Inhalation of *Yersinia pestis* results in primary pneumonic plague, a highly lethal and rapidly progressing necrotizing pneumonia. The disease begins with a period of extensive bacterial replication in the absence of disease symptoms, followed by the sudden onset of inflammatory responses that ultimately prove fatal. Very little is known about the bacterial and host factors that contribute to the rapid biphasic progression of pneumonic plague. In this work, we analyzed the *in vivo* transcriptional kinetics of 288 bacterial open reading frames previously shown by microarray analysis to be dynamically regulated in the lung. Using this approach combined with bacterial genetics, we were able to identify five *Y. pestis* genes that contribute to the development of pneumonic plague. Deletion of one of these genes, *ybtX*, did not alter bacterial survival but attenuated host inflammatory responses during late-stage disease. Deletion of *ybtX* in another lethal respiratory pathogen, *Klebsiella pneumoniae*, also resulted in diminished host inflammation during infection. Thus, our *in vivo* transcriptional screen has identified an important inflammatory mediator that is common to two Gram-negative bacterial pathogens that cause severe pneumonia.

*Yersinia pestis* is responsible for at least three major pandemics, most notably the Black Death of the Middle Ages. Due to its pandemic potential, ease of dissemination by aerosolization, and a history of its weaponization, *Y. pestis* is categorized by the Centers for Disease Control and Prevention as a tier 1 select agent most likely to be used as a biological weapon. To date, there is no licensed vaccine against *Y. pestis*.

Little is known about the bacterial and host factors that contribute to pathogenesis is essential to understanding the progression of pneumonic plague and developing or enhancing treatment options.

*Yersinia pestis* is the causative agent of plague and one of the deadliest human pathogens. Primary pneumonic plague resulting from the inhalation of *Y. pestis* is the most severe manifestation of plague, with mortality rates approaching 100% in the absence of timely delivery of antibiotics (1). Its low infectious dose, capacity for aerosol transmission, and history of weaponization have led to the classification of *Y. pestis* as a tier 1 select agent requiring biosafety level 3 containment.

Our laboratory and others have characterized a mouse model of pneumonic plague using fully virulent *Y. pestis* that closely mimics human disease (2–5). Progression of pneumonic plague is biphasic, with an initial “preinflammatory” phase in the lung highlighted by a lack of disease symptoms or detectable host immune responses. After 36 to 48 h, there is an abrupt switch to a “proinflammatory” phase characterized by the rapid onset of symptoms, induction of proinflammatory cytokines, and the dramatic accumulation of immune infiltrate in the airways. Progression into the proinflammatory phase of disease leads to the severe necrotizing pneumonia that is the hallmark of pneumonic plague and invariably proves fatal within the following 24 to 36 h.

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**RESULTS**

Transcriptional analysis of highly regulated *Y. pestis* genes during pulmonary infection. In 2005, Lathem et al. identified 410 *Y. pestis* open reading frames (ORFs) that were significantly up- or downregulated in the bronchoalveolar lavage fluid of mice 48 h after intranasal inoculation with *Y. pestis* (5). Unfortunately, performing similar analysis at earlier time points proved technically difficult. We sought to use real-time quantitative reverse transcription PCR (qRT-PCR) to evaluate in detail the expression kinetics of highly regulated *Y. pestis* genes throughout the duration of infection. We hypothesized that this analysis would reveal open reading frames that are regulated in a phase-specific manner in the lung, as well as genes that are highly induced at multiple time points during infection. Phase-specific regulation or constitutive expression may indicate that these genes are important to the progression of pneumonic plague. After elimination of a number of metabolic genes and genes with well-defined functions, 288 *Y. pestis* ORFs were chosen from the previous 410 genes for further transcriptional profiling. To this end, total RNA was isolated from the lungs of mice infected with *Y. pestis* CO92 at 24, 36, 48, and 60 hpi and analyzed by qRT-PCR. Fold difference was calculated for each gene by comparing its expression to that of RNA isolated from broth-grown culture. We focused our attention on genes showing one of the following three patterns of transcription (Fig. 1A): (i) gene was upregulated greater than 5-fold during at least one of the early time points (24 h or 36 h) compared to the later time points (48 h or 60 h), (ii) gene was upregulated greater than 5-fold during at least one of the later time points compared to early time points, or (iii) gene was upregulated greater than 5-fold relative to broth-grown culture throughout the duration of infection. From this analysis, we identified 38 genes/loci of interest that we sought to target for further analysis (see Table S1 in the supplemental material).

Identification of *Y. pestis* genes that contribute to pneumonic plague. Other than the well-established type III secretion system (T3SS), very few *Y. pestis* virulence determinants of pneumonic plague have been identified. We predicted that mutation of ORFs demonstrating dynamic or constitutive upregulation *in vivo* would reveal genes that contribute to the pathogenesis of *Y. pestis* in the lung. To test this, we deleted each of the 38 genes/loci of interest by allelic exchange mutagenesis and evaluated each mutant strain in a mouse model of pneumonic plague. This analysis identified four genes whose deletion resulted in a greater than 1-log difference in bacterial burden in the lung and/or spleen compared to wild-type infection. Deletion of the locus YPO2349-YPO2352 had the most dramatic effect, resulting in a greater than 4-log decrease in bacterial burden in the lung and a 3-log decrease in the spleen (Fig. 1B). Deletion of the open reading frame YPO0159 resulted in a similar but less dramatic effect (Fig. 1B). Interestingly, deletion of YPO0862 had no effect on bacterial growth in the lung but resulted in a roughly 1-log decrease in colonization of the spleen (Fig. 1B and C). In contrast, deletion of YPO1501 resulted in a roughly 1-log increase in colonization of the spleen (Fig. 1C). Of the loci of interest, YPO1501 exhibited early induction relative to the later disease phase, the YPO2349-YPO2351 locus was induced later during disease, and YPO0862 was highly induced throughout the duration of infection (Fig. 1D).

In 2005, Lathem et al. identified 410 *Y. pestis* genes that contribute to the pathogenesis of *Y. pestis* during primary pneumonic plague and one gene whose deletion results in enhanced dissemination to the bloodstream as reflected by recovery of bacteria from the spleen. Three of the four genes showing an *in vivo* phenotype demonstrated highly increased expression throughout the duration of infection, suggesting that this pattern of expression may be the most indicative of importance for pneumonic plague. Further delineating the functions of these genes during pulmonary infection will help to shed light on the mechanisms underlying the biphasic progression of pneumonic plague.

Deletion of ybtX alters progression into the inflammatory disease phase without affecting bacterial viability. The ultimate goal of the transcriptional analysis was to identify genes whose deletion altered the biphasic progression of pneumonic plague. In addition to the phenotypes described above, we observed that mice inoculated with a strain harboring a deletion in the gene YPO1915 (common name ybtX) showed minimal disease symptoms at 48 hpi compared to those inoculated with wild-type *Y. pestis*. Whereas most animals infected with wild-type *Y. pestis* showed lethargy, diminished response to stimuli, and in some cases ruffled or matted fur, mice inoculated with the Δ*ybtX* strain behaved similarly to uninfected animals. The ybtX gene, also known as irp8, is found within the high-pathogenicity island (HPI) of *Y. pestis* responsible for the synthesis and utilization of the siderophore yersiniabactin (Ybt), which is important for bacterial virulence during pneumonic and bubonic plague (6–8). ybtX encodes a predicted inner membrane protein with weak similarity to importers/exporters of siderophores in other bacteria and was recently shown to be a *Yersinia* Zn²⁺ importer (6, 7, 9). Transcription of ybtX was found to be highly induced in the lung throughout the duration of infection, with a greater than 100-fold increase in expression relative to broth-grown culture during three of the four time points tested (Fig. 2A). We anticipated that the near absence of disease symptoms at 48 hpi would correlate with reduced bacterial burdens in the lungs of infected animals. Surprisingly, deletion of ybtX did not alter bacterial viability in the lungs or spleens compared to mice infected with wild-type *Y. pestis* (Fig. 2B and C). Histopathological analysis of infected lungs at 48 hpi and 60 hpi during the proinflammatory disease phase revealed reduced inflammatory focus formation in mice infected with the Δ*ybtX* strain compared to those infected with wild-type *Y. pestis* (Fig. 2D and E). Importantly, complementation of Δ*ybtX* restored wild-type levels of inflammation in the lungs (Fig. 2D and E). All animals eventually succumbed to disease in approximately the same time frame, presumably due to the continued accumulation of bacterial burden and the onset of lethal sepsis (Fig. 2F). These results suggest that ybtX contributes to progression into the proinflammatory phase of pneumonic plague.

Infection with the Δ*ybtX* strain implicates a key role for neutrophil chemotaxis in the progression of pneumonic plague. Transition into the proinflammatory phase of pneumonic plague corresponds with the increased expression of proinflammatory cytokines beginning at 36 to 48 hpi (5). As infection with the Δ*ybtX* strain showed diminished inflammation in the lungs, we hypothesized that comparing proinflammatory cytokine induc-
tion in Δybtx mutant-infected animals with that in wild-type
Y. pestis-infected animals would reveal dysregulation of key cyto-
kines/chemokines that are important for initiating the proinflam-
matory disease phase. To test this, we evaluated the transcription
of a panel of proinflammatory cytokines/chemokines by qRT-
PCR analysis of infected lungs at 60 hpi. Transcription levels of the
proinflammatory cytokines gamma interferon (IFN-γ), CXCL2,
CCL3, interleukin-23 (IL-23), and tumor necrosis factor alpha

FIG 1 Identifying genes important to pneumonic plague. (A) Examples of in vivo transcription patterns of interest. Lungs from mice (n = 10) infected with
Y. pestis were harvested, and 288 ORFs were evaluated by qRT-PCR. Fold difference was calculated by comparing expression to RNA isolated from broth culture
grown at 37°C for 16 h. (B) Lung bacterial burdens 48 h after infection with wild-type Y. pestis or mutant strains. (C) Spleen bacterial burdens 48 h after
inoculation with wild-type Y. pestis or mutant strains. For panels B and C, horizontal lines represent median values of data points. All mutant values were
significantly different from wild-type values via Mann-Whitney test (*, P < 0.05; **, P < 0.0001). (D) Expression of genes of interest shown in panels B and C
in the lung relative to broth-grown culture (data shown are from initial screen).
Infection with *Y. pestis* Δ*ybtX* mutant. (A) *ybtX* transcription in the lung during infection. Transcription was evaluated by qRT-PCR compared to broth-grown culture. (B) Lung bacterial burdens in mice infected with wild-type *Y. pestis* CO92 or the *Y. pestis* Δ*ybtX* mutant. (C) Spleen bacterial burdens in mice infected with wild-type *Y. pestis* CO92 or the *Y. pestis* Δ*ybtX* mutant. Panels B and C show data from a representative experiment repeated in triplicate, where horizontal lines designate median values. (D) H&E staining of mouse lungs infected with wild-type *Y. pestis*, the *Y. pestis* Δ*ybtX* mutant, and the complemented mutant. The image is representative of an experiment performed at least three times with a minimum of three mice per strain. (E) Total area of inflammation per lung section of infected mice. Twenty-four fields from six mice (4 fields per mouse section) per condition were analyzed to calculate average area per section using ImageJ software. Asterisks signify statistical significance by two-way analysis of variance (*P* < 0.005). (F) Survival of mice (*n* = 10) infected with wild-type, Δ*ybtX*, or complemented Δ*ybtX* strains. The graph is representative of an experiment repeated in triplicate. Mean time-to-death values were as follows: wild-type strain, 74 h; Δ*ybtX* strain, 86 h; complemented Δ*ybtX* strain, 72 h. Differences in mean time-to-death values were not statistically significant.
(TNF-α) and the anti-inflammatory cytokine IL-10 were similar in animals infected with the ΔybtX mutant to those in animals infected with wild-type Y. pestis (Fig. 3). There was a significant decrease in the transcription of proinflammatory cytokines IL-6 and IL-17 and the chemokines CXCL1 and CCL2 in response to infection with the ΔybtX strain compared to infection with wild-type Y. pestis, indicating that these molecules may be involved in the onset of the proinflammatory phase of pneumonic plague (Fig. 3).

The cytokines IL-6 and IL-17 and chemokines CXCL1 and CCL2 influence neutrophil chemotaxis during bacterial infection (10–14). Previously, we showed that depletion of host neutrophils prior to intranasal inoculation with Y. pestis dramatically reduced inflammation in the lung (15). Thus, we hypothesized that the diminished inflammatory response to infection with the ΔybtX mutant would correlate with decreased neutrophil chemotaxis to the lung. To evaluate this, we analyzed the innate immune cell populations of the bronchoalveolar lavage fluid (BALF) of mice inoculated with wild-type Y. pestis and the Y. pestis ΔybtX mutant using flow cytometry as we have previously described (15). Dele-
tion of \textit{ybtX} did not result in significant changes in levels of macrophages, dendritic cells, or inflammatory monocytes in the airways during infection (Fig. 4). In contrast, there was a significant decrease in the levels of neutrophils found in the airways of \textit{ybtX} mutant-infected mice during the proinflammatory disease phase by 60 hpi (Fig. 4). Complementation of the \textit{ybtX} deletion restored this neutrophil response to levels seen in infections with the wild-type strain (Fig. 4). In summary, infection with a \textit{Y. pestis} strain lacking \textit{ybtX} results in diminished neutrophil chemotaxis to the lung, indicating that \textit{ybtX} is important to initiate the intense and ultimately lethal proinflammatory response during primary pneumonic plague.

\textit{ybtX} contributes to inflammation during pulmonary infection with \textit{Klebsiella pneumoniae}. We next sought to determine if \textit{ybtX} contributes to inflammation during infection with other bacterial pathogens, or if this phenomenon is specific to \textit{Y. pestis}. Like \textit{Y. pestis}, \textit{Klebsiella pneumoniae} is a Gram-negative, extracellular pathogen of the \textit{Enterobacteriaceae} family that causes acute pneumonia when introduced into the lungs. \textit{K. pneumoniae} is a leading cause of Gram-negative bacterial nosocomial infections and is associated with high mortality rates (16–18). Importantly, some strains of \textit{K. pneumoniae} also harbor a functional \textit{ybt} locus, including the \textit{ybtX} ORF (19, 20). To examine if \textit{ybtX} plays a role in the onset of inflammation during \textit{K. pneumoniae}-induced pneumonia, we evaluated pulmonary infection with a \textit{ΔybtX} strain of \textit{K. pneumoniae} VK148. Similar to \textit{Y. pestis}, deletion of \textit{ybtX} in \textit{K. pneumoniae} did not alter bacterial viability in the lung (Fig. 5A) but resulted in differences in pathology during late-stage pneumonia (Fig. 5B and C). By 96 hpi, the lungs of mice inoculated with wild-type \textit{K. pneumoniae} showed severe edema and loss of alveolar architecture typical of \textit{K. pneumoniae} pulmonary infection (Fig. 5B). In contrast, the lungs of mice inoculated with the \textit{K. pneumoniae} \textit{ΔybtX} mutant showed limited observable edema with relatively intact alveolar architecture (Fig. 5B). Consistent with a decrease in pulmonary edema, lung weights from \textit{ΔybtX} mutant-infected mice at 96 hpi were significantly lower than those from mice infected with wild-type \textit{K. pneumoniae} (Fig. 5C). These results indicate that \textit{ybtX} may contribute to the pathogenesis of...
severe, acute pneumonia for multiple species of bacteria harboring the ybt locus.

DISCUSSION

In order to cause pneumonic plague, Y. pestis must adapt to two very different disease phases in the lung. The first phase involves avoiding and/or suppressing immune detection and clearance mechanisms to allow for rapid bacterial replication, while the second phase involves surviving the massive onslaught of host innate immune responses. In the work presented here, we initially sought to identify bacterial genes that demonstrated phase-specific regulation during pneumonic plague and then to test their role in the kinetics of biphasic disease progression. To this end, we analyzed the transcription of highly regulated bacterial genes in vivo throughout the course of infection. This represents an important departure from the vast majority of infection transcriptional studies; rather than evaluate a single time point, our goal was to follow the full kinetics of expression of a large number of Y. pestis genes previously shown to be differentially regulated during the proinflammatory phase (5). By using RT-PCR for all time points, we were able to assay bacterial gene expression as early as 24 hpi, when bacterial numbers are too small to yield reliable transcriptome data using microarray or RNA sequencing analysis of infected lung tissue (our unpublished results).

Our analysis identified 38 genes/loci of interest that we mutated and tested in a mouse model of pneumonic plague. From this analysis, we identified three genes for which deletion resulted in a greater than 1-log reduction of bacterial burden in the lung and/or spleen and one for which deletion resulted in enhanced dissemination to the bloodstream. The most striking phenotype was observed after deletion of the YPO2349-YPO2351 locus encoding PspC, PspB, and PspA, all key proteins of the phage-shock locus that has been characterized in detail in the enteric species Yersinia enterocolitica (21, 22). This system is part of a stress response that is essential for bacterial viability by protecting Yersinia from damage caused by mislocalization of the secretin component of the T3SS (21, 23). This is the first examination of this locus in Y. pestis and highlights its importance to Yersinia pathogenesis as well as the role that the T3SS plays during pulmonary infection.

The YPO0862 open reading frame encodes a putative protein of unknown function that has loose identity to a helicase-like transcription factor. Its deletion resulted in a roughly 1-log decrease in bacterial burden in the spleen, suggesting a defect in dissemination from the lungs or a defect in survival in the spleen. In contrast, deletion of YPO1501, predicted to encode a putative esterase, resulted in increased numbers of bacteria in the spleen. Evaluating the roles of both of these genes may help to identify factors important for dissemination of Y. pestis following lung infection. Finally, deletion of the YPO0159 open reading frame predicted to encode the nitrite transporter NirC reduced bacterial burdens in both the lung and the spleen. NirC has been shown to contribute to Salmonella virulence in macrophages; however, its role in Yersinia species is yet to be defined (24). Though their regulation changed during the progression of disease, three of the four genes shown to contribute to pathogenesis were highly induced throughout the duration of infection, indicating that this pattern of expression may be indicative of a role in disease. Further defining the role of each of these genes during infection will improve our understanding of the host-pathogen interactions that occur in the lung during primary pneumonic plague.

FIG 5 K. pneumoniae ΔybtX strain infection. (A) Lung bacterial burden of mice inoculated with wild-type K. pneumoniae strain VK148. Data shown are from two individual experiments; median values are indicated by horizontal lines. (B) H&E staining of mice inoculated with wild-type or ΔybtX K. pneumoniae. (C) Total lung weights of mice inoculated with wild-type or ΔybtX K. pneumoniae. The experiment was performed in duplicate with at least five mice. For panels A and C, mice that did not survive to the designated time point are represented by an “X.” The asterisk signifies a significant difference by two-way analysis of variance (P < 0.05).
We observed that deletion of the ybtX gene resulted in diminished disease symptoms during the proinflammatory disease phase but had no effect on bacterial burden or dissemination. Detailed examination of pulmonary infection with the ΔybtX strain revealed reduced inflammation in the lung as demonstrated by histopathological analysis. The ybtX open reading frame is the third gene in the ybtPQXS operon located in the 36-kb high-pathogenicity island (HPI) that is nearly identical in all pathogenic Yersinia species (6, 25). With the exception of ybtD, all genes required for the regulation, synthesis, and transport of the siderophore yersiniabactin (Ybt) are carried within the HPI. Y. pestis mutants unable to transport or synthesize Ybt are attenuated in virulence models of bubonic and pneumonic plague (8). The products of the two genes immediately upstream of ybtX, ybtP and ybtQ, form an inner membrane ABC transporter necessary for the uptake of Ybt bound to iron (7). ybtS, the gene immediately downstream of ybtX, encodes a salicylate synthase for the synthesis of Ybt (26, 27). The ybtX open reading frame is predicted to encode a hydrophobic inner membrane protein that belongs to the major facilitator superfamily (MFS) (7). Deletion of ybtX in Yersinia species has no impact on Ybt synthesis, uptake, utilization of iron in a Ybt-dependent manner, or virulence in mice (7, 28). This is somewhat surprising, as deletion of any of the other ybt genes within the HPI results in attenuated bacterial growth in the absence of iron and during infection.

Recently, YbtX was shown to be involved in the import of Zn^{2+} in Y. pestis (9). Bobrov et al. showed that yersiniabactin is required for growth of Y. pestis under Zn^{2+}-deficient conditions in strains lacking the zinc acquisition locus ZnuABC and that zinc acquisition under these circumstances was dependent on yersiniabactin and YbtX (9). Our results are the first to identify a role for ybtX in disease. These results suggest a potential link between zinc in the induction of inflammatory responses in the lung during primary pneumonic plague. The anti-inflammatory effects of zinc within the airways have been known for some time, and it is understood that zinc deficiency in tissues can be highly inflammatory, particularly during infection (29–33). Thus, the role of ybtX in importing zinc may alter host immunity and contribute to inflammation within the lung.

The severely diminished host response in the lung allowed for use of the ΔybtX strain to probe the mouse model of pneumonic plague to identify key host mediators of inflammation. qRT-PCR evaluation revealed the decreased transcription of key proinflammatory cytokines IL-6 and IL-17 and the chemokines CXCL1 and CCL2 in the lungs of mice infected with ΔybtX, indicating that these cytokines and chemokines may be important to the biphasic progression of pneumonic plague. This is the first study to pinpoint cytokines/chemokines that may be of specific importance to the transition to the proinflammatory phase of pneumonic plague. IL-6 is a pleiotropic cytokine involved in the induction of fever and stimulation of acute-phase responses, as well as neutrophil production in the bone marrow (34–36). IL-6 is an established biomarker of pneumonia (37) and has been previously shown to be highly induced during pneumonic plague (4, 5). IL-17 is a proinflammatory cytokine known to be involved in host defense against a variety of microbes and is important for recruiting neutrophils to mucosal surfaces (11, 38, 39). IL-17 has been shown to be highly induced during pneumonic plague and is also speculated to be involved in cell-mediated defense against Yersinia infection (4, 5, 12, 40). The chemokine CCL2, otherwise known as MCP-1, is an important chemoattractant for monocytes, macrophages, and neutrophils to sites of inflammation (41, 42). CCL2 is highly induced during pneumonic plague, and it has been suggested that CCL2 interactions with its receptor on innate immune cells are important for defense against Y. pestis and are thus targeted for inhibition by the Yersinia T3SS (5, 43, 44). CXCL1 is a known chemoattractant of neutrophils that has been shown to be induced during Yersinia infection (45, 46). A common function of these four molecules is the recruitment of neutrophils to sites of inflammation, and their dysregulation in response to pulmonary infection with ΔybtX suggests an impaired ability to stimulate neutrophil chemotaxis to the lung. Design of treatments aimed at blocking these chemokines/ cytokines, with the goal of limiting neutrophil accumulation in the lung, may aid in expanding the short time window during which antibiotic treatment is effective against pneumonic plague.

Infection with a ΔybtX strain revealed a significant and strikingly specific decrease in the number of neutrophils in the airways, indicating that YbtX is involved in initiating host responses that stimulate neutrophil migration to the lung. Previously, we showed that antibody-mediated depletion of neutrophils prior to inoculation with Y. pestis resulted in significantly diminished inflammation in the lung, ultimately attenuating pneumonia without altering bacterial survival (15). Here, we demonstrate deletion of a bacterial gene that results in a similar effect. Together, these data highlight that the pathology of pneumonic plague is largely host mediated, the consequence of recruitment of neutrophils to the lung and their continued accumulation in the airways. Further, this response does not appear to be dependent solely on a threshold of bacterial burden in the lung, as deletion of ybtX attenuated host inflammatory responses without affecting bacterial burden. The unchanged bacterial burdens in the lung and spleen despite a severely hindered neutrophil response indicate that neutrophils play a limited role in controlling Y. pestis during pulmonary infection and are thus highly damaging to the host without any immediately obvious benefit. Therefore, the ability of Y. pestis to resist neutrophil-mediated killing is likely key to the progression of pneumonic plague.

Deletion of ybtX also altered infection with K. pneumoniae, resulting in decreased inflammation as indicated by histopathological examination and decrease in lung weight compared to wild-type-infected lungs. K. pneumoniae also causes severe acute pneumonia, albeit via different mechanisms. Infection with K. pneumoniae leads to early inflammatory responses in the lung that ultimately result in severe edema and a near-complete loss of alveolar architecture in localized areas in the lung. The finding that ybtX contributes to this response is intriguing and indicates that its function may be conserved among a number of bacterial species that cause pneumonia and carry the ybt gene locus. This is important, as evaluating the role of ybtX may help to elucidate the mechanisms that contribute to the onset of severe pneumonia caused by multiple pathogens and may represent a point for therapeutic targeting.

MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The fully virulent Yersinia pestis strain CO92 was obtained from the U.S. Army, Fort Detrick, MD. Y. pestis strains were grown on brain heart infusion (BHI) agar (Difco Laboratories) at 26°C for 2 days. For infections, liquid cultures of Y. pestis CO92 were grown in BHI broth for 6 to 12 h at 26°C. The cultures were then
diluted to an optical density at 620 nm (OD620) of 0.05 to 0.1 in BHI supplemented with 2.5 mM CaCl2 and grown for 12 to 16 h at 37°C with constant shaking.

VK148, a streptomycin- and rifampin-resistant mutant of K. pneumoniae ATCC 43816, was grown on LB agar with 30 μg/ml rifampin at 26°C for 24 h. For infections, a liquid culture of K. pneumoniae was grown in LB broth overnight at 37°C.

Deletion and complementation of ybtX. For Y. pestis, the ybtX open reading frame was deleted using a modified form of lambda red recombination described by Latham et al. (47). Briefly, upstream and downstream sequences of ybtX were amplified and combined in splicing by overlap extension (SOE) PCRs with a Kan’ cassette flanked by FLP recombination target (FRT) sites (48) for allelic replacement of the wild-type ybtX ORF. The Kan’ cassette was resolved by the introduction of pSKippy, a Tet‘ derivative of pPFLP3 (49) harboring an Amp’ cassette and suc∞ and carrying the FLP recombinase gene under the control of the lac promoter.

A complementing strain for ΔybtX was constructed by using Tn7-based integration of the ybtX open reading frame under the control of the ybtPQX5 promoter in single copy into the chromosomal glmS–psS intergenic region. Briefly, the ybtX open reading frame was cloned immediately downstream of the ybtPQX5 promoter in the multiple-cloning site of pUC18R6K-mini-Tn7-T-Kan (49). The resulting plasmid was electroporated along with pTNS2 (49), a plasmid carrying the TnsABC-D specific transposition pathway, into the ΔybtX strain, and transformants were selected on BHI plates containing kanamycin. The Kan’ cassette was then resolved via the introduction of pSKippy as described above.

For K. pneumoniae, the ybtX open reading frame was deleted using double recombination as previously described (50). Briefly, approximately 1-kb upstream and downstream flanking regions of the ybtX gene were PCR amplified and combined using SOE PCR for cloning into the pKAS6 vector. The resulting vector was mated into K. pneumoniae, and merodiploids were selected on LB agar with rifampin (30 μg/ml) and kanamycin (50 μg/ml). Merodiploids were grown in LB broth without antibiotics for 4–6 h and then plated on LB agar with streptomycin (1 mg/ml). Kanamycin-susceptible colonies were tested by colony PCR for loss of the ybtX gene before sequencing to confirm deletion.

Animals and animal infections. All animal studies were approved by the University of North Carolina at Chapel Hill Office of Animal Care and Use, protocol 09-057. Six- to 8-week-old female C57BL/6J mice were obtained from Jackson Laboratories. Mice were provided with food and water ad libitum and maintained at 25°C and 15% humidity with alternating 12-h periods of light and dark. For animal infections, groups of four to five mice were lightly anesthetized with ketamine-xylazine and inoculated intranasally with a lethal dose of bacteria suspended in 20 μl phosphate-buffered saline (PBS); a dose of 104 CFU was used for Y. pestis and 107 CFU was used for K. pneumoniae. Moribund animals were euthanized with an overdose of sodium pentobarbital. For determination of bacterial burden, lungs or spleens were removed at the indicated times and homogenized in 1 ml PBS using an Omni Tissue Tearer. Total RNA was purified from lung homogenates using the TRIzol reagent manufacturer protocol, treated with Turbo DNase (Ambion), and reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer’s instructions. cDNAs were used as the templates for amplification and detection of the mouse genes encoding IL-17, IL-6, IL-23, TNF-α, IFN-γ, CCL2, CCL3, CXCL1, CXCL2, IL-10, and IL-12 and with SYBR green dye (Bio-Rad) in an iCycler thermocycler (Bio-Rad). For each gene, the calculated threshold cycle (Ct) was normalized to that of the g3′β gene from the same sample before calculating fold change relative to sample isolated from broth-grown culture using the ΔΔCt method (51, 52).

Cytokine analysis. Groups of five mice were inoculated intranasally with 1 × 103 CFU of Y. pestis CO92, Y. pestis CO92ΔybtX, or the complemented Y. pestis CO92 ΔybtX. Uninfected mice and mice infected for 24, 48, and 60 h were sacrificed, and the lungs were removed, immediately minced, and submersed in 2 ml of TRIzol reagent (Ambion). Lungs were then homogenized with an Omni Tissue Tearer. Total RNA was purified from lung homogenates using the TRIzol reagent manufacturer protocol, treated with Turbo DNase (Ambion), and reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer’s instructions. cDNAs were used as the templates for amplification and detection of the mouse genes encoding IL-17, IL-6, IL-23, TNF-α, IFN-γ, CCL2, CCL3, CXCL1, CXCL2, IL-10, and IL-12 and with SYBR green dye (Bio-Rad) in an iCycler thermocycler (Bio-Rad). For each gene, the calculated threshold cycle (Ct) was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from the same sample prior to calculating the fold change using the ΔΔCt method (51, 52).

Lavage of lungs for flow cytometry. For generating a lung single-cell suspension of the BALF, mice were euthanized with an overdose of sodium pentobarbital, and the hepatic portal vein was cut and bled. The tracheas were cannulated with a 22-gauge catheter, and lungs were inflated with 1 ml PBS using a 1-ml syringe, which was then drawn out slowly. This was repeated twice for a total of 3 ml. BALF suspensions were spun at 500 × g for 5 min at 4°C and then resuspended in 1 ml red blood cell lysis solution (0.15 M NH4Cl, 10 mM KHCO3, 0.1 mM EDTA). After incubation at room temperature for 1 min, 9 ml PBS was added and cells were pelleted as described above for staining.

Staining of lung cell suspensions for flow cytometry. After red blood cell lysis, cell suspensions from each animal were resuspended in 100 μl of 2.4G2 hybridoma supernatant and incubated at room temperature for 20 min to block macrophage Fc receptors. Cell suspensions were then pelleted at 500 × g for 5 min at 4°C. Cells were resuspended and incubated for 30 min at 4°C with the following fluorescently labeled antibodies in flow cytometry buffer (2% fetal bovine serum in PBS) for staining of cell surface markers (1:500 dilutions): CD11b-phycocerythrin (clone M1/70.15; Invitrogen), CD11c-phycocerythrin-Texas Red (clone N418; Invitrogen), Ly-6G-phycocerythrin-Cy7 (clone 1A8; BD Bioscience), LIVE/DEAD fixable aqua dead cell stain (clone L34957; Invitrogen), and F4/80-allophycocyanin (clone BM8; BioLegend). Stained cells were analyzed based on fluorescence staining patterns to identify alveolar macrophages
(4/80° C CD11bmed/low CD11chigh), CD11bhigh interstitial/exudate macrophages (4/80° C CD11bhigh CD11clow/medium), monocytes (4/80° C CD11bhigh CD11clow Ly-6G−), CD11bhigh and CD11b low dendritic cells (4/80° CD11bhigh CD11clow or low), and neutrophils (4/80° CD11clow CD11bhigh Ly-6G+). Five hundred microliters of PBS was then added to samples, followed by centrifugation at 500 x g at 4°C. Cell suspensions were then resuspended in 2% formalin and incubated at room temperature for 15 min for fixation, followed by centrifugation and resuspension in PBS with gentamicin (100 µg/ml) for removal from the biosafety level 3 laboratory.

**SUPPLEMENTAL MATERIAL**
Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02302-14/-/DCSupplemental.

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