Intranasal Inoculation of Mice with *Yersinia pseudotuberculosis* Causes a Lethal Lung Infection That Is Dependent on *Yersinia* Outer Proteins and PhoP[▽]

Michael L. Fisher, Cynthia Castillo, and Joan Mecsas*

Department of Microbiology and Molecular Biology, Tufts University, Boston, Massachusetts 02111

Received 10 August 2006/Returned for modification 25 September 2006/Accepted 13 October 2006

Yersinia pseudotuberculosis infects many mammals and birds including humans, livestock, and wild rodents and can be recovered from the lungs of infected animals. To determine the *Y. pseudotuberculosis* factors important for growth during lung infection, we developed an intranasal model of infection in mice. Following intranasal inoculation, we monitored both bacterial growth in lungs and dissemination to systemic tissues. Intranasal inoculation with as few as 18 CFU of *Y. pseudotuberculosis* caused a lethal lung infection in some mice. Over the course of 7 days, wild-type *Y. pseudotuberculosis* replicated to nearly 1×10^8 CFU/g of lung in BALB/c mice, induced histopathology in lungs consistent with pneumonia, but disseminated sporadically to other tissues. In contrast, a $\Delta yopB$ deletion strain was attenuated in this model, indicating that translocation of *Yersinia* outer proteins (Yops) is essential for virulence. Additionally, a $\Delta yopH$ null mutant failed to grow to wild-type levels by 4 days postintranasal inoculation, but deletions of any other single effector YOP did not attenuate lung colonization 4 days postinfection. Strains with deletions in *yopH* and any one of the other known effector *yop* genes were more attenuated than the $\Delta yopH$ strain, indicating a unique role for *yopH* in lungs. In summary, we have characterized the progression of a lung infection with an enteric *Yersinia* pathogen and shown that YopB and YopH are important in lung colonization and dissemination. Furthermore, this lung infection model with *Y. pseudotuberculosis* can be used to test potential therapeutics against *Yersinia* and other gram-negative infections in lungs.

Yersinia pseudotuberculosis and *Yersinia enterocolitica* are gram-negative animal and human pathogens most commonly associated with acute gastroenteritis and lymphadenitis (11, 37). Both pathogens are generally transmitted by the fecal-oral route of infection and infect the gastrointestinal tract and associated lymph tissues (37, 65), but both can be isolated from the lungs of infected animals (7, 12, 50, 60, 61). In these cases, multiple organs are usually colonized, indicating that dissemination occurred from gastrointestinal or systemic sites to the lungs. Human lung infections by enteric *Yersinia* pathogens are rare, although there are reports of such cases (7, 33). In contrast, lung infections by the third pathogenic *Yersinia* species, *Yersinia pestis*, is well documented in both human and animal hosts and results in a highly lethal pneumonia (21, 47). While there are cases of experimentally induced lung infection with enteric *Yersinia* species (14, 20, 59, 80, 82), no systematic study of pneumonic yersiniosis induced by enteric *Yersinia* in mice has been completed to date.

All three pathogenic *Yersinia* species carry a 70-kb virulence plasmid, which encodes a type III secretion system (TTSS) and the secreted effector proteins, *Yersinia* outer proteins (Yops) (19, 67, 78). The TTSS is induced by growth at 37°C (18). Effector Yops—YopH, YpkA/YopO, YopM, YopE, YopT, and YopJ/P—are translocated into host cells where they interfere with normal cellular processes (84). Effector Yops require YopB, LcrV, and YopD for translocation into mammalian cells

but not for their secretion into the extracellular milieu (84). In studies on cultured cells, most effector Yops target and disrupt functions of macrophages and neutrophils (8, 19, 30, 53, 57, 62, 63, 85), cells that play critical roles in the initial defense against invading pathogens. Specifically, YopE, YopT, YopO, and YopH have all been shown to prevent phagocytosis by macrophages and neutrophils (8, 19, 30), while YopJ induces programmed cell death in macrophages (57) and inhibits the mitogen-activate protein kinase pathway (62, 63). YopM interacts with the host protein RSK-1 (54), alters the transcriptional profile of several cytokines in macrophages isolated from *Y. pestis*-infected spleens, and reduces the number of natural killer cells (45). YopM- β -lactamase fusions are translocated into granulocytes, macrophages, and dendritic cells in spleens following intravenous infection (53), suggesting that YopM also targets phagocytes.

The virulence properties of each of the Yops have been studied in animal models of one or more pathogenic *Yersinia* species. Deletion of *yop* genes reduces the ability of *Yersinia* to colonize host tissues and/or cause death after infection by one or more routes (48, 49, 77, 79, 80). For instance, orogastric infection with a *Y. pseudotuberculosis* strain lacking the tyrosine phosphatase, YopH, results in diminished colonization of the mesenteric lymph nodes and spleen compared to wild-type *Y. pseudotuberculosis* (49). Orogastric infection with *Y. enterocolitica* $\Delta yopH$ mutants results in similar attenuation in the small intestine, spleen, and liver (79). $\Delta yopO$ mutants of *Y. enterocolitica* are defective in colonization of the liver during intravenous infections (79) and in colonization of the spleen during orogastric infection of *Y. pseudotuberculosis* (49). YopM and YopE play a significant role in intravenous infections of mice

* Corresponding author. Mailing address: Department of Microbiology, 136 Harrison Ave., Tufts University, Boston MA 02111. Phone: (617) 636-2742. Fax: (617) 636-0337. E-mail: joan.mecsas@tufts.edu.

[▽] Published ahead of print on 30 October 2006.

with *Y. pestis* (48). In orogastric infections of *Y. pseudotuberculosis*, a $\Delta yopE$ mutant is attenuated in the spleen as well as the Peyer's patches (49), and a $\Delta yopJ$ mutant has a 64-fold increase in its 50% lethal dose (LD_{50}) compared to wild-type *Y. pseudotuberculosis* (56). In addition to their role in gastric and intravenous infection, there is evidence that Yops are important during lung infection (80). All three pathogenic *Yersinia* organisms were recovered from lungs of intravenously infected mice, and their numbers increased over time (80). In contrast, strains lacking a virulence plasmid were initially recovered in lungs after intravenous inoculation, but their numbers decreased over time. Additionally, mice intranasally inoculated with *Y. pestis* lacking the virulence plasmid were cleared within 72 h (47). Together, these data suggest that genes encoded on the plasmid are important for persistence in the lungs.

In addition to the Yops and TTSS, other *Yersinia* virulence factors are known. PhoP, which is the response regulator of the PhoP/Q two-component signal transduction system, plays a crucial role in the ability of *Y. pseudotuberculosis* to replicate in macrophages (27). Furthermore, *Y. pseudotuberculosis* $\Delta phoP$ mutants have a 75-fold increase in the LD_{50} following intravenous inoculation of mice (27). *Y. pseudotuberculosis* also produces the adhesions invasins and YadA, which are important during mouse infection (34, 55). *inv* is preferentially expressed at 26°C and binds to $\beta 1$ integrins of eukaryotic cells (39). *inv* mutants are defective for colonizing the cecum and Peyer's patches following orogastric infection of mice but are not defective in colonizing the spleen following intraperitoneal infection of mice (55). *yadA* is preferentially expressed at 37°C, and its deletion results in about a 10-fold increase in the LD_{50} of intraperitoneally infected mice (34).

We investigated the dynamics of *Y. pseudotuberculosis* infection in lungs of mice after intranasal inoculation with wild-type IP2666pIB1 and an isogenic $\Delta yopB$ mutant. Strikingly, we found that mice infected with as little as 18 CFU of wild-type *Y. pseudotuberculosis* developed a fatal lung infection, whereas mice infected with 3×10^2 CFU of the $\Delta yopB$ mutant displayed no visible signs of illness although the bacteria persisted in lungs more than a month and granulomatous lesions were observed.

MATERIALS AND METHODS

Bacterial strains and strain construction. *Y. pseudotuberculosis* strains used in this study are listed in Table 1. *Yersinia* strains were grown in LB broth for mouse infections and in Luria (L) broth for conjugation and other protocols (see reference 49). The IP2666pIB1yopH(NdeI) strain was the wild-type and/or parental *Y. pseudotuberculosis* strain (41) used for all experiments, unless otherwise stated. $\Delta yopH$, $\Delta yopO$, $\Delta yopM$, $\Delta yopJ$, and $\Delta yopE$ mutants and multiple *yop* deletion strains were generated in this strain with plasmids and methods described previously (49). $\Delta yopH$, $\Delta yopO$, $\Delta yopM$, and $\Delta yopE$ mutants were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of trichloroacetic acid-precipitated supernatants of each mutant grown under YOP secretion conditions (49). $\Delta yopH$, $\Delta yopM$, and $\Delta yopE$ mutants were also confirmed by immunoblot analysis of trichloroacetic acid-precipitated supernatants. To generate the $\Delta yopJ$ strain, the following primers were used to amplify flanking regions of the *yopJ* gene: YopJ1C, GAGACAGTCGACGTCGACGACCATC GCCGAG; YopJ2B, CCAATACATTACTCGAGCATTTATTTATCCTTAT TCAGGG; YopJ3B, GGATAAAATAAATGCTCGAGTAATGTATTTTGGGA AATCTTGC; and YopJ4A, GAGACAGCATGCCTGGGTATCGGTGCTAT GATCGGC. The fragments generated by the primer pair YopJ1C and YopJ2B and the pair YopJ3B and YopJ4A were purified and combined in a second PCR with primers YopJ1C and YopJ4A. The resulting fragment was cloned into the

TABLE 1. *Yersinia* strains

Strain name	Genotype	Reference or source
Wild-type	<i>Y. pseudotuberculosis</i> serotype 3;	41
IP2666pIB1	IP2666c pIB1 ⁺ yopH(NdeI)	
LL-89	<i>Y. pseudotuberculosis</i> strain IP2666c pIB1 ⁺ yopH(NdeI); Kan ^r	L. Logsdon and J. Mecsas
MLF-13	LL89 $\Delta lacZ$	This study
MLF-5	$\Delta yopH$	L. Logsdon and J. Mecsas
MLF-10	$\Delta yopO$	This study
MLF-3	$\Delta yopM$	This study
MLF-2	$\Delta yopE$	L. Logsdon and J. Mecsas
MLF-36	$\Delta yopJ$	This study
MLF-11	$\Delta yopB$	This study
MLF-20	$\Delta yopE yopO$	This study
MLF-29	$\Delta yopE yopO yopM$	This study
MLF-4	$\Delta yopH yopE$	L. Logsdon and J. Mecsas
MLF-41	$\Delta yopH yopO$	This study
MLF-50	$\Delta yopH yopM$	This study
MLF-34	$\Delta yopH yopJ$	This study
	<i>yopH</i> R409A	41
	<i>yopH</i> Q11R	41
	<i>yopH</i> V31G	41
	<i>yopH</i> K342A	41
	<i>yopH</i> $\Delta 223-6$	41
IP2666pIB1	Serogroup O3, pIB ⁺	J. Bliska
IP2666 $\Delta phoP$	IP2666pIB1 $\Delta phoP$	J. Bliska; see reference 27 for deletion
YPIIIpIB1 ⁺	<i>Y. pseudotuberculosis</i> strain serotype 3	55
JMB-111	<i>Y. pseudotuberculosis</i> strain IP2666pIB1 ⁻	4

suicide vector pCVD442 using the SalI and SphI sites. The deletion was generated as described previously (49). $\Delta yopJ$ deletions were verified by PCR with YopJ1C and YopJ4A and confirmed by testing for cytotoxicity in macrophages using a CytoTox 96 Non-Radioactive cytotoxicity assay (Promega) as previously described (57).

The IP2666pIB1 $\Delta lacZ$ strain was generated by first amplifying flanking fragments of the *lacZ* gene in *Y. pseudotuberculosis* by PCR using the following primers: P31, GCATTAGTCGACGGTGAACCTGAGTCTTCC; P32, CGCCA CGCATGCCACCTGCGGATCATTTGAG; P33, CAGGTGGCATGCGTGGC GGATAAACCGATG; and P34, TGCCGTGAGCTCTTGGTACTGATAGGT TTCAT. The fragments were cloned into pCVD442 as described for *yopJ* using primers P31, P32, P33, and P34. Clones were verified for a $\Delta lacZ$ phenotype on L plates containing X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; Sigma). On such medium, the wild type is blue, while the $\Delta lacZ$ mutant remains white. The *lacZ* knockout construct was introduced into a kanamycin-marked IP2666pIB1 strain LL89. To generate LL89, pUTminiTN5Kn2 was introduced into IP2666pIB1 by conjugation as previously described (55). Three kanamycin-resistant exconjugants were tested for virulence in competition experiments with IP2666pIB1 in BALB/c mice. Two exconjugants were as fit as wild type in colonizing the intestinal tract, lymph tissues, and the spleen, and one, named LL-89, was chosen to use as a control in these studies. Sequence analysis of the transposon insertion indicated that the transposon was in the intergenic region between locus numbers YPTB3287 and YTPB3288 in *Y. pseudotuberculosis* IP32953, which encode a hypothetical protein and a putative GntP gluconate family transporter, respectively. The IP2666pIB1 $\Delta phoP$ mutant, its parental strain (27), and *yopH* mutants (41) were provided by James Bliska.

Mouse infections. *Yersinia* strains were grown with aeration overnight at 26°C in LB broth as previously described (49) or at 37°C in LB broth supplemented with 5 mM CaCl₂ to prevent lysis of bacteria. The following morning, cultures were diluted 1:40 and grown for 8 h at 26°C or 37°C. Cultures were then diluted to an optical density at 600 nm of 0.025 and grown at 26°C or 37°C for approx-

FIG. 1. *Y. pseudotuberculosis* colonization of lungs occurs at low doses and is dependent on *phoP*. (A) In vivo growth of *Y. pseudotuberculosis* in BALB/c or Swiss Webster mice. Mice were inoculated intranasally with wild-type *Y. pseudotuberculosis* with 7.8×10^1 CFU (▲), 8.4×10^2 (■), or 8.4×10^3 (●). Four days postinoculation lungs were harvested, weighed, homogenized, and plated for CFU. (B) Strains lacking PhoP are attenuated in colonization of lungs. BALB/c mice were intranasally inoculated with 8.0×10^2 CFU of IP2666pIB1, 6.0×10^2 CFU of IP2666pIB1 Δ phoP, or 2.5×10^3 CFU of YPIIIpIB1. Four days postinoculation, lungs were weighed, homogenized, and plated for CFU. Each dot represents output from one mouse. Bars represent the geometric mean. *P* values were determined by a two-tailed Student's *t* test. *, *P* < 0.01.

imately 16 h to an optical density at 600 nm of 4 to 6. Bacteria were diluted in sterile phosphate-buffered saline (PBS) at 26°C or 37°C to the appropriate CFU/ml for intranasal infection and maintained at their growth temperature until inoculation into animals. Six- to eight-week-old female BALB/c (Taconic Labs, Charles River Labs, or National Cancer Institute) or Swiss Webster (Taconic Labs) mice were anesthetized with isoflurane and infected intranasally with 40 μ l of the bacterial suspension (35). Appropriate dilutions of inocula were plated on L plates to determine the doses administered, which are indicated in figure legends and tables. Each strain was used to infect 2 to 4 mice at a time, and each experiment was repeated two to four times with the exception of the data shown in Fig. 1A and the *yopH* time to morbidity experiment. At appropriate times after infection, mice were sacrificed by CO₂ asphyxiation. Tissues were harvested, weighed, homogenized, and plated on L plates containing 1 μ g/mg

irgasan (49) to determine the number of CFU. Data are reported as the number of CFU/g of organ for lung, liver, spleen, and trachea; number of CFU/ml of blood; and number of CFU/organ for the lymph nodes and nasopharynx as the small size of the organs and the association of lymph nodes with connective tissue made determination of weights inaccurate. Homogenization of tissues was accomplished using a variable-speed tissue tearor (Biospec Products Inc.) using established methods described previously (2, 13, 49). Samples were blended until the solution appeared homogeneous, which occurred after 10 to 45 s of homogenization, depending on the tissue.

For competition experiments, the IP2666kan^r Δ lacZ (MLF-13) strain was used as the reference strain. This strain was grown separately and then mixed with the unmarked wild type IP2666pIB1yopH(NdeI) strain or an isogenic Δ yop mutant strain generating a mixed inoculum. The mixtures were plated onto L plates containing X-Gal, and blue and white colonies were counted to obtain the ratio of mutant to wild-type *Y. pseudotuberculosis* in the input inocula. Likewise, after infection appropriate dilutions of tissue homogenates were plated on L plates containing X-Gal to determine the ratio of mutant to wild-type *Y. pseudotuberculosis* colonies in tissues. A minimum of 100 colonies were counted for each competitive index (CI). The CI was calculated as follows: CI = (number of mutant colonies/number of wild-type colonies)_{output ratio}/(number of mutant colonies/number of wild-type colonies)_{input ratio}.

For time-to-morbidity experiments, 1 week prior to infection, blood from tail veins of some mice was collected into Microtainer serum separator tubes (Becton Dickinson) according to the manufacturer's protocols and stored at -20°C for comparison to postinfection bleeds. Mice were inoculated intranasally with various doses of wild-type *Y. pseudotuberculosis* or Δ yopH mutant and monitored daily for signs of morbidity such as scruffiness, lack of response to touch lethargy, or a hunched appearance. Mice that moved only upon nudging were sacrificed by CO₂ asphyxiation. All surviving mice were sacrificed after 28 days, and blood was collected by cardiac puncture and frozen for future analysis.

Histology. Mice were inoculated with 300 CFU/mouse of wild-type *Y. pseudotuberculosis* or the Δ yopB strain or with PBS alone. For infections with wild-type *Y. pseudotuberculosis*, two mice were sacrificed by CO₂ asphyxiation at days 1, 2, 4, 6, and 7. For the Δ yopB strain infections, two mice were sacrificed at 1, 2, 4, 6, 7, 14, 21, 28, 35, and 42 days postinoculation. PBS-treated control mice were sacrificed at 1, 4, 7, 14, 28, and 42 days postinoculation for comparison. The lungs were perfused via the trachea with 10% neutral buffered formalin (NBF), harvested, incubated in 10% NBF at 26°C for 24 h, and placed in fresh 10% NBF at 4°C until further processing. Lungs were sliced, transferred to histocassettes (Fisherbrand), incubated at 26°C in 70% ethanol for at least 24 h, and embedded in paraffin. Eight- to 10-micrometer sections were stained with hematoxylin and eosin or prepared for immunohistochemistry (see below). Slides were evaluated in a blind fashion by veterinary pathologist Lauren Richey at Tufts University Department of Laboratory Animal Medicine. Pictures were taken using a color camera (Nikon DS-M5) and an inverted microscope (Nikon TE2000).

For immunohistochemistry staining, the paraffin was extracted from the sections with a 5-min wash in 100% xylene. Samples were treated for 10 min with 70% ethanol, followed by 5-min treatments each with 95% ethanol, 100% ethanol, and distilled water. Immunostaining was carried out using the LSAB 2 System-HRP (DakoCytomation K0673) according to the manufacturer's protocol. Briefly, samples were incubated with a 1:300 dilution of rabbit anti-*Y. pseudotuberculosis* antibodies (gift of Ralph Isberg) for 30 min, rinsed with PBST (PBS plus 0.5% Tween 20), incubated with a 1:300 dilution of goat anti-rabbit immunoglobulin G (IgG)-biotin (DakoCytomation E0432) for 30 min, rinsed with PBST, and incubated with streptavidin-horseradish peroxidase (HRP). Samples were then incubated for five min with chromogen solution and rinsed with distilled water. Slides were counterstained with hematoxylin for 3 min, rinsed with distilled water, and incubated in automation buffer (NM30; Biomedica Corp.).

All mice were handled according to protocols approved by the Institutional Animal Care and Use Committee of Tufts University.

Immunodetection assays of anti-*Y. pseudotuberculosis* IgGs from mouse serum. A total of 50 μ l of heat-killed *Yersinia* (2.2 μ g of protein/ml) cells in PBS was incubated in 96-well flat bottom MaxiSorp Immuno Plates (Nunc) at 4°C overnight. Wells were rinsed six times with PBST, blocked with PBSF (PBS plus 10% fetal bovine serum) for 1 h at room temperature, and washed six times with PBST. Serum from mice was diluted 1:100 in PBSF, and 100 μ l was added to the wells and incubated for 1 h at room temperature; wells were then washed six times in PBST. Goat anti-mouse HRP-conjugated antibodies (Sigma) were diluted 1:5,000 in PBSF and incubated at room temperature for 1 h. After six washes with PBST, 100 μ l of TMB (3,3',5,5'-tetramethylbenzidine) substrate (eBioscience) was added for 6 to 12 min, followed by the addition of 50 μ l of 1 M phosphoric acid to quench the reaction. Plates were read at 450 nm and 540

nm, and the background (540 nm) was subtracted from the 450-nm reading. All dilutions were performed in duplicate, and the experiment was repeated three times. Postinfection samples were scored as positive if their values were greater than the average plus two times the error of the preinfection samples.

RESULTS

Low doses of *Y. pseudotuberculosis* colonize the lungs of infected mice. *Y. pseudotuberculosis* is generally considered an enteric pathogen; however, it has been recovered from the lungs of infected animals (50, 60). Given this observation and that *Y. pseudotuberculosis* is closely related to *Y. pestis* (1), we determined whether *Y. pseudotuberculosis* IP2666pIB1 was capable of colonizing the lungs of mice following intranasal inoculation. In preliminary experiments, BALB/c or Swiss Webster mice were inoculated intranasally with different doses of IP2666pIB1 to determine at which dose, if any, *Y. pseudotuberculosis* could establish an infection. Mice were monitored daily for signs of illness. Four days postinoculation, mice appeared ill and were sacrificed to determine bacterial loads (Fig. 1A). An initial inoculum of less than 100 CFU grew to almost 1×10^6 CFU/g of lung in BALB/c mice and 1×10^4 CFU/g of lung in Swiss Webster mice (Fig. 1A). Higher levels of colonization were observed when more bacteria were used in the initial inoculum. In mice inoculated with 8.4×10^2 or 8.4×10^3 CFU, *Y. pseudotuberculosis* levels reached an average of 1×10^7 or 5×10^7 CFU/g of lung in BALB/c mice and 5×10^6 or 5×10^7 CFU/g of lung in Swiss Webster mice, respectively. In additional experiments, CD-1 and C57BL/6 mice were intranasally infected with 500 CFU and showed similar levels of colonization to that of BALB/c (data not shown). Together, these data indicate that *Y. pseudotuberculosis* can colonize lungs of inbred and outbred strains of mice at low inoculation doses, although the bacteria replicates more efficiently in BALB/c mice than Swiss Webster mice.

To determine if other strains of *Y. pseudotuberculosis* colonized lungs as proficiently as IP2666pIB1, mice were inoculated with YPIIpIB1, and infection was allowed to proceed for 4 days. Mice inoculated with the YPIIpIB1 strain showed fewer and milder symptoms by visual observation and harbored fewer bacteria in lungs than mice infected with IP2666pIB1 (Fig. 1B). Shortly after these experiments were completed, the YPIIpIB1 strain was shown to carry an inactivating point mutation in *phoP* (27). PhoP is the response regulator of the PhoP/Q two-component signal transduction system, which is essential for intracellular growth of *Y. pseudotuberculosis* in macrophages (27, 29). We tested whether PhoP was important for colonizing lungs by inoculating mice intranasally with the IP2666pIB1 Δ *phoP* deletion strain and comparing it to its isogenic IP2666pIB1 parental strain and YPIIpIB1 (Fig. 1B). The Δ *phoP* deletion strain was on average 100 times more deficient in colonizing lungs than the isogenic wild-type

IP2666pIB1 strain (Fig. 1B), indicating that PhoP-regulated processes are important for lung colonization. The IP2666pIB1 strain background was chosen for use in subsequent experiments because of its ability to highly colonize the lungs.

Growth temperature has little effect on kinetics of growth or dissemination. Many *Yersinia* virulence factors including Yops, lipopolysaccharide (LPS), invasins, YadA, and LcrF are differentially regulated at high and low temperatures (3, 40, 44, 46). Expression or repression of any of these factors prior to inoculation could alter the kinetics, dissemination, or outcome of infection. To assess the effect of growth temperature of the inoculum of *Y. pseudotuberculosis* on these facets of infection, mice were infected with *Y. pseudotuberculosis* grown at either 26°C or 37°C. Lungs; respiratory-associated tissues, including trachea, nasopharynx, and tracheobronchial lymph nodes; and systemic tissues, including liver, spleen, and blood were harvested at 1, 2, 4, 6, and 7 days postinoculation, and the bacterial load in each tissue was determined.

Prior growth at either 26°C or 37°C had little effect on the bacterial burden or dissemination in any tissue. After an initial dose of 3×10^2 to 5×10^2 CFU, bacterial loads from both growth conditions in the lungs increased to an average of 1×10^3 to 2×10^3 CFU/g of lung at 1 day postinoculation and to 1×10^4 at 2 days postinoculation (Fig. 2A). Bacteria continued to replicate in the lungs until day 6, when they reached an average of 1×10^7 to 3×10^7 CFU/g of lung. From day 6 to day 7, bacterial loads remained steady, and most mice were moribund at day 7. No statistically significant differences between bacterial loads in lungs inoculated with bacteria grown at 26°C versus 37°C were observed in the lungs at any day except day 6, when a fourfold defect was statistically significant.

Colonization of the trachea, bronchial lymph nodes, and nasopharyngeal tissue was consistently lower than that observed in the lungs, and as in lungs, preinoculation growth temperature had little effect on the levels of *Y. pseudotuberculosis* in these respiratory tissues (Fig. 2B, C, and D). In the trachea, *Y. pseudotuberculosis* was not recovered until 4 days postinfection, and bacterial loads reached 2×10^4 CFU/g of trachea by day 6 (Fig. 2B). It should be noted, however, that no evidence of primary tracheal colonization was observed by histology (data not shown). Rather, it appeared that the bacteria recovered in the trachea were due to their high growth in the lungs and subsequent spread into the bronchial tracheal tubes. Bacteria started to disseminate to tracheobronchial lymph nodes by 4 days postinfection, about half the tracheobronchial lymph nodes were colonized by 6 days postinfection, and the majority of lymph nodes were colonized at 7 days postinfection with average levels of 2×10^2 CFU/lymph node (Fig. 2C). Bacteria were only sporadically recovered from the nasopharynx, indicating that this tissue is not a major niche of *Y. pseudotuberculosis* after intranasal inoculation (Fig. 2D).

FIG. 2. Intranasal inoculation with *Y. pseudotuberculosis* results in a lethal lung infection. BALB/c mice were inoculated with 300 to 500 CFU of *Y. pseudotuberculosis* grown at 26°C (●) or 37°C (■). Mice were sacrificed 1, 2, 4, 6, and 7 days postinoculation and lungs (A), trachea (B), lymph nodes (C), nasopharynx (D), liver (E), spleen (F), and blood (G) were harvested, homogenized, and plated for CFU. Each dot represents data from one mouse. Open symbols indicate that bacteria were below the limit of detection (note, the limit of detection per gram of trachea recovered is generally higher than in the lungs, liver, and spleen because the weight of the trachea is smaller). Bars represent the geometric mean. *, $P < 0.01$ (Student's *t* test). NP, nasopharynx; Trach, trachea.

Dissemination to the spleen, liver, and blood occurred sporadically, and levels of colonization were much lower than levels observed in the lungs. *Y. pseudotuberculosis* was detected at very low levels in some of the livers at 1 and 2 days postinfection but not in the blood and in only one spleen (Fig. 2E, F, and G). By 6 days postinfection, all but one liver harbored *Y. pseudotuberculosis* although levels ranged from 1×10^1 to 7×10^4 CFU/g of liver (Fig. 2E). No substantial colonization of either the spleen or blood was observed before 4 days postinoculation, after which it occurred sporadically (Fig. 2F and G). No significant differences were observed in any systemic tissue between bacteria grown at either 26°C or 37°C, except in the liver at day 4, when a defect in bacteria grown at 37°C was observed. These data demonstrate that following intranasal inoculation with *Y. pseudotuberculosis*, dissemination starts to occur from the lungs to other respiratory and systemic tissues by 4 days postinoculation and that bacterial growth at 26°C versus 37°C prior to inoculation has little effect on the outcome of infection. The growth within all other tissues is lower than that observed in the lungs, and the range in numbers of CFU of *Y. pseudotuberculosis* recovered in those tissues is generally much greater. Combined, these data indicate that the lungs are the primary site of infection and suggest that the morbidity reached at day 7 is most likely due to bacterial loads in the lungs.

Histopathology of lung infection with *Y. pseudotuberculosis* is consistent with pneumonia. Histological analysis was performed on lungs of mice inoculated with *Y. pseudotuberculosis* to determine the location and types of immune cells recruited to lungs and damage caused by infection over time. Lungs were harvested 1, 2, 4, 6, and 7 days postinoculation, and sections were stained with hematoxylin and eosin to visualize cells (Fig. 3). One- and two-day postinoculation lungs infected with *Y. pseudotuberculosis* appeared similar to PBS-inoculated lungs (Fig. 3A, B, F, and G, and data not shown). The alveoli were open and contained no large areas of inflammation, although occasionally small areas of congestion were observed in both PBS-treated and infected lungs (data not shown). In contrast, by 4 days postinoculation, the normal architecture of the lung was disrupted in many areas as lungs contained multifocal, suppurative, necrotic lesions, and fibrination in alveolar spaces (Fig. 3C and H). In addition, large colonies of blue-purple staining rods were observed at high magnification in regions of inflammation and necrosis. The size of the inflammatory lesions and size of the colonies increased over the course of the infection (Fig. 3D, E, I, and J), consistent with the increased bacterial loads observed during the 7-day infection. The presence of *Y. pseudotuberculosis* in these disruptive lesions was confirmed by immunohistochemistry using serum against *Y. pseudotuberculosis* (Fig. 4).

Time and dose to morbidity after *Y. pseudotuberculosis* intranasal inoculation. To determine at what dose *Y. pseudotuberculosis* infections resulted in 50% of the mice reaching morbidity, groups of 3 or 4 BALB/c mice were inoculated with different doses of IP2666pIB1 grown at 26°C or 37°C (Table 2). Mice were monitored daily for signs of illness, and those that appeared moribund were sacrificed. Fifty percent of mice inoculated with a dose of 18 CFU of *Y. pseudotuberculosis* grown at 26°C became moribund between 16 to 18 days postinfection, and 2 out of 3 mice inoculated with 48 CFU of *Y. pseudo-*

FIG. 3. Histological analysis of lungs from BALB/c mice inoculated with wild-type *Y. pseudotuberculosis*. Mice were inoculated with 300 to 500 CFU of *Y. pseudotuberculosis*, and lungs were harvested 1 (A and F), 2 (B and G), 4 (C and H), 6 (D and I), and 7 (E and J) days postinoculation, sectioned, and stained with hematoxylin and eosin. Slides are shown at a magnification of $\times 10$ (A to E) or $\times 60$ (F to J).

tuberculosis grown at 37°C became moribund (Table 2). These data indicate that the dose for 50% of mice to reach morbidity is below 50 CFU. Higher doses of *Y. pseudotuberculosis* resulted in a faster time to morbidity as mice inoculated with greater than 2×10^3 CFU of *Y. pseudotuberculosis* were moribund between 5 to 7 days postinfection whereas mice inoculated with less than 100 CFU were moribund between 11 to 18 days postinfection (Table 2).

Since all surviving mice received very low doses of *Y. pseudotuberculosis*, it was possible that they did not receive any CFU. Some surviving mice were tested for the presence of anti-*Yersinia* antibodies by enzyme-linked immunosorbent assay

FIG. 4. Immunohistochemical analysis of lung sections. Sections from mice 4 days postinoculation with PBS (A and C) or with 300 to 500 CFU of *Y. pseudotuberculosis* grown at 26°C (B and D) were stained with anti-*Y. pseudotuberculosis* antibodies. Goat anti-mouse HRP-conjugated antibody was used as a secondary stain. Brown areas represent areas of HRP activity; lighter brown/gray areas are red blood cells. Samples shown are at a magnification of $\times 10$ (A and B) and $\times 60$ (C and D). Arrow (D) indicates an area of colocalization of antibody with bacterial colonies. Boxed sections in panels A and B indicate the areas magnified in panels C and D, respectively.

(Table 2). Surviving mice that received a dose of 18 CFU or 33 CFU developed IgG antibodies to *Y. pseudotuberculosis*, indicating that the inoculation of these mice successfully delivered bacteria to the nasal cavities and/or lungs and that the mice were able to control the infection. Likewise, several mice receiving a lower inoculation dose also mounted an immune response. However, IgG antibodies to *Y. pseudotuberculosis* were detected in only $\sim 10\%$ of the tested mice inoculated at doses below 18 CFU (Table 2), indicating that the bacteria either never reached the nasal cavities or lungs or were cleared before a significant infection and/or immune response occurred.

Translocation of Yops is necessary for wild-type lung colonization. In other routes of infection, translocation of Yops is important for full virulence of *Y. pseudotuberculosis* (49). Translocation, but not secretion, of effector Yops is dependent on YopB, a component of the translocon of the TTSS. To assess the importance of translocation of Yops during lung infection, mice were infected with *Y. pseudotuberculosis* $\Delta yopB$ grown at 26°C and monitored for 42 days or infected with bacteria grown at 37°C and monitored for 14 days. Bacterial loads were determined in the lungs, trachea, bronchial lymph nodes, nasopharynx, spleen, liver, and blood. Throughout the course of infection, none of the mice displayed visible symptoms of illness. At days 1 and 2 postinfection, *Y. pseudotuberculosis* $\Delta yopB$ colonization levels were similar to wild type in the lungs; however, by day 4 little growth had occurred in the *yopB* mutant (Fig. 5A), and there was a significant difference between wild-type and $\Delta yopB$ strains. Colonization peaked at 5×10^5 CFU/g of lung 14 days postinoculation of bacteria, which was approximately 100-fold lower than peak wild-type levels when mice were moribund at day 7 (Fig. 2A). *Y. pseudotuberculosis* $\Delta yopB$ bacteria grown at 37°C were

cleared from some lungs by 14 days and by 42 days for bacteria grown at 26°C. Since mice were not monitored beyond 14 days when infected with the $\Delta yopB$ mutant grown at 37°C, it is unclear if initial growth temperature significantly affects the time of persistence in the lungs. Nonetheless, these observations indicated that the $\Delta yopB$ mutant induces a less virulent infection of lungs, which can persist for several weeks, before bacteria are cleared from infection.

The $\Delta yopB$ strain was also deficient in colonizing the trachea (Fig. 5B) and liver (Fig. 5C), and no bacteria were detected in the spleen, blood, and nasopharynx at any time point (data not shown). The $\Delta yopB$ strain reached similar, albeit low levels, as wild type in the lymph tissues at 7 days postinoculation (Fig. 5D). Together, these data indicate that translocation of Yops is critical for virulence, for efficient replication in lungs, and for dissemination to and/or colonization of systemic tissues after intranasal inoculation.

Lungs of mice infected with the $\Delta yopB$ strain were examined by histology and showed several marked differences in their histopathology compared to those infected with the wild type. Notably, the $\Delta yopB$ mutant-infected lungs showed only mild pathology by 7 days postinoculation (Fig. 6B and F). Inflammatory lesions had no significant necrosis or cell debris at that time. By 28 days postinoculation, two types of lesions were

TABLE 2. LD₅₀ of *Y. pseudotuberculosis* in intranasal infection of BALB/c mice

Strain (culture condition)	Dose (no. of CFU/mouse)			No. of dead mice/total no. of mice	Day of death(s)	IgG ⁺ ^a
	Expt. 1	Expt. 2	Expt. 3			
IP2666pIB1 (26°C)	0.8	0.8		0/7		1/4
			1.6	0/3		0/3
		3		0/3		1/4
	4		4	2/6	15, 17	0/3
		6		0/4		0/4
		18		2/4	16, 18	2/2
	26		26	5/6	12, 13, 14, 15, 17	
			61	3/3	11, 11, 16	
		93		4/4	11, 11, 11, 13	
	182			3/3	11, 12, 13	
IP2666pIB1 (37°C)			460	3/3	9, 9, 10	
	496			3/3	8, 8, 8	
		9,280		4/4	5, 5, 5, 7	
	1.3			0/3		
		1.7		0/3		0/3
	2.2			1/3	18	
		4.0		0/3		0/3
		33		1/3	11	2/2
	48			2/3	11, 12	
	140			3/3	9, 9, 10	
IP2666 $\Delta yopH$ (26°C)		2,360		3/3	5, 5, 6	
	3,800			3/3	5, 5, 5	
	3			0/3		0/3
	16			0/3		1/1
	182			1/3	16	2/2
	1,192			2/3	10, 12	1/1
	9,900			3/3	9, 10, 10	

^a No. of seropositive mice/total no. of mice. The number of seropositive mice was determined by enzyme-linked immunosorbent assay against heat-killed *Yersinia*.

FIG. 5. YopB is essential for high levels of colonization and dissemination. BALB/c mice were infected with 300 to 500 CFU of *Y. pseudotuberculosis* $\Delta yopB$ grown at 26°C (●) or 37°C (■). Mice inoculated with the $\Delta yopB$ mutant grown at 26°C were sacrificed at 1, 2, 4, 6, 7, 14, 21, 28, 35, and 42 days postinoculation, and mice inoculated with $\Delta yopB$ strain grown at 37°C were sacrificed at 1, 2, 4, 7, and 14 days postinoculation. Lungs (A), trachea (B), liver (C), and lymph nodes (D) were harvested, homogenized, and plated for CFU. Each circle represents the CFU from a mouse inoculated with the $\Delta yopB$ strain grown at 26°C, and each square represents CFU from a mouse inoculated with the $\Delta yopB$ strain grown at 37°C. Open symbols indicate that no bacteria were recovered at that limit of detection. Bars represent the geometric mean. P values were determined between the levels

FIG. 6. Histological analysis of lungs from BALB/c mice inoculated with $\Delta yopB$ *Y. pseudotuberculosis*. Mice were inoculated with 300 to 500 CFU of *Y. pseudotuberculosis*, and lungs were harvested 1 (A and E), 7 (B and F), 28 (C and G), and 42 (D and H) days postinoculation. Lungs were sectioned and stained with hematoxylin and eosin. Slides show lungs at a magnification of $\times 10$ (A to D) or $\times 60$ (E to H).

found in the lungs of the *yopB* strain-infected mice. Large granulomatous lesions were found that contained foamy macrophages surrounding a necrotic center (Fig. 6C) which increased in size through 42 days postinoculation (Fig. 6D). In addition, several smaller lesions, similar to those observed at day 7, were found scattered throughout the lungs. Combined, these data indicate that the *yopB* mutant induces a slower and more controlled, though chronic, state of inflammation compared to that observed with wild-type *Y. pseudotuberculosis* infection in lungs.

A *yopH* mutant strain is attenuated in intranasal infections. To investigate which of the translocated, effector Yops are important for lung colonization, mice were intranasally inocu-

of the $\Delta yopB$ strain recovered as shown here and levels of wild-type *Y. pseudotuberculosis* shown in Fig. 2 using a two-tailed Student's *t* test. *, $P < 0.01$ between wild type and *yopB* mutant grown at 26°C; +, $P < 0.01$ between wild type and *yopB* mutant grown at 37°C.

FIG. 7. Colonization of lungs of BALB/c mice with *yop* mutant strains 4 days after intranasal inoculation in single-strain and competition infections. (A) Mice were inoculated with $\sim 8 \times 10^2$ CFU wild-type *Y. pseudotuberculosis* or an isogenic mutant of *yopB*, *yopH*, *yopO*, *yopM*, *yopE*, *yopJ*, or a *yopEOM* triple mutant or a strain of IP2666 lacking the virulence plasmid (pIB1⁻). Four days postinoculation lungs were harvested, homogenized, and plated for CFU. (B) BALB/c mice were inoculated with $\sim 8 \times 10^2$ CFU of an equal mixture of MLF-13 and either IP2666pIB1 or a *yopB*, *yopH*, *yopO*, *yopM*, *yopE*, or *yopJ* mutant or a *yopEOM* triple mutant. Four days postinoculation lungs were harvested, homogenized, and plated on L plates containing X-Gal, and the ratios of wild type to mutants were determined. Each dot represents data from one mouse. Open circles indicate that no bacteria were recovered at the limit of detection. Bars represent the geometric mean. *P* values were calculated by a two-tailed Student's *t* test. *, *P* < 0.05 for the number of CFU of wild-type *Y. pseudotuberculosis* recovered versus the number of CFU of a mutant strain.

lated with *Y. pseudotuberculosis* IP2666pIB1 strains containing deletions of one or more *yop* genes (Fig. 7). Mice were inoculated with 8×10^2 CFU of either wild-type IP2666pIB1, $\Delta yopB$, IP2666pIB1⁻ (lacking the virulence plasmid), $\Delta yopH$, $\Delta yopO$, $\Delta yopM$, $\Delta yopE$, $\Delta yopJ$, or the $\Delta yopEOM$ triple mutant strain. Mice were sacrificed 4 days postinoculation, a time point chosen because colonization levels were high with wild-type infection and the $\Delta yopB$ strain was severely attenuated. The IP2666pIB1⁻ strain, like the $\Delta yopB$ mutant, grew approximately 1,000-fold less than wild-type *Y. pseudotuberculosis* mice and showed no signs of illness over the course of the 4-day infection (Fig. 7A). These results suggest that no other factors encoded on pIB1 play additional, significant roles in the absence of Yop translocation in infected lungs. Although mice infected with the *yopH* strain became ill, this strain had a

FIG. 8. Colonization of *Y. pseudotuberculosis yopH* mutants in competition with wild-type *Y. pseudotuberculosis* 4 days postinoculation. BALB/c mice were inoculated with $\sim 8 \times 10^2$ CFU of an equal mixture of MLF-13 and either the MLF-9, *yopH*(R409A), *yopH*(K342A), *yopH*(V31G), *yopH*(Q11R), or *yopH* Δ 223–226 strain. Bacteria from lungs were grown on L plates containing X-Gal, and the competitive index was calculated as described in Materials and Methods. Black circles represent the CI from individual mice, open circles indicate that no mutants were detected, and bars represent the geometric mean. *P* values were determined by a two-tailed Student's *t* test. *, *P* < 0.05.

10-fold defect in colonization relative to wild-type *Y. pseudotuberculosis*, indicating that YopH enhances replication in lungs. Single *yopJ*, *yopE*, *yopO*, or *yopM* deletion strains were not attenuated 4 days postinoculation. Furthermore, the *yopEOM* triple mutant showed no observable attenuation in a single-strain infection (Fig. 7A), indicating that these three Yops do not function redundantly in this model of infection.

To assess whether the single *yop* mutant strains were more attenuated in the presence of wild-type *Y. pseudotuberculosis*, a phenomenon which has been observed in other routes of infection (49), coinfection experiments were performed. Mice were intranasally inoculated with an equal mixture of the reference strain, IP2666pIB1 Δ lacZ and either wild-type IP2666pIB1 or the $\Delta yopB$, $\Delta yopH$, $\Delta yopO$, $\Delta yopM$, $\Delta yopE$, $\Delta yopJ$, or $\Delta yopEOM$ mutant. As expected, the reference strain was as fit as the unmarked wild-type *Y. pseudotuberculosis* strain, with an average CI of 1 (Fig. 7B). As in single-strain infections, the *yopB* mutant was severely attenuated, and the $\Delta yopH$ strain was attenuated 10-fold relative to wild-type *Y. pseudotuberculosis* (Fig. 7B). Furthermore, the $\Delta yopO$, $\Delta yopM$, $\Delta yopE$, $\Delta yopJ$, or $\Delta yopEOM$ strain competed as well as wild-type *Y. pseudotuberculosis* (Fig. 7B). Thus, in contrast to competition studies after orogastric inoculation, none of the single *yop* mutants was more attenuated in the presence of wild-type *Y. pseudotuberculosis* than in the absence of wild-type *Y. pseudotuberculosis* (Fig. 7A) at this time point.

To further investigate the role of YopH in lung colonization, two additional types of infections were carried out. First, coinfection studies were performed with a series of *yopH* point mutations or small deletions with wild-type *Y. pseudotuberculosis* (Fig. 8). The *yopH* mutations studied were *yopHR409A*, a catalytically inactive mutant (41, 89); *yopHQ11R* and *yopHV31G*, which are deficient in binding to the focal adhesion protein p130CAS (58); *yopHK342A* which participates in binding of phosphopeptides to a second site in YopH (41); and

FIG. 9. Colonization of *Y. pseudotuberculosis* *yopH* double mutants. Mice were intranasally inoculated with $\sim 8 \times 10^2$ CFU of wild-type *Y. pseudotuberculosis* or mutants of the *yopHE*, *yopHO*, *yopHM*, *yopHJ*, or *yopEOMJ* strains. Four days postinoculation lungs were weighed, homogenized, and plated for CFU. Black circles represent the CFU recovered from individual mice, open circles indicate that the number of CFU was below the limit of detection, and bars represent the geometric mean. *P* values were determined by a two-tailed Student's *t* test. *, *P* < 0.05.

*yopH*Δ223–226 which is defective in binding to focal adhesion complexes in cultured cells (68). The catalytically inactive mutant was 100-fold attenuated compared to wild-type *Y. pseudotuberculosis* (Fig. 8). In contrast, none of the other mutations showed significant colonization defects in this model of infection, indicating that these functions of YopH are not required for lung colonization at day 4. Second, the estimated LD₅₀ of a *yopH* mutant was determined to be approximately 575 CFU (Table 2) by the method of Reed and Muench (72), which is ~20- to 30-fold higher than that of wild-type *Y. pseudotuberculosis*. This indicates that YopH is involved in causing morbidity as well as in colonization of the lungs.

The observation that the $\Delta yopB$ strain was more defective in lung colonization than the $\Delta yopH$ strain (*P* < 0.01), suggested that one or more additional effector Yops may play a significant role in lung colonization in the absence of YopH. Thus, a series of double *yop* mutants was tested for their ability to colonize lungs after intranasal inoculation. All four double mutants, $\Delta yopHO$, $\Delta yopHE$, $\Delta yopHM$, and $\Delta yopHJ$, were more attenuated in their ability to colonize lungs than the $\Delta yopH$ strain (Fig. 9 and 7A), indicating that each of these Yops contributes to colonization in the absence of YopH. Moreover, the $\Delta yopEOMJ$ strain was significantly more attenuated than the $\Delta yopEOM$ strain (Fig. 7A), suggesting that *yopJ* plays an important role in lung colonization in the absence of *yopE*, *yopO*, and *yopM*.

DISCUSSION

Here, we demonstrate that intranasal inoculation with *Y. pseudotuberculosis* causes a fulminant lung infection in mice.

Bacterial growth at 26°C or 37°C prior to inoculation had no effect on initial colonization levels, dissemination, or time to morbidity, indicating that virulence factors upregulated at either temperature are equally capable of initiating infection. For instance, Yops are expressed at 37°C, but our data indicate that bacteria not expressing *yop* genes immediately prior to inoculation are as fit as those that do. It is clear, however, that translocation of Yops is essential for virulence by 96 h postinoculation as the $\Delta yopB$ mutant was significantly attenuated at this time. Expression of *yadA* and *inv* is also regulated by temperature; *yadA* is expressed at 37°C but not 26°C (10, 43), and *inv* is inversely regulated (10, 40). Our data indicate that bacteria expressing either of these factors immediately prior to inoculation are equally fit to cause lung infection. It is of interest to determine whether removing these factors from *Y. pseudotuberculosis* altered the course of intranasal infection of mice. Finally, the LPS of *Y. pseudotuberculosis* has different modifications depending on growth temperature (6, 70), but these changes did not alter initial colonization levels or dissemination to other tissues.

Low doses of *Y. pseudotuberculosis* resulted in robust lung colonization, illness, and lethality, as 50% of the mice infected with 18 CFU and 100% of mice infected with 61 CFU of *Y. pseudotuberculosis* became moribund within 17 days. Interestingly, the LD₅₀ of *Y. pestis* in C57BL/6 mice after intranasal inoculation is $\sim 3.0 \times 10^2$ CFU, which is about 10-fold higher than what we observed with *Y. pseudotuberculosis* (47, 86). However, the course of infection with *Y. pestis* is much faster as mice succumb to infection by day 3 or 4 postinoculation. Furthermore, *Y. pestis* levels in the lungs were higher, and dissemination to other tissues occurred sooner (by day 3) and more uniformly (36, 47). The lower levels of colonization of *Y. pseudotuberculosis* and slower time to morbidity relative to *Y. pestis* are unlikely due to the difference in mouse background as *Y. pseudotuberculosis* colonization of C57BL/6 mice is similar to that of BALB/c mice (data not shown). Furthermore, both Swiss Webster and BALB/c mice succumb to aerosolized or intranasal inoculation of lethal doses of *Y. pestis* within 4 days (36, 42, 71, 86). Combined, these data indicate that the more rapid morbidity associated with *Y. pestis* infection is likely a result of a physiological difference between *Y. pseudotuberculosis* and *Y. pestis*. For example, *Y. pestis* has a shorter LPS (66) and a different lipid A composition that is modified during growth at 37°C (70), elaborates a peptide capsule (16), and lacks functional copies of *inv* (75) and *yadA* (76). Furthermore, while *Y. pestis* has a functional copy of *yopT*, some infectious *Y. pseudotuberculosis* serotypes, including the serotype tested here, do not (83). One or more of these differences could affect the host response to infection, which, in turn, may alter the course of infection.

Our histopathological analysis indicated that *Y. pseudotuberculosis*-induced pneumonia had features resembling those caused by *Y. pestis*, although inflammation arose at later time points after infection. The lungs of mice inoculated with low doses of both *Yersinia* species were quiescent early in infection (47), whereas later—day 2 for *Y. pestis* and day 4 for *Y. pseudotuberculosis*—pathology became evident. Specifically, lungs of mice intranasally inoculated with *Y. pestis* had an influx of neutrophils, extracellular bacteria, and hemorrhaging at foci of infection by 2 days postinoculation (47, 71), similar to effects

observed with *Y. pseudotuberculosis* by 4 days postinoculation. In human cases, pneumonic plague is associated with high bacterial loads, multifocal pneumonia, increased abundance of neutrophils, fibrination of alveolar spaces, and pulmonary edema (31). The lungs of cats and African green monkeys infected with *Y. pestis* demonstrate nearly identical pathologies (23, 87). One difference between the *Y. pestis* and *Y. pseudotuberculosis* histopathology is that in *Y. pseudotuberculosis* infections, we often observe large colonies of bacteria growing in tight masses such as those depicted in Fig. 3I as well as smaller, more dispersed microcolonies (Fig. 4D). These large, tightly packed colonies are reminiscent of what we observe with *Y. pseudotuberculosis* infections in other organs, such as the mesenteric lymph nodes after oral infection (4) and spleen after intravenous infection (M. McCoy and J. Meccas, unpublished data).

The low infectious dose of *Y. pseudotuberculosis* in mice is particularly striking compared to the LD₅₀ of other respiratory pathogens. For instance, *Streptococcus pneumoniae* has an LD₅₀ of 1×10^5 CFU/mouse in intranasally infected MF-1 mice (17), but the same dose is ineffective in causing disease in the Swiss Webster mouse line, indicating an even higher lethal dose in this background (35). Similarly, the LD₅₀ of *Pseudomonas aeruginosa* is approximately 1×10^7 CFU/mouse in the C3H/HeJ background (25). Sublethal doses of *P. aeruginosa* fail to grow in the lungs of mice and are cleared within 14 days (25, 26), whereas high doses result in death within 48 h (26). Murine lung models have also been used to study other gram-negative pathogens such as *Vibrio cholerae* and *Shigella* spp. (24, 51, 69, 73). As with non-*Yersinia* respiratory pathogens, these organisms generally require high intranasal doses between 1×10^6 and 2×10^8 CFU/mouse. Despite the fact that mice become ill following exposure to these organisms, the bacteria decrease in number within hours to days following inoculation. Generally, lungs from these mice demonstrate mild to moderate pathology between 6 and 24 h postinoculation. In the *V. cholerae* model, half the mice do not survive 24 h postinoculation, and those that do survive have moderate pneumonia and fibrination in the lungs (24). Mice intranasally infected with *Shigella flexneri* develop moderate inflammation within 6 h but then resolve it within 48 h postinoculation (73). In the model presented here, low inoculation doses of *Y. pseudotuberculosis* grow to high levels in the lung and induce a pronounced pneumonia over the course of a week. These aspects of *Y. pseudotuberculosis* lung infection will aid in the study of both bacterial virulence factors involved in the development of pneumonia, in the development of host defenses in lungs, and in the testing of prophylactic therapeutics and therapeutics delivered after infection has occurred.

Mice infected with the $\Delta yopB$ strain showed no visible signs of illness up to 42 days postinoculation, but bacteria were recovered in some lungs 42 days after inoculation. Consistently, the histopathology of wild-type *Y. pseudotuberculosis*-infected mouse is vastly different from that of infection with the $\Delta yopB$ strain. Lungs infected with wild type showed severe pathology, while lungs infected with the $\Delta yopB$ strain appeared healthy up to 6 days postinfection. Several weeks postinoculation with the $\Delta yopB$ strain, infected lungs presented large, structured granulomas containing lymphocytes and foamy macrophages not observed in a wild-type infection. The histo-

pathology of the $\Delta yopB$ strain infection demonstrates chronic elements, such as collagen deposits and lymphocytic, granulomatous formations reminiscent of structures formed in the intragranulomatous necrosis in a pulmonary granulomas model (32). Although the $\Delta yopB$ strain has not been tested in a *Y. pestis* background in a mouse model of pneumonic plague, a strain lacking the virulence plasmid was cleared after 3 days postintranasal inoculation (47). A *Y. pestis* $\Delta yopB$ strain might induce a similar inflammatory state as its *Y. pseudotuberculosis* counterpart. On the other hand, *Y. pseudotuberculosis* may have other factors that allow for its persistence, as a plasmid-minus strain of *Y. pseudotuberculosis* is cleared from lungs less rapidly than either *Y. pestis* or *Y. enterocolitica* following intravenous inoculation (80).

Fewer Yops appeared to be required for colonization of lungs after intranasal inoculation than with other routes of infection as single mutants of $\Delta yopE$, $\Delta yopO$, $\Delta yopM$, and $\Delta yopJ$ did not show any significant attenuation in lungs 4 days after intranasal inoculation. However, all are attenuated in at least one other route of infection with various *Yersinia* species (48, 49, 56, 79). Although deletions of these *yop* genes had little effect on lung colonization at day 4 postinoculation, a role for these Yops might be observed in colonization or morbidity studies if the infection proceeded longer or if lower inoculation doses were given to mice. Our data suggest that YopE, YopO, or YopM enhances or augments the role of YopJ in lung colonization since in the absence of all four Yops, the $\Delta yopEOMJ$ strain colonized the lungs poorly, whereas a $\Delta yopEOM$ strain colonized lungs efficiently. While YopJ, YopO, YopM, and YopE have different biochemical activities and bind to different host proteins (5, 8, 54, 63), most of these Yops alter the ability of macrophages and/or neutrophils to respond normally to bacteria (30, 45, 57), supporting the idea that these Yops may all inactivate the same cell type(s) during lung infection, albeit by different mechanisms. In fact, our data with double mutants consisting of YopH and these other effector Yops suggest that these other Yops play redundant roles in infection.

The observation that YopH is required for productive infection after many different routes of inoculation (9, 41, 45, 49, 79) implies that YopH has multiple roles in infection and/or that its function is required for survival in many different tissues. Curiously, the catalytically inactive *yopH* mutant, *yopH(R409A)*, was even more attenuated than the null mutant. Translocation of this mutant could reduce the translocation levels of the other effector Yops in vivo, and thus there are fewer Yops in key cells to counteract host responses, rendering the bacteria more sensitive to host defenses. Alternatively, the presence of the mutant protein may sequester intracellular host targets, leading to an unfavorable environment for *Yersinia*. When other effector *yop* deletions were introduced into the $\Delta yopH$ strain background, colonization was attenuated by approximately 10-fold compared to the $\Delta yopH$ strain, indicating that in the absence of YopH, the lack of these Yops renders the bacteria more susceptible to host defenses and/or less capable of establishing an infection. One possible explanation for this result is that in the absence of YopH, the host can mount a more aggressive response to *Yersinia* (74), and a concomitant loss of other Yops leaves *Yersinia* more vulnerable to these host defenses than when the bacteria express YopH.

During our analysis it became apparent that PhoP is important in lung colonization as IP2666 Δ phoP was attenuated for growth in lungs by 100-fold compared to wild type. PhoP is important for virulence of *Y. pseudotuberculosis* and *Y. pestis* when infection is initiated by other routes of inoculation. Subcutaneous inoculation of a *Y. pestis* Δ phoP mutant demonstrated that there was a 75-fold increase in the LD₅₀ compared to wild-type *Y. pestis* (64). Similarly, oral inoculation of a *Y. pseudotuberculosis* Δ phoP mutant showed that the LD₅₀ was higher than that of an isogenic wild-type *Y. pseudotuberculosis* strain (27). Both *Y. pseudotuberculosis* and *Y. pestis* require PhoP to replicate in macrophages (27, 64). Although histological analysis showed that *Y. pseudotuberculosis* was amassed in large extracellular colonies at 4 days postinoculation, it is possible that replication in macrophages early in infection is necessary for the establishment of this fulminant infection (15). Alternatively, since PhoP regulates a number of genes (28, 52, 64, 88, 90), a Δ phoP strain may be deficient in lung colonization due to its inability to appropriately regulate genes involved in other facets of *Yersinia* physiology.

Given the low infectious dose of *Y. pseudotuberculosis* in mice, it is remarkable that we could find only one case in the literature of pneumonia in humans that may have been triggered by aerosol-borne *Y. pseudotuberculosis* (33). The infrequency of documented human cases of pneumonia triggered by enteric *Yersinia* pathogens (7, 33, 81) may reflect differences in lung anatomy between mice and humans (38), differences in response to infection with enteric *Yersinia* or gram-negative pathogens between mice and humans (22), or the infrequency of encountering aerosolized enteric *Yersinia*. Alternatively, humans may be more resistant to infection because humans are outbred, and the mice used for most of the studies are inbred. Indeed, the outbred strain of mice used here was more resistant to *Y. pseudotuberculosis* replication. It is noteworthy that experimentally induced pneumonia with aerosolized enteric *Yersinia* has been documented in several other mammals (20, 82), and there are cases of the recovery of enteric *Yersinia* from the lungs of infected animals (7, 12, 50, 60, 61), although in such cases spread to the lungs most likely occurred after oral ingestion and subsequent systemic spread.

In conclusion, we have described an intranasal model system of lung infection in mice using *Y. pseudotuberculosis*. There are several noteworthy differences between *Y. pseudotuberculosis* and *Y. pestis* which may account for the differences in the rates of growth in the lungs and kinetics of dissemination in mice, including the LPS structure, the absence of YdaA and Inv in *Y. pestis*, and the presence of two additional virulence factors. Determining whether these or other differences between *Y. pestis* and *Y. pseudotuberculosis* affect dissemination and/or alter the need for different Yops during lung infection will give insights into host defenses in lungs, as well as effective and/or redundant bacterial mechanisms to counteract host defenses during infection. In addition, it is of interest to determine whether virulence factors such as PhoP, YopB, and YopH are also important in *Y. pestis* infection of lungs. PhoP is important for virulence of both *Y. pestis* and *Y. pseudotuberculosis* when infection is initiated by other routes of inoculation (27, 64). Given the similarities and differences between the course of infection of *Y. pestis* and *Y. pseudotuberculosis*, it is a reasonable hypothesis that some of the virulence factors found to be

important in *Y. pseudotuberculosis* infection will also be involved in lung infection with *Y. pestis*, while others may be important only in one species. By understanding the infection of lungs with *Y. pseudotuberculosis*, we can begin identifying common virulence factors between these two species of *Yersinia* and test potential therapeutics and/or vaccines directed against features common to both.

ACKNOWLEDGMENTS

We thank members of the Mecas Laboratory for strains, advice, and critical reading of the manuscript; Andrew Camilli and Julianne LeMieux for demonstrating intranasal inoculations, helpful discussions, and critical reading of the manuscript; Lauren Richey for professional analysis of histological samples; James Bliska for providing the phoP mutant and yopH point mutants and their isogenic parental strains prior to publication; and Molly Bergman for the enzyme-linked immunosorbent assay protocol.

This work was funded by NIH grant R01-AI056068 to J.M. M.L.F. was funded by the NIH Molecular Analysis of Microbial Pathogens 5T32GM07310 and Molecular Genetics of Basic Cell Function 5T32GM007310 training grants. C.C. was funded by training grant GM66567. We are grateful to the Center for Gastroenterology Research on Absorptive and Secretory Processes funded by NIDDK P30-34928 and their help in preparing histological samples and reagents.

REFERENCES

- Achtman, M., K. Zurth, G. Morelli, G. Torrea, A. Guiyoule, and E. Carniel. 1999. *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. *Proc. Natl. Acad. Sci. USA* **96**:14043–14048.
- Angelichio, M. J., D. S. Merrell, and A. Camilli. 2004. Spatiotemporal analysis of acid adaptation-mediated *Vibrio cholerae* hyperinfectivity. *Infect. Immun.* **72**:2405–2407.
- Anisimov, A. P., S. V. Dentovskaya, G. M. Titareva, I. V. Bakhteeva, R. Z. Shaikhutdinova, S. V. Balakhonov, B. Lindner, N. A. Kocharova, S. y. N. Senchenkova, O. Holst, G. B. Pier, and Y. A. Knirel. 2005. Intraspecies and temperature-dependent variations in susceptibility of *Yersinia pestis* to the bactericidal action of serum and to polymyxin B. *Infect. Immun.* **73**:7324–7331.
- Balada, J. M., and J. Mecas. 2006. *Yersinia* has a tropism for B and T cell zones of lymph node that is independent of the type III secretion system. *PLOS Pathog.* in press.
- Barz, C., T. N. Abahji, K. Trulzsch, and J. Heesemann. 2000. The *Yersinia* Ser/Thr protein kinase YpkA/YopO directly interacts with the small GTPases RhoA and Rac-1. *FEBS Lett.* **482**:139–143.
- Bengoechea, J., B. Lindner, U. Seydel, R. Diaz, and I. Moriyon. 1998. *Yersinia pseudotuberculosis* and *Yersinia pestis* are more resistant to bactericidal cationic peptides than *Yersinia enterocolitica*. *Microbiology* **144**:1509–1515.
- Bigler, R. D., R. R. Atkins, and E. J. Wing. 1981. *Yersinia enterocolitica* lung infection. *Arch. Intern. Med.* **141**:1529–1530.
- Black, D. S., and J. B. Bliska. 2000. The RhoGAP activity of the *Yersinia pseudotuberculosis* cytotoxin YopE is required for antiphagocytic function and virulence. *Mol. Microbiol.* **37**:515–527.
- Bliska, J., K. Guan, J. Dixon, and S. Falkow. 1991. Tyrosine phosphate hydrolysis of host proteins by an essential *Yersinia* virulence determinant. *Proc. Natl. Acad. Sci. USA* **88**:1187–1191.
- Bolin, L., 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.
- Bottomone, E. J. 1997. *Yersinia enterocolitica*: the charisma continues. *Clin. Microbiol. Rev.* **10**:257–276.
- Bresnahan, J. F., U. G. Whitworth, Y. Hayes, E. Summers, and J. Pollock. 1984. *Yersinia enterocolitica* infection in breeding colonies of ruffed lemurs. *J. Am. Vet. Med. Assoc.* **185**:1354–1356.
- Butler, S. M., and A. Camilli. 2004. Both chemotaxis and net motility greatly influence the infectivity of *Vibrio cholerae*. *Proc. Natl. Acad. Sci. USA* **101**:5018–5023.
- Carnoy, C., C. Mullet, H. Muller-Alouf, E. Leteurtre, and M. Simonet. 2000. Superantigen YPMa exacerbates the virulence of *Yersinia pseudotuberculosis* in mice. *Infect. Immun.* **68**:2553–2559.
- Cavanaugh, D. C., and R. Randall. 1959. The role of multiplication of *Pasteurella pestis* in mononuclear phagocytes in the pathogenesis of flea-borne plague. *J. Immunol.* **83**:348–363.
- Chen, T. H., and S. S. Elberg. 1977. Scanning electron microscopic study of virulent *Yersinia pestis* and *Yersinia pseudotuberculosis* type 1. *Infect. Immun.* **15**:972–977.

17. Chiavolini, D., G. Memmi, T. Maggi, F. Iannelli, G. Pozzi, and M. Oggioni. 2003. The three extra-cellular zinc metalloproteinases of *Streptococcus pneumoniae* have a different impact on virulence in mice. *BMC Microbiol.* **3**:14.
18. Cornelis, G., J.-C. Vanootegem, and C. Sluiter. 1987. Transcription of the *yop* regulon from *Y. enterocolitica* requires trans acting pYV and chromosomal genes. *Microb. Pathog.* **2**:367–379.
19. Cornelis, G. R. 2000. Molecular and cell biology aspects of plague. *Proc. Natl. Acad. Sci. USA* **97**:8778–8783.
20. Di Genaro, M. S., M. E. Escudero, C. Aguilera, L. Scardapane, and A. M. de Guzman. 1998. Intranasal immunization with *Yersinia enterocolitica* O:8 cellular extract protects against local challenge infection. *Microbiol. Immunol.* **42**:781–788.
21. Doll, J. M., P. S. Zeitz, P. Ettestad, A. L. Bucholtz, T. Davis, and K. Gage. 1994. Cat-transmitted fatal pneumonic plague in a person who traveled from Colorado to Arizona. *Am. J. Trop. Med. Hyg.* **51**:109–114.
22. Eskay, R. L., M. Grino, and H. T. Chen. 1990. Interleukins, signal transduction, and the immune system-mediated stress response. *Adv. Exp. Med. Biol.* **274**:331–343.
23. Fingold, J. M. 1969. Pneumonic plague in monkeys. An electron microscopic study. *Am. J. Pathol.* **54**:167–183.
24. Fullner, K. J., J. C. Boucher, M. A. Hanes, G. K. Haines, III, B. M. Meehan, C. Walchle, P. J. Sansonetti, and J. J. Mekalanos. 2002. The contribution of accessory toxins of *Vibrio cholerae* O1 El Tor to the proinflammatory response in a murine pulmonary cholera model. *J. Exp. Med.* **195**:1455–1462. doi:10.1084/jem.20020318.
25. George, S. E., M. J. Kohan, M. I. Gilmour, M. S. Taylor, H. G. Brooks, J. P. Creason, and L. D. Claxton. 1993. Pulmonary clearance and inflammatory response in C3H/HeJ mice after intranasal exposure to *Pseudomonas* spp. *Appl. Environ. Microbiol.* **59**:3585–3591.
26. George, S. E., M. J. Kohan, D. A. Whitehouse, J. P. Creason, C. Y. Kawanishi, R. L. Sherwood, and L. D. Claxton. 1991. Distribution, clearance, and mortality of environmental pseudomonads in mice upon intranasal exposure. *Appl. Environ. Microbiol.* **57**:2420–2425.
27. Grabenstein, Jens P., M. Marceau, C. Pujol, M. Simonet, and J. B. Bliska. 2004. The response regulator PhoP of *Yersinia pseudotuberculosis* is important for replication in macrophages and for virulence. *Infect. Immun.* **72**:4973–4984.
28. Grabenstein, Jens P., H. S. Fukuto, L. E. Palmer, and J. B. Bliska. 2006. Characterization of phagosome trafficking and identification of PhoP-regulated genes important for survival of *Yersinia pestis* in macrophages. *Infect. Immun.* **74**:3727–3741.
29. Groisman, E. A. 2001. The pleiotropic two-component regulatory system PhoP-PhoQ. *J. Bacteriol.* **183**:1835–1842.
30. Grosdent, N., I. Maridonneau-Parini, M. P. Sory, and G. R. Cornelis. 2002. Role of Yops and adhesins in resistance of *Yersinia enterocolitica* to phagocytosis. *Infect. Immun.* **70**:4165–4176.
31. Guarner, J., W. Shieh, P. Greer, J. Gabastou, M. Chu, E. Hayes, K. Nolte, and S. Zaki. 2002. Immunohistochemical detection of *Yersinia pestis* in formalin-fixed, paraffin-embedded tissue. *Anat. Pathol.* **117**:205–209.
32. Guirado, E., S. Gordillo, O. Gil, J. Diaz, G. Tapia, C. Vilaplana, V. Ausina, and P.-J. Cardona. 2006. Intragranulomatous necrosis in pulmonary granulomas is not related to resistance against *Mycobacterium tuberculosis* infection in experimental murine models induced by aerosol. *Int. J. Exp. Pathol.* **87**:139–149.
33. Hagiwara, S., Y. Ishii, Y. Sugiyama, and S. Kitamura. 1995. Hypersensitivity pneumonitis caused by a home humidifier. *Nihon Kyobu Shikkan Gakkai Zasshi* **33**:1024–1029. [In Japanese.]
34. Han, Y., and V. Miller. 1997. Reevaluation of the virulence phenotype of the *inv yadA* double mutants of *Yersinia pseudotuberculosis*. *Infect. Immun.* **65**:327–330.
35. Hava, D. L., and A. Camilli. 2002. Large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors. *Mol. Microbiol.* **45**:1389–1406.
36. Honko, A. N., N. Sriranganathan, C. J. Lees, and S. B. Mizel. 2006. Flagellin is an effective adjuvant for immunization against lethal respiratory challenge with *Yersinia pestis*. *Infect. Immun.* **74**:1113–1120.
37. Hubbard, W. T., C. W. Petenyi, L. A. Glasgow, C. T. Uyeda, and S. A. Creighton. 1971. *Yersinia pseudotuberculosis* infection in the United States. Septicemia, appendicitis, and mesenteric lymphadenitis. *Am. J. Trop. Med. Hyg.* **20**:679–684.
38. Irvin, C. G., and J. H. Bates. 15 May 2003. Measuring the lung function in the mouse: the challenge of size. *Respir. Res.* **4**:4. [Epub ahead of print.]
39. Isberg, R. R., and J. M. Leong. 1990. Multiple beta 1 chain integrins are receptors for invasive, a protein that promotes bacterial penetration into mammalian cells. *Cell* **60**:861–871.
40. Isberg, R. R., A. Swain, and S. Falkow. 1988. Analysis of expression and thermoregulation of the *Yersinia pseudotuberculosis inv* gene with hybrid proteins. *Infect. Immun.* **56**:2133–2138.
41. Ivanov, M. I., J. A. Stuckey, H. L. Schubert, M. A. Saper, and J. B. Bliska. 2005. Two substrate-targeting sites in the *Yersinia* protein tyrosine phosphatase co-operate to promote bacterial virulence. *Mol. Microbiol.* **55**:1346–1356.
42. Jones, T., J. J. Adamovicz, S. L. Cyr, C. R. Bolt, N. Bellerose, L. M. Pitt, G. H. Lowell, and D. S. Burt. 2006. Intranasal protollin (TM)/F1-V vaccine elicits respiratory and serum antibody responses and protects mice against lethal aerosolized plague infection. *Vaccine* **24**:1625–1632.
43. 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.
44. Kawahara, K., H. Tsukano, H. Watanabe, B. Lindner, and M. Matsuura. 2002. Modification of the structure and activity of lipid A in *Yersinia pestis* lipopolysaccharide by growth temperature. *Infect. Immun.* **70**:4092–4098.
45. Kerschen, E. J., D. A. Cohen, A. M. Kaplan, and S. C. Straley. 2004. The plague virulence protein YopM targets the innate immune response by causing a global depletion of NK cells. *Infect. Immun.* **72**:4589–4602.
46. Lambert de Rouvroit, C., C. Sluiter, and G. R. Cornelis. 1992. Role of the transcriptional activator, VirF, and temperature in the expression of the pYV plasmid genes of *Yersinia enterocolitica*. *Mol. Microbiol.* **6**:395–409.
47. Lathem, W. W., S. D. Crosby, V. L. Miller, and W. E. Goldman. 2005. Progression of primary pneumonic plague: a mouse model of infection, pathology, and bacterial transcriptional activity. *Proc. Natl. Acad. Sci. USA* **102**:17786–17791.
48. Leung, K. Y., B. S. Reisner, and S. C. Straley. 1990. YopM inhibits platelet aggregation and is necessary for virulence of *Yersinia pestis* in mice. *Infect. Immun.* **58**:3262–3271.
49. Logsdon, L. K., and J. Mecsas. 2003. Requirement of the *Yersinia pseudotuberculosis* effectors YopH and YopE in colonization and persistence in intestinal and lymph tissues. *Infect. Immun.* **71**:4595–4607.
50. Mair, N. S., and G. S. Ziffo. 1974. Isolation of *Y. pseudotuberculosis* from a foal. *Vet. Rec.* **94**:152–153.
51. Mallett, C. P., T. L. Hale, R. W. Kaminski, T. Larsen, N. Orr, D. Cohen, and G. H. Lowell. 1995. Intranasal or intragastric immunization with proteosome-*Shigella* lipopolysaccharide vaccines protects against lethal pneumonia in a murine model of *Shigella* infection. *Infect. Immun.* **63**:2382–2386.
52. Marceau, M., F. Sebbane, F. Ewann, F. Collyn, B. Lindner, M. A. Campos, J.-A. Bengoechea, and M. Simonet. 2004. The pmrF polymyxin-resistance operon of *Yersinia pseudotuberculosis* is upregulated by the PhoP-PhoQ two-component system but not by PmrA-PmrB, and is not required for virulence. *Microbiology* **150**:3947–3957.
53. Marketon, M. M., R. W. DePaolo, K. L. DeBord, B. Jabri, and O. Schneewind. 2005. Plague bacteria target immune cells during infection. *Science* **309**:1739–1741.
54. McDonald, C., P. O. Vacratsis, J. B. Bliska, and J. E. Dixon. 2003. The *Yersinia* virulence factor YopM forms a novel protein complex with two cellular kinases. *J. Biol. Chem.* **278**:18514–18523.
55. Mecsas, J., I. Bilis, and S. Falkow. 2001. Identification of attenuated *Yersinia pseudotuberculosis* strains and characterization of an orogastric infection in BALB/c mice on day 5 postinfection by signature-tagged mutagenesis. *Infect. Immun.* **69**:2779–2787.
56. Monack, D. M., J. Mecsas, D. Bouley, and S. Falkow. 1998. *Yersinia*-induced apoptosis in vivo aids in the establishment of a systemic infection of mice. *J. Exp. Med.* **188**:2127–2137.
57. Monack, D. M., J. Mecsas, N. Ghori, and S. Falkow. 1997. *Yersinia* signals macrophages to undergo apoptosis and YopJ is necessary for this cell death. *Proc. Natl. Acad. Sci. USA* **94**:10385–10390.
58. Montagna, L. G., M. I. Ivanov, and J. B. Bliska. 2001. Identification of residues in the N-terminal domain of the *Yersinia* tyrosine phosphatase that are critical for substrate recognition. *J. Biol. Chem.* **276**:5005–5011.
59. Najdenski, H., A. Vesselinova, E. Golkocheva, S. Garbom, and H. Wolf-Watz. 2003. Experimental infections with wild and mutant *Yersinia pseudotuberculosis* strains in rabbits. *J. Vet. Med. Series B* **50**:280–288.
60. Nikolova, S., Y. Tzvetkov, H. Najdenski, and A. Vesselinova. 2001. Isolation of pathogenic yersiniae from wild animals in Bulgaria. *J. Vet. Med. Series B* **48**:203–209.
61. Obwolo, M. J., and T. J. Gruffydd-Jones. 1977. *Yersinia pseudotuberculosis* in the cat. *Vet. Rec.* **100**:424–425.
62. Orth, K. 2002. Function of the *Yersinia* effector YopJ. *Curr. Opin. Microbiol.* **5**:38–43.
63. Orth, K., Z. Xu, M. B. Mudgett, Z. Q. Bao, L. E. Palmer, J. B. Bliska, W. F. Mangel, B. Staskawicz, and J. E. Dixon. 2000. Disruption of signaling by *Yersinia* effector YopJ, a ubiquitin-like protein protease. *Science* **290**:1594–1597.
64. Oyston, P. C. F., N. Dorrell, K. Williams, S.-R. Li, M. Green, R. W. Titball, and B. W. Wren. 2000. The response regulator PhoP is important for survival under conditions of macrophage-induced stress and virulence in *Yersinia pestis*. *Infect. Immun.* **68**:3419–3425.
65. Paff, J. R., D. A. Triplett, and T. N. Saari. 1976. Clinical and laboratory aspects of *Yersinia pseudotuberculosis* infections, with a report of two cases. *Am. J. Clin. Pathol.* **66**:101–110.
66. Parkhill, J., B. W. Wren, N. R. Thomson, R. W. Titball, M. T. G. Holden, M. B. Prentice, M. Sebahia, K. D. James, C. Churcher, K. L. Mungall, S. Baker, D. Basham, S. D. Bentley, K. Brooks, A. M. Cerdeno-Tarraga, T. Chillingworth, A. Cronin, R. M. Davies, P. Davis, G. Dougan, T. Feltwell, N. Hamlin, S. Holroyd, K. Jagels, A. V. Karlyshev, S. Leather, S. Moule, P. C. F. Oyston, M. Quail, K. Rutherford, M. Simmonds, J. Skelton, K. Stevens, S.

- Whitehead, and B. G. Barrell. 2001. Genome sequence of *Yersinia pestis*, the causative agent of plague. *Nature* **413**:523–527.
67. Perry, R. D., and J. D. Fetherston. 1997. *Yersinia pestis*—etiologic agent of plague. *Clin. Microbiol. Rev.* **10**:35–66.
 68. Persson, C., R. Nordfelth, K. Andersson, A. Forsberg, H. Wolf-Watz, and M. Fallman. 1999. Localization of the *Yersinia* PTPase to focal complexes is an important virulence mechanism. *Mol. Microbiol.* **33**:828–838.
 69. Phalipon, A., M. Kaufmann, P. Michetti, J. Cavaillon, M. Huerre, P. Sansonetti, and J. Kraehenbuhl. 1995. Monoclonal immunoglobulin A antibody directed against serotype-specific epitope of *Shigella flexneri* lipopolysaccharide protects against murine experimental shigellosis. *J. Exp. Med.* **182**:769–778.
 70. Reibel, R., R. K. Ernst, B. B. Gowen, S. I. Miller, and B. J. Hinnebusch. 2004. Variation in lipid A structure in the pathogenic yersiniae. *Mol. Microbiol.* **52**:1363–1373.
 71. Reed, D. S., and M. J. Martinez. 2006. Respiratory immunity is an important component of protection elicited by subunit vaccination against pneumonic plague. *Vaccine* **24**:2283–2289.
 72. Reed, L. J., and H. A. Muench. 1938. A simple method for estimating fifty percent end points. *Am. J. Hyg.* **27**:493–497.
 73. Sansonetti, P. J., A. Phalipon, J. Arondel, K. Thirumalai, S. Banerjee, S. Akira, K. Takeda, and A. Zychlinsky. 2000. Caspase-1 activation of IL-1 β and IL-18 are essential for *Shigella flexneri*-induced inflammation. *Immunity* **12**:581–590.
 74. Sauvonnnet, N., I. Lambermont, P. v. d. Bruggen, and G. R. Cornelis. 2002. YopH prevents monocyte chemoattractant protein 1 expression in macrophages and T-cell proliferation through inactivation of the phosphatidylinositol 3-kinase pathway. *Mol. Microbiol.* **45**:805–815.
 75. Simonet, M., B. Riot, N. Fortineau, and P. Berche. 1996. Invasin production by *Yersinia pestis* is abolished by insertion of an IS200-like element within the *inv* gene. *Infect. Immun.* **64**:375–379.
 76. Skurnik, M., and H. Wolf-Watz. 1989. Analysis of the yopA gene encoding the Yop1 virulence determinants of *Yersinia* spp. *Mol. Microbiol.* **3**:517–529.
 77. Straley, S. C., and M. L. Cibull. 1989. Differential clearance and host-pathogen interactions of YopE[−] and YopK[−] YopL[−] *Yersinia pestis* in BALB/c mice. *Infect. Immun.* **57**:1200–1210.
 78. Titball, R. W., J. Hill, D. G. Lawton, and K. A. Brown. 2003. *Yersinia pestis* and plague. *Biochem. Soc. Trans.* **31**:104–107.
 79. Trulzsch, K., T. Sporleder, E. I. Igwe, H. Russmann, and J. Heesemann. 2004. Contribution of the major secreted Yops of *Yersinia enterocolitica* O:8 to pathogenicity in the mouse infection model. *Infect. Immun.* **72**:5227–5234.
 80. Une, T., and R. R. Brubaker. 1984. In vivo comparison of avirulent Vwa[−] and Pgm[−] or Pstr phenotypes of yersiniae. *Infect. Immun.* **43**:895–900.
 81. van Zonneveld, M., J. M. Droogh, M. W. J. A. Fieren, I. C. Gyssens, T. van Gelder, and W. Weimar. 2002. *Yersinia pseudotuberculosis* bacteraemia in a kidney transplant patient. *Nephrol. Dial. Transplant.* **17**:2252–2254.
 82. Velianov, D., S. Nikolova, K. Naidenski, T. Raducheva, and V. Kusovski. 1993. Studies on aerosol *Yersinia pseudotuberculosis* infection of guinea-pigs. *Acta Microbiol. Bulg.* **30**:3–10.
 83. Viboud, G. I., and J. B. Bliska. 2001. A bacterial type III secretion system inhibits actin polymerization to prevent pore formation in host cell membranes. *EMBO J.* **20**:5373–5382.
 84. Viboud, G. I., and J. B. Bliska. 2005. *Yersinia* outer proteins: role in modulation of host cell signalling responses and pathogenesis. *Annu. Rev. Microbiol.* **59**:69–89.
 85. Visser L. G., A. Annema, and R. van Furth. 1995. Role of Yops in inhibition of phagocytosis and killing of opsonized *Yersinia enterocolitica* by human granulocytes. *Infect. Immun.* **63**:2570–2575.
 86. Wang, S., D. Heilman, F. Liu, T. Giehl, S. Joshi, X. Huang, T.-h. Chou, J. Goguen, and S. Lu. 2004. A DNA vaccine producing LcrV antigen in oligomers is effective in protecting mice from lethal mucosal challenge of plague. *Vaccine* **22**:3348–3357.
 87. Watson, R. P., T. W. Blanchard, M. G. Mense, and P. W. Gasper. 2001. Histopathology of experimental plague in cats. *Vet. Pathol.* **38**:165–172.
 88. Winfield, M. D., T. Latifi, and E. A. Groisman. 2005. Transcriptional regulation of the 4-amino-4-deoxy-L-arabinose biosynthetic genes in *Yersinia pestis*. *J. Biol. Chem.* **280**:14765–14772.
 89. Zhang, Z. Y., Y. Wang, L. Wu, E. B. Fauman, J. A. Stuckey, H. L. Schubert, M. A. Saper, and J. E. Dixon. 1994. The Cys(X)5Arg catalytic motif in phosphoester hydrolysis. *Biochemistry* **33**:15266–15270.
 90. Zhou, D., Y. Han, L. Qin, Z. Chen, J. Qiu, Y. Song, B. Li, J. Wang, Z. Guo, and Z. Du. 2005. Transcriptome analysis of the Mg²⁺-responsive PhoP regulator in *Yersinia pestis*. *FEMS Microbiol. Lett.* **250**:85–95.