The Proinflammatory Response Induced by Wild-Type
Yersinia pseudotuberculosis Infection Inhibits Survival
of yop Mutants in the Gastrointestinal Tract and Peyer’s Patches

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Single-strain infections and coinfections are frequently used to assess roles of virulence factors in infected tissues. After oral inoculation of mice, Yersinia pseudotuberculosis yopE and yopH mutants colonize the intestines and Peyer’s patches in single-strain infections but fail to persist in competition with wild-type Y. pseudotuberculosis, indicating that these two infection models provide different insights into the roles of Yops. To determine how wild-type Y. pseudotuberculosis hinders yop mutant survival, yop mutant colonization and host responses were investigated in several different infection models that isolated specific features of wild-type Y. pseudotuberculosis infection. Infection with wild-type Y. pseudotuberculosis caused significantly more inflammation than yop mutants. Results from coinfections of gamma interferon (IFN-γ)/−/− mice revealed that IFN-γ-regulated defenses target these mutants, suggesting that YopE and YopH protect Y. pseudotuberculosis from these defenses in BALB/c mice. We developed an oral-intraperitoneal infection model to evaluate the effects of spleen and liver colonization by Y. pseudotuberculosis on yop mutants in the intestines. Spleen and liver infection increased inflammation and decreased yop mutant survival in the intestines, indicating that infection of these organs has consequences in intestinal tissues. Finally, competition infections with Y. pseudotuberculosis mutants with various abilities to induce inflammation demonstrated that survival of the yopE, but not the yopH, mutant was consistently decreased in inflamed tissues. In summary, infection with Y. pseudotuberculosis in intestinal and systemic sites induces intestinal inflammation, which decreases yop mutant survival. Thus, competition studies with wild-type yersiniae reveal critical roles of Yops in combating host responses to a normal virulent infection.

During infection, a host initially senses and responds to a pathogen, at the site of its entry and/or at sites of tissue damage, by initiating cascades of proinflammatory signals that activate resident host defenses and recruit additional immune effectors (41, 54, 58). Many enteric bacterial pathogens, such as Listeria, Salmonella, and enteric Yersinia species, spread from initial sites of infection in the intestinal tract to systemic sites, while others, such as vibrios, clostridia, and enteropathogenic Escherichia coli, rarely leave the intestinal tract (24, 39, 53, 59). Following infection with gram-negative enteric Yersinia pathogens, sites of inflammation include different regions of the intestines, as well as the associated lymphoid tissues of the Peyer’s patches (PP) and mesenteric lymph nodes (MLN), and distal systemic sites, such as the spleen and liver (10, 17). To establish infection and subvert host defenses at these sites, enteric Yersinia pathogens require a type III secretion system, which translocates virulence factors, called Yops (for Yersinia outer proteins), into host cells (5, 9, 19, 29, 30, 52, 57).

Both the type III secretion system and the Yops are conserved among all three pathogenic Yersinia species, the enteric pathogens Yersinia pseudotuberculosis and Yersinia enterocolitica and Yersinia pestis, the causative agent of plague. Yops inhibit critical features of normal mammalian cell responses to bacteria; in cell culture, YopE, YopO, and YopH prevent phagocytosis by macrophages and neutrophils (18, 23, 44–46); YopH also blocks Ca2+ signaling in neutrophils (2), the oxidative burst of macrophages (6, 22), and T-cell signaling, proliferation, and activation (1, 20, 48, 62). YopJ suppresses TNF-α in cultured macrophages, presumably by deubiquitinating TRAF2, TRAF6, and IKKB, which prevents activation of NF-κB and mitogen-activated protein kinase pathways (38, 40, 63), and promoting apoptosis of macrophages in cell culture and in the MLN of mice (36). YopM causes a systemic depletion of natural killer (NK) cells after infection administered by intravenous injection and alters expression of interleukin-15 and the interleukin-1 receptor (25). Thus, Yops target multiple features of the host immune response to promote survival of yersiniae during infection.

Since the dynamic interplay between host defenses and pathogens cannot be completely mimicked by cell culture systems, animal infection models provide valuable insights into how virulence factors thwart host defenses and reveal interactions among host defenses during infection (8, 25, 27, 36, 47). We previously reported differences in the levels of attenuation of yopE and yopH mutants in single-strain mouse infections compared to competition infections with wild-type Y. pseudotuberculosis (30). In single-strain infections, yopE and yopH mutants rarely colonized the spleen but generally colonized the intestines and PP at or near wild-type levels at 5 days postin-
fection (30). In contrast, when mice were coinfected with an equal mixture of yop mutant and wild-type Y. pseudotuber-
culos is bacteria, yopE and yopH mutants initially colonized the intestines and PP at 2 days postinfection but failed to survive to 5 days postinfection (30). The decrease in yop mutant survival between days 2 and 5 correlated with systemic colonization of the spleen and liver by wild-type Y. pseudotuberculosis and with increasing signs of disease, such as weight loss and scruffiness (30). While multiple mechanisms may explain how wild-type Y. pseudotuberculosis outcompetes yop mutants, the correlation between the development of systemic symptoms and yop mutant clearance suggests that coinfection with wild-type Y. pseudotuberculosis elicits a heightened host response that inhibits growth of strains lacking YopE or YopH.

Our findings from a variety of animal infection models indicate that yop mutants are outcompeted by wild-type Y. pseudotuberculosis because the mutants cannot combat the stronger proinflammatory response generated by infection with wild-type Y. pseudotuberculosis. Furthermore, by using novel animal infection models that isolate the effects of wild-type Y. pseudotuberculosis colonization in the spleen and liver from those due to wild-type Y. pseudotuberculosis present in the intestinal tract and PP, we identified an unexpected consequence of infection of the spleen and liver, which was that infection of these organs caused increased inflammation in the intestines, a connection that has been previously unrecognized.

MATERIALS AND METHODS

Bacterial strains. Y. pseudotuberculosis YPIII pB1 (30) was used as the wild-type strain for all experiments, and all mutants were generated with this strain. This strain is virulent in mice but has a mutation in phoP (21). The yopE and yopH unmarked deletion strains (30), the kanamycin-resistant (Kan') wild-type YPIII strain (30, 34), and the inv mutant strain (34) have been previously described. The yopM mutant was constructed by mating a Y. pseudotuberculosis yopE mutant (36) with an E. coli SM10 pir strain containing the suicide plasmid pCDV424 carrying the yopM deletion as previously described (30).

Mouse infections. All mice were 7- to 9-week-old females. BALB/c mice were purchased from Taconic, Germantown, NY, or the National Cancer Institute, Frederick, MD; gamma interferon (IFN-γ)-resistant (IFN-γR−/−) mice were purchased from The Jackson Laboratory, Bar Harbor, ME (stock no. 002286; IFN-γ deletion on the BALB/c background). Oropharyngeal infections were performed as previously described (30). For oral-intraperitoneal (i.p.) infections, mice were orogastrically inoculated with 2 × 10^8 CFU of wild-type or yopE or yopH mutant Y. pseudotuberculosis on day 0 and received an i.p. injection of 100 µl of either phosphate-buffered saline (PBS) or 1 × 10^6 CFU of the inv strain 2 days after oral inoculation. Mice were sacrificed by CO2 asphyxiation 5 days post oral inoculation, and tissue samples were processed as previously described (30). For mice infected with the inv mutant, samples were collected in RPMI medium (Gibco, Carlsbad, CA), and blood samples from cardiac puncture were collected in Microtainer brand tubes with EDTA (BD, Franklin Lakes, NJ). Tissue samples were washed through a 70-um cell strainer (BD Falcon, Bedford, MA) with the rubber end of a 12-ml syringe, generating a single-cell suspension, and washed one time with RPMI medium. Cells were resuspended in fluorescence-activated cell sorter (FACS) buffer (PBS supplemented with 0.01% sodium azide and 1% fetal bovine serum) at a cell density of approximately 1 to 2 × 10^6 cells/ml, and all further steps were performed at 4°C. Fe receptors were blocked with rat anti-mouse CD16/CD32 antibody (BD Pharmingen, San Diego, CA) and then stained with fluorescein isothiocyanate-conjugated anti-GR1 (Ly-6G; BD Pharmingen) and PE-Cy7-conjugated anti-CD11b (BD Pharmingen) for 30 min to identify specific cell types. Samples were washed in FACS buffer, fixed in 1% paraformaldehyde for 15 min, and resuspended in FACS buffer. After blocking and staining, red blood cells in blood samples were lysed in PharMlyse (BD Pharmingen) and then fixed as described above. A FACScalibur (Becton Dickinson) was used for flow cytometry analysis of all samples. Cells were gated based on forward and side scatter to distinguish live from dead cells, and dead cells were eliminated from analysis. All data are expressed as the percentage positive of all live cells. For analysis of granulocytes and macrophages, cells were gated as CD11b+/GR1hi or CD11b+/GR1lo, respectively, as previously described (28, 36). To eliminate B cells that contaminate the CD11b+ GR1lo population, this population was then reanalyzed by forward and side scatter, cells with low forward and side scatter were not included in the results.

Statistics. Statistical differences were calculated on logarithmically transformed data by the two-tailed unpaired Student t test.

RESULTS

yopE is susceptible to IFN-γ-mediated responses elicited by wild-type Y. pseudotuberculosis. One possible mechanism for inhibition of yop mutants by wild-type Y. pseudotuberculosis is that infection with wild-type Y. pseudotuberculosis elicits more inflammation, which decreases yop mutant survival. In preliminary experiments, a fivefold increase in serum IFN-γ levels occurred 5 days after oral inoculation of BALB/c mice with wild-type Y. pseudotuberculosis (data not shown). To determine whether yop mutants survive as well as wild-type Y. pseudotuberculosis in the absence of this proinflammatory signal, IFN-γ knockout (IFN-γ−/−) mice were coinfected with the wild-type or the yopE or yopH mutant strain. Competitive indices (CIs) were determined 5 days postinoculation and compared to our previous results with BALB/c mice (30). Immune cell functions altered in these mice include decreased activation of macrophages and NK cells, altered T-cell proliferation, and altered activation of cytokines and chemokines (14, 27).

In IFN-γ−/− mice, the CIs for yopE were 4- to 22-fold higher than those observed previously in BALB/c mice (30) in the intestinal tract and associated lymph tissues (Fig. 1A). Moreover, the yopE mutant was recovered at the same level as wild-type Y. pseudotuberculosis, as indicated by a CI of 1, in the ceca, colons, and MLN of the IFN-γ−/− mice. The lower CI in the IFN-γ−/− mice compared to the BALB/c mice is likely due to a lower limit of detection (1:250 to 1:500) in the IFN-γ experiments compared to the limit of detection (1:100 to 1:200) in the previous experiments (30). These results suggest that in BALB/c mice, the yopE mutant is susceptible to IFN-γ-regulated responses and that YopE protects Y. pseudotuberculosis from these responses.

In contrast to the yopE− mutant, the absence of IFN-γ did not completely restore the ability of the yopH mutant to compete with wild-type Y. pseudotuberculosis, although the CIs for the yopH mutant in the IFN-γ−/− mice were 5- to 7.5-fold higher in the cecum and colon than previously seen in BALB/c mice (Fig. 1B). These results indicate that other factors present in the IFN-γ−/− mice also inhibit survival of the yopH mutant.
FIG. 1. IFN-γ responses inhibit yopE and yopH mutants in the intestines during competition infections with wild-type *Y. pseudotuberculosis*. IFN-γ−/− mice were infected with equal mixtures of wild-type *Y. pseudotuberculosis* and the yopE mutant (A) or the yopH mutant (B) or with yopE (C), yopH (D), or wild-type (E) bacteria. The CIs (A and B) or colonization levels (C to E) were determined 5 days postinoculation. CI = (yop mutant/wild type) output/(yop mutant/wild type) input. The symbol × denotes the levels of colonization or CI previously reported in BALB/c mice (30). Each symbol represents data from one mouse, and bars indicate geometric means of the CIs or the average for the log CFU/g of tissue. The *n* -fold difference between the colonization of IFN-γ−/− mice and the previously reported colonization of BALB/c mice was calculated by dividing the average CI or CFU/g of tissue in IFN-γ−/− mice by the average CI or CFU/g of tissue in BALB/c mice and are listed at the bottom of each graph.
The differences between the yopE and yopH mutants in the IFN-γ−/− mice suggest that the yopE strain is more susceptible to IFN-γ-mediated responses than the yopH strain during co-infection with wild-type Y. pseudotuberculosis.

To determine if the lack of IFN-γ responses increased the survival of the yop mutants in the absence of wild-type infection, single-strain oral infections with the yopE or yopH mutant or wild-type strain were performed with IFN-γ−/− mice. Colonization by each strain was determined 5 days postinoculation and compared to our previous results with BALB/c mice (30). Both yop mutant strains colonized the intestines of IFN-γ−/− and BALB/c mice at similar levels (Fig. 1C and D). Thus, in the intestines, IFN-γ-mediated responses only deter the yop mutants in competition infections, indicating that these responses are elicited by wild-type Y. pseudotuberculosis. In contrast, levels of the yopE mutant in the PP and MLN and the yopH mutant in the MLN were higher in the IFN-γ−/− mice than in the BALB/c mice (Fig. 1C and D), suggesting that IFN-γ-mediated responses hinder the yop mutants in the absence of a wild-type infection in lymph nodes. Levels of colonization of the spleen by the yopE mutant and the wild type, but not the yopH mutant, were significantly (240-fold and 50-fold, respectively) higher in IFN-γ−/− mice than in BALB/c mice (Fig. 1C, D, and E). Hence, IFN-γ plays a significant role in inhibiting the yopE mutant and the wild type from reaching and/or surviving in the spleen. Despite increased systemic colonization, the IFN-γ−/− mice did not succumb to infection earlier or show more severe signs of illness, such as weight loss and scruffiness, than BALB/c mice during the 5-day infection. In summary, during coinfections with wild-type Y. pseudotuberculosis, yop mutants survive better in the intestines in the absence of IFN-γ, suggesting that in BALB/c mice, wild-type infection triggers inflammatory defenses that hinder yop mutant survival.

Wild-type Y. pseudotuberculosis causes more inflammation than yop mutants in the intestines, PP, and blood. To verify that infection with wild-type Y. pseudotuberculosis elicits more inflammation than yop mutant strains, intestinal inflammation was measured following infection with these strains. Levels of lactoferrin, a protein secreted from the secondary granules of activated neutrophils (33, 43), were measured in the feces of mice 1 day prior to infection with wild-type or yop mutant Y. pseudotuberculosis and on subsequent days postinfection. Maximal lactoferrin levels were reached 3 days after oral infection with wild-type Y. pseudotuberculosis (Fig. 2A). At this point, the feces of wild-type-infected mice exhibited significantly higher concentrations of lactoferrin than mice infected with the yopE or yopH mutants (Fig. 2A). Between days 3 and 5, lactoferrin levels in the feces of wild-type-infected mice fluctuated but remained significantly higher than in uninfected mice (Fig. 2B and C). Mice receiving a mixed inoculum of the wild-type strain and a yop mutant strain were also tested to determine if wild-type Y. pseudotuberculosis elicits the same inflammatory response during competition infections. These mice developed elevated concentrations of lactoferrin in feces, which peaked at day 5 and were similar to those of wild-type-infected mice (Fig. 2C). In contrast to mice infected with the wild-type strain, lactoferrin concentrations in the feces of mice infected with yopE or yopH mutant bacteria were never significantly different from those of uninfected mice (Fig. 2).

FIG. 2. Infection with wild-type (wt) Y. pseudotuberculosis causes elevated intestinal inflammation as measured by fecal lactoferrin levels. Mice were orally inoculated with wild-type Y. pseudotuberculosis or yopE, yopH, or yopMJ mutant bacteria or a mixture of wild-type and yopE mutant bacteria. Fecal samples were collected daily and lactoferrin levels determined by ELISA. Each circle represents data from one mouse, and bars indicate the geometric means, which are also listed below the graph. Statistically significant differences (†) compared to uninfected (un) mice were determined by unpaired Student t test (P < 0.01).

A yopMJ deletion strain, which was used in the following experiments, was also tested in this assay. The yopMJ mutant strain has a colonization pattern similar to that of the yopE and yopH mutants, in that it colonizes the gastrointestinal (GI)
tract and PP but rarely spreads to the spleen and liver. Mice infected with the yopMJ mutant had significantly higher amounts of lactoferrin than uninfected mice at day 3; however, this spike in levels was not sustained on day 4 or 5 (Fig. 2).

To further assess the host response to infection with wild-type or yop mutant Y. pseudotuberculosis, granulocyte and macrophage or monocyte levels were measured in the PP and blood by flow cytometry analysis following oral inoculation of wild-type Y. pseudotuberculosis, various yop mutant strains, or a mixture of the wild type and a yopE mutant. The cellular markers CD11b and GR-1 (Ly-6G) were used to detect granulocytes (CD11b^\text{hi}/Gr-1^{hi}) and blood monocytes or tissue macrophages (CD11b^\text{lo}/Gr-1^{lo}) (Fig. 3A) (28). At 5 days postinfection, granulocyte levels in the PP were significantly higher in mice infected with the wild type, a yopMJ mutant, or a mixture of wild-type and yopE mutant strains than in mice infected with the yopE or yopH mutant strain (Fig. 3B). The granulocyte levels in the blood were significantly higher in mice infected with wild-type Y. pseudotuberculosis or a mixture of wild-type and yopE mutant bacteria than in mice infected with only yopE, yopH, or yopMJ mutant bacteria. Elevated levels of granulocytes in the blood correlated with the ability of wild-type Y. pseudotuberculosis, but not that of these yop mutants, to colonize systemic sites (Fig. 3C). Although granulocyte levels in the PP and blood following infection with the yopE and yopH mutants did not reach the levels caused by wild-type infection, they were higher than in uninfected mice, indicating that infection with the yopE and yopH mutants triggered some inflammation (Fig. 3B and C).

At 3 days postinoculation, granulocyte levels in the PP of all infected mice were higher than in those of uninfected mice; however, significant differences were not detected between mice infected with the wild-type strain or the yop mutant strains (data not shown). Similarly, at both 3 and 5 days post-
inoculation levels of macrophage in the PP and monocytes in the blood of all infected mice were higher than in uninfected mice, with no significant differences between mice infected with different strains (data not shown). Collectively, these results indicate that late in infection more granulocytes trafficked to the PP and blood following infection with wild-type \textit{Y. pseudotuberculosis} than with \textit{yopE} or \textit{yopH} mutant bacteria. These observations, that infection with wild-type \textit{Y. pseudotuberculosis}, either in single-strain infection or in coinfections, elicited more inflammation than infection with the \textit{yopE} or \textit{yopH} mutant bacteria, support the model that \textit{yop} mutant bacteria are inhibited in competition with wild-type \textit{Y. pseudotuberculosis} because of a heightened inflammatory response.

\textbf{Colonization of the spleen and liver reduces yop mutant survival in the GI tract.} In coinfections, \textit{yop} mutant bacteria must compete with wild-type \textit{Y. pseudotuberculosis} in the intestines and regional lymph tissues and must also confront and survive global effects from disseminated infection of the spleen and liver by wild-type \textit{Y. pseudotuberculosis}. Since significantly more granulocytes were detected in the blood after infection with wild-type \textit{Y. pseudotuberculosis}, we investigated whether colonization of the spleen and liver by \textit{yopE} and \textit{yopH} mutant, but not wild-type, \textit{Y. pseudotuberculosis}. The log CFU/g of tissue of the \textit{inv} strain (■) recovered on day 5 from mice orally inoculated on day 0 with the \textit{yopE} strain and injected i.p. on day 2 with the \textit{inv} strain was calculated (A). Open symbols indicate that colonization by the \textit{inv} mutant strain was below the limit of detection of 1 \textit{inv} bacterium per 200 to 250 \textit{yopE} bacteria. Colonization by the \textit{yopE} (B) or \textit{yopH} (C) mutant or wild-type (D) bacteria recovered on day 5 from mice orally inoculated on day 0 and injected i.p. on day 2 with PBS (▲) or the \textit{inv} mutant strain (■) was determined. Each symbol represents data from one mouse, and bars indicate averages. The \textit{n}-fold differences were calculated by dividing the average number of CFU/g of tissue of \textit{yopE} or \textit{yopH} mutant or wild-type bacteria in mice injected with PBS by the average number of CFU/g of tissue in mice injected with the \textit{inv} mutant strain and are listed below each graph. Significant differences (asterisks) between levels of colonization in mice treated with PBS versus mice treated with \textit{inv} were determined by the unpaired Student \textit{t} test (\textit{P} < 0.05). The log CFU/g of tissue of \textit{yopE} and \textit{yopH} mutant bacteria in oral competition infections with wild-type \textit{Y. pseudotuberculosis} is represented by the symbol × in panels B and C for comparison. These values were calculated from the previously reported competitive indices and in many cases are overestimates because the \textit{yop} mutants were below the limit of detection (30).
PP poorly, if at all (Fig. 4A and data not shown). In the intestinal tract, the inv strain was detected at 2 to 3 log units of bacteria per g of tissue; in most cases, fewer than 50 inv colonies were detected in the intestinal contents collected (Fig. 4A). This is significantly lower than the 4 to 6 log units of wild-type bacteria per g of tissue or 1,000 to 100,000 wild-type bacteria in the intestinal contents collected (Fig. 1E) (30). Because wild-type Y. pseudotuberculosis was first detected in the spleen 2 days after oral inoculation (30), i.p. inoculums were administered at this time. Thus, in this oral-i.p. model the consequences of infection of the spleen and liver are reproduced with very low levels Y. pseudotuberculosis in the GI tract or PP.

Mice were orally inoculated on day 0 with yopE, yopH, or wild-type Y. pseudotuberculosis. Two days later, half the mice received i.p. injections of the inv strain and half received injections of PBS. Five days after oral inoculation, colonization levels of the yopE, yopH, or wild-type bacteria were compared between mice that received an i.p. inoculation of the inv strain and those that received PBS. If infection of the spleen and liver hindered the survival of yop mutants in the intestinal tract, colonization by the yop mutants should be lower in mice that received the inv strain compared to those that received PBS. Indeed, levels of colonization by the yopE mutant decreased 7- to 11-fold throughout the intestinal tracts mice that received the inv strain (Fig. 4B, circles) compared to mice that received PBS (Fig. 4B, triangles). The levels of yopE colonization in the intestinal tracts of mice that received the i.p. injection of the inv strain are similar to the levels of yopE colonization in oral competition experiments with the wild-type strain (30). However, infection by the inv strain of the spleen and liver did not affect the ability of the yopE mutant to colonize the PP, and levels were higher than in oral competition infections with the wild-type strain. The yopH mutant colonization was also 10-fold lower in the ceca of mice that received the inv strain (Fig. 4C, circles) than in mice that received PBS (Fig. 4C, triangles) but did not decrease significantly in the rest of the intestines or the PP. This is in contrast to the lower levels of yopH colonization in the ileum and colon in oral competition infection with the wild-type strain (30). In control experiments, colonization by wild-type yersinia in the intestines remained the same or increased after i.p. injection of the inv strain (Fig. 4D). Thus, yop mutant, but not wild-type, Y. pseudotuberculosis bacteria were susceptible to changes in the intestines due to infection of the spleen and liver.

To determine if bacterial products, such as lipopolysaccharide, present after i.p. injection altered yop mutant survival in the intestines, oral-i.p. infections were performed with heat-killed wild-type Y. pseudotuberculosis. The heat-killed Y. pseudotuberculosis did not alter intestinal survival of the yopE mutant, suggesting that active infection in the spleen and liver, not merely the presence of bacterial products, is required to decrease intestinal survival of the yopE mutant (data not shown).

Infection of the spleen and liver triggers increased inflammation in the intestines and bloodstream. To verify that spleen and liver infection with the inv strain increased levels of intestinal inflammation, fecal lactoferrin levels were measured in mice during oral-i.p. infections. At 5 days after oral inoculation, fecal lactoferrin levels were four- to fivefold higher in mice that received an oral inoculation of the yopE strain followed by an i.p. injection of the inv strain than in those that received PBS or in uninfected mice (Fig. 5). Furthermore, infection by the inv strain in the spleen and liver induced levels of intestinal inflammation comparable to those caused by infection with wild-type Y. pseudotuberculosis (Fig. 2).

To further examine how infection of the spleen and liver affects inflammation in different tissues, FACS analysis was used to measure granulocyte and monocyte/macrophage levels in the blood and PP of oral-i.p.-infected mice. Leukocyte levels in mice orally inoculated with the yopE mutant and i.p. injected with either the inv strain or PBS were compared to those in uninfected mice (Fig. 6). Mice inoculated with the yopE strain and injected with the inv strain had significantly more granulocytes in the blood than the PBS-injected control group (Fig. 6A). These levels were similar to those seen in mice orally inoculated with the wild-type strain (Fig. 3C), indicating that oral-i.p. infection generated systemic responses similar to those following oral inoculation with wild-type Y. pseudotuberculosis.

Levels of monocytes in the blood and of macrophages and granulocytes in the PP were not significantly different between uninfected and infected mice (Fig. 6B and data not shown). These data, combined with the observation that colonization by the yopE mutant in the PP was not affected following i.p. inoculation of the inv strain, are consistent with the model in which yopE mutant survival decreases in tissues with increased levels of inflammation.

**yopE mutants are attenuated in the PP in the presence of inflammation-inducing Y. pseudotuberculosis strains.** Infection of the spleen and liver caused increased intestinal inflammation and decreased yop mutant survival in the GI tract but did not alter either inflammation or yop mutant survival in the PP. These results suggested that during oral competition infections, the presence of wild-type Y. pseudotuberculosis in the PP inhibited yop mutant survival, either by inducing inflammation...
or by competing for colonization niches, for example, by competing for access to the PP through M cells or competing for a limiting nutrient. To assess whether yop mutant growth was inhibited by increased inflammation or by competition for niches in the PP, yop mutant levels were measured in infections in which equivalent numbers of Y. pseudotuberculosis bacteria were competing for colonization of the PP in the presence or absence of increased inflammation. In these experiments, colonization by the yopE and yopH mutants was compared in single-strain infections, in competition with the yopMJ strain, and in competition with each other. These three yop mutant strains rarely colonized systemic sites (Fig. 7) (30) but colonized the intestines and PP at similar levels (Fig. 7); however, the yopMJ mutant induced significantly more inflammation in these tissues than the yopE or yopH mutant (Fig. 2 and 3). Thus, if colonization by the yopE or yopH mutant bacteria was inhibited in inflamed tissues, then colonization by the yopE and/or yopH mutant bacteria should decrease in the presence of the yopMJ strain but not in the presence of each other. Alternatively, if the yopE or yopH mutant bacteria were prevented from gaining access to the PP by the presence of other invasive Y. pseudotuberculosis bacteria, then colonization by these mutants would decrease in the presence of each other and the yopMJ mutant strain.

In competition with the yopMJ mutant strain, yopE mutant colonization decreased 11-fold in the ileum and 40-fold in the PP compared to single-strain infections (Fig. 7A, compare circles to triangles), consistent with the increased inflammation observed in the intestines and PP following infection with the yopMJ strain (Fig. 2 and 3). Colonization by the yopE mutant in competition with the yopH mutant was not significantly different than in single-strain infections (Fig. 7C, compare circles to triangles). The 6.5-fold difference in the ileum was largely due to a single outlier. These results indicate that yopE mutant growth was inhibited by inflammation and not by the presence of Y. pseudotuberculosis strains competing for colonization niches in the ileum and PP.

Colonization by the yopH mutant was unchanged by competition with either yopMJ or yopE mutant bacteria in all tissues (Fig. 7B and D), indicating that these strains do not outcompete yopH mutants for colonization niches. This suggests that access to niches is not the cause of the decrease in yopH mutant survival in coinfection with wild-type Y. pseudotuberculosis. Furthermore, these results indicate that the yopH mutant is not susceptible to the increased inflammation induced by the yopMJ strain and that infection by the yopMJ strain does not generate the aspect of wild-type infection that suppresses yopH mutant survival. Combined with the IFN-γ results, these data indicate that the yopE and yopH mutants are susceptible to different host factors in co-infections with wild-type Y. pseudotuberculosis.

**DISCUSSION**

A number of mutants in a variety of bacterial pathogens survive as well as the wild-type pathogen in single-strain infections but are outcompeted by the wild-type strain in competition infections (30, 36, 37, 55, 60). Possible explanations for these results include an inability of the mutant to compete for sites of colonization or key nutrients or an inability to survive the host response to infection by the wild-type pathogen. Our results that the host defenses mounted in response to wild-type Y. pseudotuberculosis infection are stronger than those mounted after yop mutant infection provide an explanation for discrepancies between single-strain and competition infections. Furthermore, they illustrate important differences between the two infection models as assays for functions of virulence factors. In essence, single-strain infections measure the ability of a strain to access nutrients or niches and overcome host defenses resident in that tissue or recruited in response to infection by the mutant. In contrast, competition infections measure the ability of a strain to access limiting nutrients or niches and grow in inflamed tissues generated in response to a wild-type infection. Thus, the role of a Yop under wild-type infection

![Graph 1](http://iai.asm.org/)

**FIG. 6.** Infection of the spleen and liver by the inv mutant strain leads to elevated levels of granulocytes in the blood but not in PP. Cells from blood (A) and PP (B) samples collected from uninfected mice or mice orally inoculated with the yopE mutant and injected with either PBS (yopE/PBS) or the inv mutant strain (yopE/inv) at day 5 were stained with antibodies for GR-1 and CD11b and analyzed by FACS. Graphed are the percentages of granulocytes among the total live cells; each diamond represents the percentage from one mouse, and bars indicate the geometric means. Statistically significant differences (*) were determined by unpaired Student t test (P < 0.01).
FIG. 7. Competition infections with yop mutant strains. Mice were orally inoculated with either yopE (A) or yopH (B) mutant bacteria singly or with an equal mixture of yopE and yopMJ (A) or yopH and yopMJ (B) mutant bacteria. On day 5, numbers of CFU/g of tissue were determined in single-strain infections of the yopE (△) or yopH (■) mutant and the yopE (○) or yopH (●) mutant in competition with the yopMJ (■) mutant. Mice were orally inoculated with either yopE (C) or yopH (D) mutant bacteria or an equal mixture of yopE and yopH mutant bacteria (C and D).
conditions is measured in competition studies, since the phenotype of a yop mutant is assessed in the tissue environment that occurs during infection with the virulent pathogen.

Differences in host responses to Haemophilus influenzae and Streptococcus pneumoniae in single-strain and polymicrobial competition infections were recently demonstrated (31). In these experiments, while both pathogens were able to colonize the nasopharynx in single-strain infections, coinfection with both pathogens led to increased immune cell recruitment and decreased survival of S. pneumoniae in the nasopharynx (31). Collectively, these two studies indicate that the host response to infection hinders mutant strains and competing pathogens and, by altering the composition of mucosal tissues, likely affects normal flora.

Surprisingly, infection of the spleen and liver by Y. pseudotuberculosis led to increased inflammation and decreased yop mutant survival in the GI tract, indicating a connection between systemic infection and the GI tract. Since a number of organisms, including Salmonella, Listeria, and Candida spp., disseminate from the intestines to distal organs (16, 24, 53, 59), this finding is important when considering host responses to disseminated infection and potential consequences for normal intestinal flora and tissues. It is interesting that the intestinal regions where yopE and yopH mutants were inhibited by systemic infection were the same tissues that these yop mutant strains colonized better in IFN-γ−/− mice than in BALB/c mice in competition infections. This suggests that IFN-γ mediates the effect systemic infection has on intestinal tissues. In fact, in preliminary experiments with IFN-γ−/− mice, systemic infection by the inv strain did not suppress yopE mutant growth in the intestinal tract (data not shown). Although it is possible that the low levels of inv colonization in the intestinal tract promoted the increased intestinal inflammation, this seems unlikely since the levels were so low that they were undetectable in some mice, yet the levels of inflammation were higher than the levels triggered by colonization with yopE or yopH bacteria. Further analysis of cytokine and chemokine levels are aimed at identifying the signaling pathways that link the initial recognition of the infection with the extensive inflammation that occurs at other sites of infection.

In clinical cases of bacteremia and septic shock, intestinal symptoms are frequently seen but it is difficult to separate cause and effect because the source of bacteremia is usually the intestinal tract (15, 56). However, in clinical cases of septicemia caused by transfusion of blood samples contaminated with Y. enterocolitica, diarrheal symptoms are observed within an hour after transfusion (4, 51). Given the rapid onset of intestinal symptoms, it is unlikely that the bacteria spread from the bloodstream, infect the intestines, and cause these symptoms, when induction of intestinal inflammation in mice takes 48 to 36 h when the bacteria are directly inoculated into the intestinal tract (Fig. 2A). These early signs of intestinal pathology support our findings that systemic infection increases intestinal inflammation.

In competition infections, yop mutants face increased inflammation due to systemic infection and the presence of wild-type yersiniae within the intestines and PP. There are several possible reasons why the presence of wild-type yersiniae in the intestines and PP induces more inflammation than the yopE and yopH mutants at 5 days postinfection, despite the observation that their levels are comparable in these tissues (30). Wild-type Y. pseudotuberculosis colonized the intestines and PP in 10-fold higher numbers than the yopE and yopH mutants at 2 days postinfection (30). This greater initial bacterial load likely fuels more inflammation potentially by stimulating Toll-like receptors (54). In addition, YopE and YopH themselves could trigger increased inflammation by activating Toll-like receptors or stimulating immune cells that elicit inflammation; however, studies with cultured cells indicate that YopE and YopH reduce proinflammatory signals (48, 49, 61, 62). Therefore, we favor the idea that the presence of YopE and YopH promotes bacterial survival, resulting in higher bacterial levels and increased inflammation.

The observation that yop mutants are more attenuated in a variety of infection models with stronger proinflammatory responses provides in vivo evidence that YopE and YopH overcome host defenses amassed in inflamed tissues. Interestingly, while cell culture data indicate that both YopE and YopH inhibit macrophage and granulocyte function (5, 23, 45), our in vivo data indicate that the yopE and yopH mutants are susceptible to different host defenses. Under three experimental conditions, coinfection with wild-type Y. pseudotuberculosis, oral-i.p. infections, and coinfection with yopMJ, increased inflammation, as indicated by increased granulocytes, correlated with an inability of the yopE mutant to grow. Conversely, under two experimental conditions, coinfection of IFN-γ−/− mice with wild-type Y. pseudotuberculosis and coinfection with the yopH mutant, a less aggressive proinflammatory response allowed the yopE mutant to grow. While granulocyte levels were measured to assess inflammation, it is likely that other mediators of inflammation also increase under these conditions. In fact, IFN-γ itself does not recruit neutrophils, although it causes increased activation of neutrophils and alters other aspects of inflammation including cytokine and chemokine profiles that affect neutrophil recruitment (7, 12, 26, 27, 42, 50). It is interesting that colonization of the yopE mutant was reduced 11-fold in the ileum at day 5 during competition with the yopMJ strain, although intestinal inflammation induced by yopMJ was only detected at day 3. It is possible that the increased granulocytes present at day 3 may hinder that yopE mutant to such a degree that colonization levels do not recover after the inflammation has resolved. Alternatively, other mediators of inflammation, such as macrophages, may be present in the intestinal tract at day 5 and inhibit survival of the

On day 5, the number of CFU/g of tissue was determined for the yopE (■) mutant in a single-strain infection or for the yopE (●) mutant in competition with yopH mutant bacteria (C) and for yopH (●) mutant bacteria in a single-strain infection and yopH (●) mutant bacteria in competition with the yopE mutant (D). The n-fold difference below each tissue equals the average number of CFU/g of yopE or yopH mutant bacteria singly versus in competition. Each symbol represents the colonization of one mouse, and bars indicate the geometric means. Statistically significant differences (*) were determined by unpaired Student t test (P < 0.05).
yopE mutants. Analysis of additional cell types, activation levels of immune cells, and examination of infection in mutant mouse strains will determine the roles of specific cell types in Yersinia infections and in targeting the yopE mutant.

Growth of the yopH mutant was impaired in some, but not all, tissues with increased granulocytes. Specifically, infection with both the wild-type and yopMJ mutant strains induced recruitment of granulocytes to the PP, but only wild-type infection inhibited survival of the yopH mutant. Since infection with the yopMJ strain does not reproduce all aspects of wild-type Y. pseudotuberculosis infection, further analysis of differences in the immune response to infection with wild-type or yopMJ bacteria should suggest the host factors that eliminate the yopH mutant in competitions with wild-type Y. pseudotuberculosis.

Our studies indicated that lower IFN-γ levels increased wild-type Y. pseudotuberculosis colonization in the spleen. These results are consistent with previous reports that IFN-γ−/− mice are more susceptible to infection with other pathogens, including Mycobacterium species, Cryptosporidium parvum, and Francisella tularensis (11, 13, 27). However, in previous studies with intravascular inoculation of Y. enterocolitica, antibody depletion of IFN-γ did not alter systemic colonization (3). This difference could be due to the use of different animal models or different routes of inoculation, or Y. pseudotuberculosis may be more susceptible to IFN-γ-mediated responses than Y. enterocolitica.

In summary, infection with wild-type Y. pseudotuberculosis triggered a stronger proinflammatory response than infection with the yopH and yopE mutants, and thus, competition infections measure the ability of a yop mutant to survive in the face of a heightened immune response. Therefore, functions of Yops in combating inflammation and other changes induced by wild-type are better assessed in competition infections. However, in cases where wild-type pathogens suppress immune function, such as during latent infections, coinfection with mutant strains could lead to increased inflammation and decreased survival of both strains. Thus, understanding how wild-type and mutant strains modulate host defenses in single-strain infections and coinfections is critical in investigating the function of virulence factors in these infection models.

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