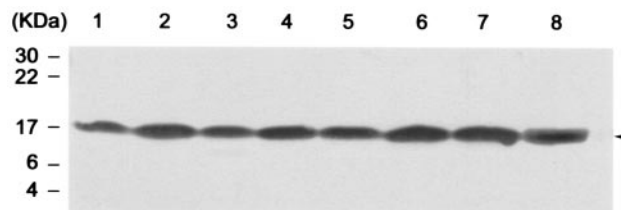


FIG. 3. Western blot analysis of F1 antigen expressed by *Y. pestis* KIM5-3001 derivatives. The arrowhead indicates the F1 antigen. Lanes: 1, KIM5-4003 (KIM5-3001.1 carrying pDG5); 2, KIM5-4004 (KIM5-3001.1 carrying pIC20R); 3, KIM5-4005 (KIM5-4002 carrying pDG5); 4, KIM5-4006 (KIM5-4002 carrying pIC20R); 5, KIM5-3001 (*psaA*⁺ pCD1⁺); 6, KIM5-3001.1 (*psaA* mutant pCD1⁺); 7, KIM5-4001 (*psaA*⁺ pCD1⁻); 8, KIM5-4002 (*psaA* mutant pCD1⁻). An equal amount of bacteria was loaded in each lane (see Materials and Methods).

Phagocytosis and adherence of *E. coli* carrying the *psaA* gene or a vector control to RAW264.7 mouse macrophages. In order to further investigate PsaA as a newly defined antiphagocytic factor, we wanted to remove other potential *Y. pestis* factors that might influence our results. We accomplished this by using *E. coli* harboring clones of *psaA* and a vector control in our macrophage infection assay. Figure 2B shows that *psaA* was expressed well in both *E. coli* HB101 and DH5 α . Figure 4 shows the results of experiments with *E. coli* HB101. Experiments performed with *E. coli* DH5 α revealed similar results (data not shown). The ability to express *psaA* had a significant effect on the phagocytosis of *E. coli* (Fig. 4B, $P < 0.05$). However, the total number of cell-associated bacteria did not show any significant difference ($P > 0.05$) as determined by plate counting. This result suggested that the ability to express the

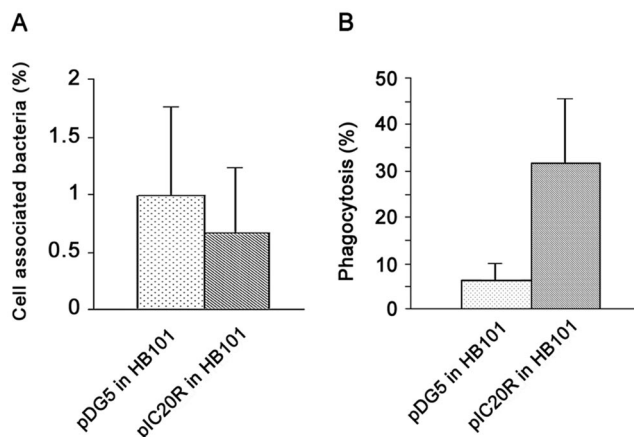


FIG. 4. RAW264.7 mouse macrophage cell-associated *E. coli* HB101 carrying the pH 6 Ag operon clone (pDG5) or the control vector (pIC20R) (percent) (A) and phagocytosis (percent) (B) of *E. coli* HB101 carrying the pH 6 Ag operon clone (pDG5) or the control vector (pIC20R) by the macrophages. The infection assay was conducted as described in Materials and Methods. Macrophage cell-associated bacteria (percent) and phagocytosis (percent) were calculated as for Fig. 1. Shown are results for *E. coli* HB101 carrying pDG5 and *E. coli* HB101 carrying vector pIC20R. Bars represent means plus standard deviations for three experiments, each with duplicate samples. The ability to express pH 6 Ag had a significant effect on the phagocytosis of *E. coli* ($P < 0.05$).

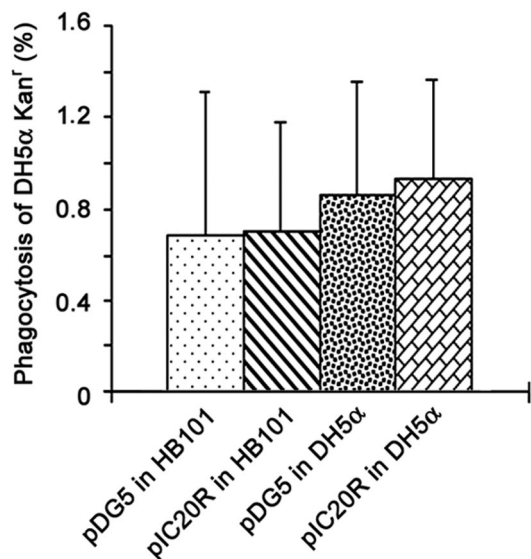


FIG. 5. Phagocytosis (percent) of *E. coli* DH5α Kan^r was calculated as the number of intracellular bacteria divided by the number of bacteria in the inoculum times 100 after coinfection with *E. coli* HB101 carrying pDG5 or pIC20 and *E. coli* DH5α carrying pDG5 or pIC20R. Bars represent means plus standard deviations of phagocytosis (percent) of *E. coli* DH5α Kan^r for two experiments, each with duplicate samples. The ratios of the DH5α Kan^r bacteria to the competitive bacteria were ~1:1 to 1:6.

antigen had no significant impact on adherence of the organism to cultured macrophages. The presence of pDG5 in both *E. coli* HB101 and DH5α resulted in an approximately fivefold lower level of phagocytosis by the macrophages compared to that of bacteria containing only the vector. These data strongly suggest that the pH 6 Ag alone is responsible for the reduced phagocytosis of both *E. coli* strains as well as of *Y. pestis*.

Coinfection and pH 6 Ag-dependent antiphagocytosis. In order to determine whether this antiphagocytosis is specific for PsaA-producing bacteria or a general effect on phagocytosis by the macrophage, we used an *E. coli* DH5α Kan^r strain in coinfection experiments with the cloned locus or vector control in macrophage infection assays. Briefly, *E. coli* DH5α Kan^r was added together with *E. coli* HB101 carrying pDG5 or pIC20R and *E. coli* DH5α carrying pDG5 or pIC20R to infect RAW264.7 mouse macrophages. After a 30-min infection, the extracellular bacteria were killed by gentamicin treatment for 2 h and the macrophage monolayers were processed as described in Materials and Methods. Since we did not note any increase in adherence due to PsaA, the efficiency of phagocytosis of *E. coli* DH5α Kan^r was calculated as the number of intracellular bacteria divided by the number of bacteria in the inoculum times 100. There was no significant difference found for phagocytosis of *E. coli* DH5α Kan^r by the macrophages during these coinfections (Fig. 5). These data demonstrate that the antiphagocytosis seen with PsaA-producing bacteria was specifically for cells expressing the antigen and did not affect the general ability of the macrophage to phagocytize *E. coli*.

Fluorescence microscopy of *Y. pestis* KIM5-3001- and KIM5-3001.1-infected RAW264.7 macrophages. In order to visually confirm the plate counting results presented above fluorescence microscopy was conducted. Figure 6 shows double-fluo-

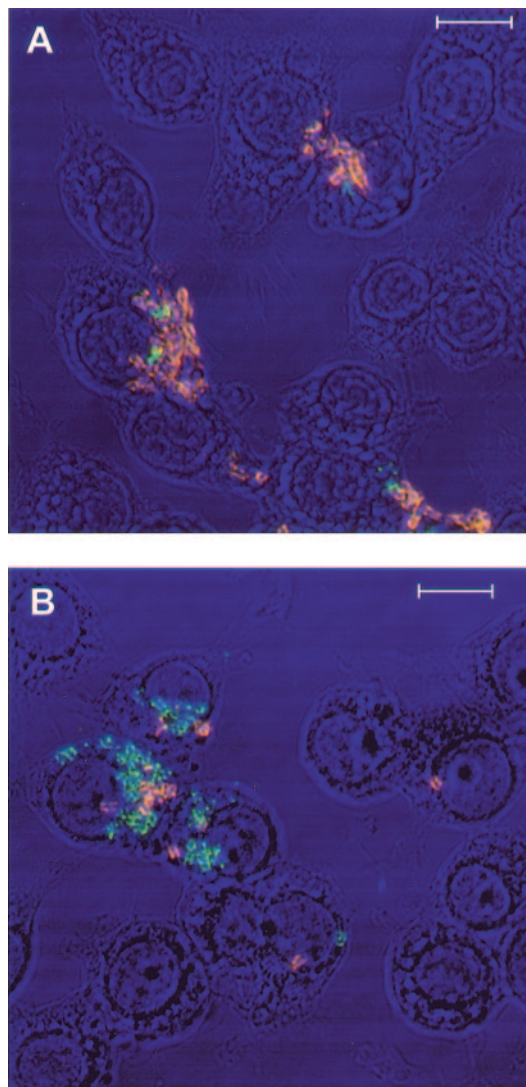


FIG. 6. Confocal fluorescence microscopy of RAW264.7 macrophages infected by *Y. pestis* KIM5-3001(*psaA*⁺) (A) and KIM5-3001.1(*psaA* mutant) (B). Extracellular bacteria labeled orange, while intracellular bacteria labeled green. Magnification, ×120. Bars, 10 μm.

rescence-labeled bacteria that are located extracellular to (red-orange) or taken up by (green) the macrophages. We examined approximately five fields in each experiment and repeated each experiment three times. In each case it was apparent that the phagocytosis of KIM5-3001.1 (*psaA* mutant) (Fig. 6B) was significantly more than that of the KIM5-3001 (*psaA*⁺) parent strain (Fig. 6A). Fields of *Y. pestis* producing PsaA always contained a higher proportion of orange-fluorescing bacteria than those of the *psaA* mutant, visually demonstrating that more *psaA*-positive bacteria remained outside the macrophages and were not phagocytosed. Table 3 shows that the numbers of phagocytosed (green labeled) KIM5-3001 (*psaA*⁺) bacteria are significantly less than those of the KIM5-3001.1 (*psaA* mutant) ($P < 0.01$) while the numbers of extracellular (red-orange labeled) KIM5-3001 (*psaA*⁺) bacteria are significantly higher than those of the KIM5-3001.1 (*psaA* mutant) ($P < 0.01$). However, there is no significant difference

TABLE 3. Numbers of *Y. pestis* KIM5 Isogenic bacteria of phagocytosed by and adherent to RAW264.7 macrophages as determined by fluorescence microscopy

Strain	No. of extracellular bacteria ^a	No. of intracellular bacteria ^a	Total no. of extra- and intracellular bacteria ^b
KIM5-3001 (pCD1 ⁺ <i>psaA</i> ⁺)	115.4 ± 27.3	65.6 ± 16.5	181.0 ± 43.8
KIM5-3001.1 (pCD1 ⁺ <i>psaA</i> mutant)	70.0 ± 9.1	129.0 ± 22.0	199.0 ± 31.1

^a Extracellular bacteria imaged as red-orange (labeled with rhodamine and Fluorescein isothiocyanate [FITO]), while intracellular bacteria imaged as green (labeled with FITC) (see Materials and Methods and Fig. 6). Values represent means ± standard deviations for five fields of bacteria in the semiconfluent mouse macrophage monolayer under a fluorescence microscope.

^b Values represent means ± standard deviations of total numbers of extra- and intracellular bacteria described in footnote *a*.

between KIM5-3001 and KIM5-3001.1 when the total numbers of bacteria (extracellular and intracellular) were added together ($P > 0.05$). This result suggested that the numbers of macrophage-associated bacteria were similar for both KIM5 derivatives. Accordingly, the chance to adhere to the macrophages for both KIM5-3001 (*psaA* mutant) and KIM5-3001 (*psaA*⁺) was similar, but their intracellular or extracellular fate was significantly different.

DISCUSSION

Previously, it has been shown that *Y. pestis*, once grown inside phagocytes, was able to resist subsequent phagocytosis (7). Several other *Yersinia* factors have been shown to be antiphagocytic. Four of the type III secretion system effector proteins, YopH, YopT, YopO, and YopE, are particularly important in the ability of *Y. pestis* to inhibit phagocytosis (9). Also, F1 capsular antigen has been reported to block phagocytosis (12). The current study adds to the antiphagocytic armament of *Y. pestis* by showing that the pH 6 Ag also has the function of inhibiting phagocytosis. In our experiments, *Y. pestis* with and without the pCD1 plasmid (KIM5-3001 and -4001) always revealed an approximately five- to eightfold lower level of phagocytosis than the *psaA*-negative isogenic strain when analyzed by plate counting. This difference was not due to differences in the level of F1 antigen expression. Furthermore, *E. coli* HB101 and DH5 α harboring a *psaA* operon clone (pDG5 plasmid) also showed significantly lower phagocytosis than the bacteria carrying the vector only. The *psaA* clone, pDG5, completely restored the ability of *Y. pestis* *psaA* mutants (KIM5-3001.1 and -4002) to resist phagocytosis. Additionally, expression of some Yops (YopE, YopN, and YopD) had been greatly reduced when growth was at pH 6. These results demonstrate that besides Yops and F1 antigen, PsaA is another factor that contributes to resistance to phagocytosis by *Y. pestis* and is Yop and F1 antigen independent. In the current study, we did not see a significant difference between the total numbers of cell-associated bacteria ($P > 0.05$) when *psaA* isogenic strains were compared. However, there was a significant difference between the extracellular and intracellular bacterial distribution between *psaA*⁺ and *psaA* mutant strains (Table 3 and Fig. 6). Under the fluorescence microscope KIM5-3001 (*psaA*⁺) showed an approximately twofold lower level of

phagocytosis than the *psaA* mutant counterpart (Table 3). The lower ratio of internalized mutant bacteria noted by microscopy may have been caused by the bacteria overlapping each other. Superimposed bacteria cannot be recognized by microscopy and would be counted as one bacterium. However, the trend for both methods was the same. Taken together, these results indicated that the pH 6 Ag does not appear to promote adherence of the bacteria to macrophages but rather functions at least partially as an antiphagocytic factor. Furthermore, our data did not show any significant difference between pCD1⁺ and pCD1⁻ strains in either *psaA* wild-type or mutant *Y. pestis* backgrounds and the ability to resist phagocytosis under pH 6 Ag-inducing conditions. Accordingly, pH 6 Ag is unlikely to be the adhesin responsible for the delivery of Yops to host cells in this species, and the adhesin responsible for delivery of Yops to host cells by *Y. pestis* remains unknown.

PsaA of *Y. pestis* has been shown to be induced inside macrophages (24, 25). It may be one of the reasons why *Y. pestis*, once grown inside phagocytes, is able to resist uptake (7). Previously it was shown that *Y. pestis* bacteria strongly producing PsaA were more rapidly fatal to mice than bacteria not expressing this antigen (1). Furthermore, animal studies have found an ~200-fold increase in the 50% lethal dose of the *psaA* mutant compared with that of the parent *Y. pestis* (23). These previous results suggest that PsaA is involved in the pathogenesis of plague. Our current results suggest that PsaA contributes to virulence by at least preventing phagocytosis of *Y. pestis*. Our demonstration that the antiphagocytic Yops may be reduced in expression under conditions that induce *psaA* expression suggest a potential role for the antigen during the early stages of *Y. pestis* infection. Given that PsaA is produced inside macrophages under acidic conditions (24), the antigen may be the first antiphagocytic factor produced by the organism after release from infected macrophages. Further experimentation will be required to define the relationship of PsaA to other previously defined antiphagocytic factors and determine if pH 6 Ag performs other functions that contribute to virulence of the organism.

Our results are consistent with the findings and hypothesis of Makoveichuk et al. (25), who showed that purified pH 6 Ag selectively binds to apoB-containing lipoproteins in human plasma. These researchers suggest that pH 6 Ag after binding to the lipoprotein could prevent recognition of the pathogen by the host defenses. The lower efficiency of uptake of pH 6 Ag-positive organisms, both *Y. pestis* and *E. coli*, by mouse macrophages compared with that of their pH 6 Ag-negative counterparts might be caused by the pH 6 Ag binding to the apoB-containing lipoprotein in fetal calf serum in cell culture media and preventing the bacteria from being phagocytosed by the macrophages. Previously, *Y. pestis* pH 6 Ag was shown to bind to β 1-linked galactosyl residues in glycosphingolipids, which are likely found on a range of host cells (27). Our observation that the pH 6 Ag did not appear to promote significant binding of *Y. pestis* to macrophages may be due to prebinding of lipoprotein and thus competition with receptors on the host cell surface. Our data is in contrast with the results of Yang et al. (39), who found that the *psa* locus is responsible for thermoinducible binding of *Y. pseudotuberculosis* to cultured cells. This may be due to the use of a different host cell type and/or culture conditions. Also *Y. pseudotuberculosis* en-

codes different adhesins than *Y. pestis*, as has been shown for the *inv*, *ail*, and *yadA* genes (11, 26, 32, 34). Accordingly, these other adhesin/invasin proteins may play an interactive role in the ability of pH 6 Ag to promote adherence of *Y. pseudotuberculosis* to host cells.

PsaA-mediated antiphagocytosis seemed specific to the pH 6 Ag-expressing organisms, as shown in our coinfection assays with differentially marked *E. coli* bacteria (Fig. 5). These results suggested that the mechanism of PsaA-mediated antiphagocytosis is different from that of Yops. YopE has been demonstrated to function as a GTPase-activating protein to downregulate multiple Rho GTPases (3, 38), which leads to disruption of actin microfilaments in the target cell (30, 31). YopH is homologous to eukaryotic protein tyrosine phosphatases (14), which induce overall dephosphorylation of host proteins and are able to interfere with early tyrosine phosphorylation signals that occur in the cell during phagocytosis. YopT and YopO both are involved in preventing actin rearrangement in host cells and inhibition of phagocytosis (13, 21, 40). The effect of these Yop proteins results in a general failure of macrophages to phagocytose extracellular bacteria. F1 antigen has been reported to block phagocytosis by a mechanism different from that of the type III secretion system, presumably by preventing bacterium-host cell receptor interaction that potentially could result in the uptake of the pathogen (12). The mechanism of pH 6 Ag-mediated phagocytosis resistance may be similar to that of the F1 antigen since it also did not have any influence on the general phagocytic ability of the macrophages. Additionally, our results indicate that there may be other *Y. pestis*-encoded factors independent of Yops (e.g., pCD1) that promote antiphagocytosis by expression of pH 6 Ag. This is suggested by the ~38-fold decrease in uptake of pDG5-complemented *Y. pestis* *psaA* mutant strains compared to the ~5-fold decrease seen when comparing *E. coli* clones to vector controls, given that the copy number of these plasmids is similar in the two different genera.

In summary, PsaA of *Y. pestis* provides antiphagocytic protection in addition to that provided by Yops and the F1 capsule antigen, which help the bacteria escape host defense mechanisms. PsaA mediates antiphagocytosis probably by preventing adhesin-receptor interaction similar to the F1 antigen or possibly by selectively binding to apoB-containing lipoproteins to avoid recognition by host macrophages. The fact that PsaA is antiphagocytic and has been shown to be induced inside macrophages suggests a possible role for the protein in the early stages of *Y. pestis* infection. Experiments to address this question are currently under way.

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