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RESEARCH ARTICLE

# A Single Amino Acid Change in the Response Regulator PhoP, Acquired during *Yersinia pestis* Evolution, Affects PhoP Target Gene Transcription and Polymyxin B Susceptibility

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**ABSTRACT** *Yersinia pestis*, the causative agent of plague, evolved from the closely related pathogen *Yersinia pseudotuberculosis*. During its emergence, *Y. pestis* is believed to have acquired its unique pathogenic characteristics through numerous gene gains/losses, genomic rearrangements, and single nucleotide polymorphism (SNP) changes. One such SNP creates a single amino acid variation in the DNA binding domain of PhoP, the response regulator in the PhoP/PhoQ two-component system. *Y. pseudotuberculosis* and the basal human-avirulent strains of *Y. pestis* harbor glycines at position 215 of PhoP, whereas the modern human-virulent strains (e.g., KIM and CO92) harbor serines at this residue. Since PhoP plays multiple roles in the adaptation of *Y. pestis* to stressful host conditions, we tested whether this amino acid substitution affects PhoP activity or the ability of *Y. pestis* to survive in host environments. Compared to the parental KIM6+ strain carrying the modern allele of *phoP* (*phoP-S215*), a derivative carrying the basal allele (*phoP-G215*) exhibited slightly defective growth under a low-$Mg^{2+}$ condition and decreased transcription of a PhoP target gene, *ugd*, as well as an ~8-fold increase in the susceptibility to the antimicrobial peptide polymyxin B. The *phoP-G215* strain showed no apparent defect in flea colonization, although a *phoP*-null mutant showed decreased flea infectivity in competition experiments. Our results suggest that the amino acid variation at position 215 of PhoP causes subtle changes in the PhoP activity and raise the possibility that the change in this residue have contributed to the evolution of increased virulence in *Y. pestis*.

**IMPORTANCE** *Y. pestis* acquired a single nucleotide polymorphism (SNP) in *phoP* when the highly human-virulent strains diverged from less virulent basal strains, resulting in an amino acid substitution in the DNA binding domain of the PhoP response regulator. We show that *Y. pestis* carrying the modern *phoP* allele has an increased ability to induce the PhoP-regulated *ugd* gene and resist antimicrobial peptides compared to an isogenic strain carrying the basal allele. Given the important roles PhoP plays in host adaptation, the results raise an intriguing possibility

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that this amino acid substitution contributed to the evolution of increased virulence in *Y. pestis*. Additionally, we present the first evidence that *phoP* confers a survival fitness advantage to *Y. pestis* inside the flea midgut.

**KEYWORDS** PhoP, *Yersinia pestis*, evolution, transcription

*Yersinia pestis* is a Gram-negative bacterium that is the causative agent of plague. It has been responsible for at least three human pandemics—the Justinian plague (1), Black Death (2), and modern plague—and has claimed lives of as many as 200 million people throughout human history (3). The infectious life cycle of *Y. pestis* is complex, alternating between two hosts: mammals (mainly rodents) and insect vectors (fleas) (4). The flea acquires *Y. pestis* by taking a blood meal from an infected mammal. The ingested bacteria can form a biofilm inside the flea gut and block the flea foregut and are eventually regurgitated into a new mammalian host during a fleabite to cause bubonic plague (4). *Y. pestis* also can be transmitted through an aerosol route to cause pneumonic plague (3). Consequently, *Y. pestis* encounters a wide range of host environments during its life cycle and must adapt to each to achieve successful propagation.

The PhoP/PhoQ two-component system plays a critical role in the adaptation of many Gram-negative bacteria to stressful host environments as well as in pathogenesis (5). In a typical prokaryotic two-component system, an environmental stimulus is detected by the sensor kinase, initiating a phosphorelay that results in the phosphorylation and the activation of the response regulator. The activated response regulator binds to the promoters of the target genes, ultimately leading to coordinated changes in global gene expression profiles (5). The PhoP/PhoQ system consists of the sensor kinase PhoQ and the response regulator PhoP and has been shown to detect several stimuli, including low-$Mg^{2+}$ conditions (6), acidic pH (7), and the presence of cationic antimicrobial peptides (8). In *Y. pestis*, *phoP* is required for growth in low-$Mg^{2+}$ environments, for survival inside macrophages, and for resistance to antimicrobial peptides (9–12). Loss of *phoP* function in *Y. pestis* results in an approximately 2- to 75-fold increase in the 50% lethal dose ($LD_{50}$) and an increased time to death in murine bubonic plague models, suggesting that *phoP* plays a key role in virulence (11, 13). In addition, *Y. pestis* strains lacking *phoP* are reported to have reduced abilities to form biofilms in a flow cell system at 25°C and to block fleas *in vivo*, indicating that PhoP/PhoQ is also important for developing a transmissible infection in the flea (14). PhoP activity affects the expression of ~200 to 700 genes according to microarray studies, and at least 30 genes, including *ugd* and the seven-gene *pmr* operon, are known to be direct targets of PhoP (15–19). The *pmrHFIJKLM* and *ugd* genes encode enzymes that are necessary for the addition of the sugar 4-amino-4-deoxy-L-arabinose (aminoarabinose) to the lipid A moiety of *Y. pestis* lipopolysaccharide (LPS) (16). The aminoarabinose modification reduces the net negative charge on the outer surface of the bacterium, leading to an increased resistance to the cationic antimicrobial peptides (9, 16, 20).

The phylogenetic analyses of various *Y. pestis* and *Y. pseudotuberculosis* strains have indicated that *Y. pestis* is a clone of *Y. pseudotuberculosis* that evolved as recently as 5,000 to 79,000 years ago (21–24). Despite their close relationships, *Y. pestis* and *Y. pseudotuberculosis* have remarkably distinct ecologies; while *Y. pseudotuberculosis* is a soil- and waterborne pathogen that normally causes self-limiting gastrointestinal disease, *Y. pestis* is a highly virulent insect vector-borne pathogen (25). A comparison of *Y. pestis* and *Y. pseudotuberculosis* genomes suggests that *Y. pestis* acquired its unique pathogenic characteristics through numerous changes in its genome, including the acquisition of two plasmids (pMT1 and pPCP1), genomic rearrangements, massive gene inactivation, and gene gains (25, 26). Small genetic changes, including single nucleotide polymorphisms (SNPs), have also contributed to the adaptation of *Y. pestis* to the insect and mammalian hosts. For example, Zimbler et al. showed that the plasminogen activator protease Pla acquired a single amino acid substitution during *Y. pestis* evolution, and this substitution enhanced the

ability of *Y. pestis* to disseminate within its mammalian host (27). Similarly, Sun et al. showed that in addition to the gain of the murine toxin gene *ymt* on the pMT1 plasmid, the stepwise acquisition of inactivating mutations in three genes controlling biofilm formation enhanced the transmissibility of *Y. pestis* by its flea vector (28). The comparative examination of ancient human *Y. pestis* genomes from archaeological sites suggests that the pandemic strains of *Y. pestis* arose from less virulent non-flea-borne strains capable of infecting humans and that the small changes in the functions of key genes may have led to the increased virulence and transmissibility of *Y. pestis* (23). However, the specific genetic changes that transformed *Y. pestis* into a highly virulent human pathogen remain elusive.

*Y. pestis* strains are classified into several phylogenetic branches on the basis of comprehensive genomic analyses (21, 22, 29). A basal/ancestral branch (termed 0) is rooted in *Y. pseudotuberculosis* and includes all human-avirulent *Y. pestis* (Pestoides and subsp. *microtus*) strains. Branches 1 and 2, which contain most of the modern human-pathogenic strains sampled worldwide, as well as the newly discovered branches 3 and 4, all diverged from the basal branch 0 (21, 22, 29). In particular, the East Smithfield strain (ESS) that was isolated from the skeletal remains of Black Death victims is placed near the base of branch 1, very close to the polytomy from which branches 1 through 4 diverged (2, 29). In contrast, the *Y. pestis* subsp. *microtus* 91001 strain belongs to the basal branch 0 and is considered one of the closest relatives of the branch 1 and 2 strains that are avirulent to humans (29, 30). Therefore, genetic differences between these two strains may represent changes that played key roles in enhancing the virulence or host specificity of *Y. pestis* during the emergence of the modern human-virulent strains (2). A comparison of the genomic sequences between *Y. pestis* subsp. *microtus* 91001 and ESS revealed SNPs in 113 genes. Notably, some of the SNPs are found in genes implicated in the virulence or the host adaptation of *Y. pestis*, including *hmsT* (biofilm formation), *iucD* (iron acquisition), *ail* (host cell adhesion and serum resistance), and *phoP* (2). The SNP located in *phoP* creates a single amino acid change at position 215 of the encoded PhoP protein; *Y. pestis* subsp. *microtus* 91001 encodes glycine at this residue, while serine is coded for in ESS (2). Given the known roles of PhoP in host adaptation, we hypothesized that the *phoP* allele encoding serine might have conferred increased fitness in the flea and/or mammalian host environments and thus have been positively selected during *Y. pestis* evolution. Herein, we tested this possibility by introducing an S-to-G amino acid substitution in PhoP of the modern *Y. pestis* strain KIM6+ and assessed its effects on protein function. We show that the S-to-G substitution indeed results in the differential regulation of the transcription of a PhoP target gene and in antimicrobial peptide susceptibility.

## RESULTS

Comparison of the *Y. pestis* ESS and *Y. pestis* subsp. *microtus* 91001 genomic sequences revealed a G/A nucleotide polymorphism inside *phoP*, which generates a serine/glycine single amino acid change at position 215 of the encoded PhoP response regulator (2). A further comparison of the *phoP* sequences from other *Yersinia* strains showed that glycine (G) is encoded in *Y. pseudotuberculosis* and other basal strains of *Y. pestis*, such as Angola and Pestoides, while serine (S) is shared by all the modern strains, including KIM and CO92 (Fig. 1A). Hence, it was concluded that this SNP was acquired on the basal branch (branch 0) of the *Y. pestis* phylogenetic tree, before the polytomy where branches 1 to 4 diverged (29). The modeling of PhoP in *Y. pestis* and of a related response regulator protein PmrA in *Salmonella* predicts that this residue is located in the DNA binding domain of the protein, within the flexible loop that is likely to contact the target DNA (Fig. 1B) (10, 31). Therefore, we hypothesized that the amino acid change at this position may alter the activity of PhoP.

To assess the effects of this amino acid variation on PhoP function, we introduced the S-to-G substitution in PhoP of the KIM6+ strain to represent the basal allele of *phoP*. The resulting strain, KIM6+ *phoP-G215*, was cultured in TMH medium containing 20 $\mu$M

A.

| Gene | Substitution | Codon change | G | S |
|---|---|---|---|---|
| *phoP* | G215S | GGT>AGT | *Y. pseudotuberculosis*<br>*Y. pestis* Microtus 91001<br>*Y. pestis* Pestoides A<br>*Y. pestis* Pestoides F<br>*Y. pestis* Angola | *Y. pestis* ESS<br>*Y. pestis* CO92<br>*Y. pestis* KIM |

B.


**FIG 1** A nonsynonymous SNP in *phoP* creates a G-to-S single amino acid substitution in PhoP between *Y. pestis* subsp. *microtus* and East smithfield strain (ESS). (A) The codon change caused by the SNP in *phoP* and the resulting amino acid substitution are listed. *Yersinia* strains are classified based on the amino acid residue (glycine or serine) at position 215 of the PhoP protein. (B) Amino acid sequences and predicted secondary structure of PhoP proteins from *Y. pestis* ESS and *Y. pestis* subsp. *microtus* 91001. The secondary structure prediction is based on the previously published work by Grabenstein et al. (10) and Perez and Groisman (33). The black strips indicate the predicted $\alpha$-helices, and the gray arrows indicate the predicted $\beta$-sheets. The location of the G-to-S amino acid substitution is indicated in a shaded box.

$MgCl_2$ (a *phoP*-activating low-$Mg^{2+}$ condition), and its growth was compared to that of the parental wild-type (*phoP-S215*) strain and the isogenic KIM6+ Δ*phoP* strain (Table 1). At 21°C, all the strains were able to grow and formed tightly packed biofilms at the liquid-air interface of the glass tubes. The thicknesses of the biofilms were visibly different among the three strains, with the wild-type strain forming the most robust aggregates and the Δ*phoP* strain forming the thinnest biofilm. The biofilm of the *phoP-G215* strain showed an intermediate appearance (Fig. 2A). At 37°C, the wild-type and *phoP-G215* strains were able to grow normally, while the growth of the Δ*phoP* strain was inhibited. No biofilm formation was observed in any of the strains at this temperature (Fig. 2B).

To see if the variable biofilm formation in the strains with different *phoP* alleles was due to differences in growth or in biofilm production, a growth curve analysis was performed at ~23°C in TMH medium supplemented with 0.2% ribose as the carbon source. In the high-$Mg^{2+}$ TMH medium containing 20 mM $MgCl_2$, the growth rates of wild-type and *phoP-G215* strains were almost identical, while the Δ*phoP* strain showed a slightly reduced growth rate. However, in the low-$Mg^{2+}$ TMH medium containing 10 $\mu$M $MgCl_2$, the *phoP-G215* strain showed slightly reduced growth compared to that of the wild-type strain. The growth of the Δ*phoP* strain was highly defective as expected, since PhoP function is essential for bacterial survival under the low-magnesium con-

**TABLE 1** *Yersinia* and *Salmonell*a strains used in this study

| Strain | Relevant characteristics | Reference |
|---|---|---|
| *Y. pestis* KIM6+ | Molecular grouping 2.MED, $pCD1^-$ | 48 |
| *Y. pestis* KIM6+ Δ*phoP* | KIM6+ *phoP*Δ127–429 | 15 |
| *Y. pestis* KIM6+ *phoP-G215* | KIM6+ *phoP-G215* | This work |
| *Y. pestis* KIM6+/pGFP | KIM6+/p67GFP3.1 $Amp^r$ $Carb^r$ | 49 |
| *Salmonella* Typhimurium | *Salmonella enterica* serovar Typhimurium ATCC 14028 | 50 |

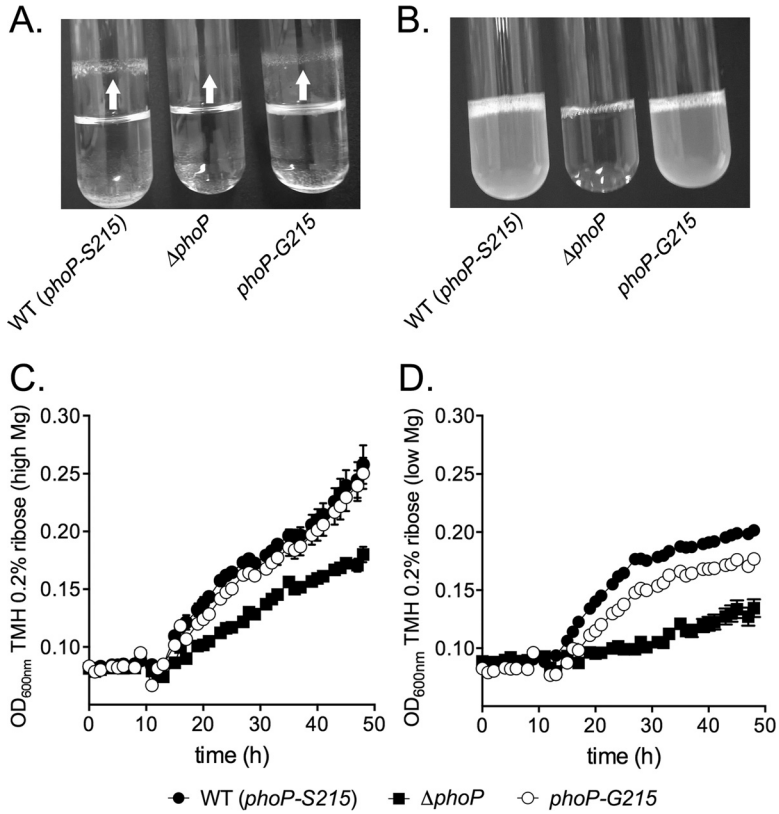


**FIG 2** Growth characteristics of *Y. pestis* strains. (A and B) *Y. pestis* KIM6+ (wild type [WT]) or the indicated KIM6+-derived strains were cultured overnight in TMH medium containing 20 $\mu$M $Mg^{2+}$ (*phoP*-activating low-$Mg^{2+}$ condition) in glass tubes at 21°C (A) or 37°C (B), and their gross growth phenotypes were compared. The arrows indicate the biofilms formed on the surfaces of the glass tubes. The biofilms appear to have formed above the liquid-air interface, because the liquid was reaching higher levels in the tubes during incubation in a rotary shaker. The pictures are representatives of three independent experiments. (C and D) Growth curve analysis was performed at ambient room temperature (~23°C) using TMH supplemented with 0.2% ribose as the carbon source. The indicated *Y. pestis* KIM6+-derived strains were cultured successively over two nights, first in brain heart infusion medium and then in TMH ribose containing 20 mM $Mg^{2+}$. On day three, the strains were diluted to 1:1,000 in TMH ribose with 20 mM $Mg^{2+}$ (high $Mg^{2+}$) (C) or TMH ribose with 10 $\mu$M $Mg^{2+}$ (low $Mg^{2+}$) (D) and growth was recorded on a Bioscreen C (Growth Curves, USA) for 48 h. Data represent the average from three biological replicates.

ditions (10). These results suggested that the subtle difference in biofilm formation at this temperature is likely due to the reduced growth rate of the *phoP-G215* bacteria and that the S-to-G substitution in PhoP may have caused a small change in the functionality of the protein.

To determine if the amino acid change in PhoP has any effect on the activity of the protein, we compared the expression levels of a PhoP target gene, *ugd*, in the *phoP* variant strains. RNA samples were collected from the overnight cultures of *Y. pestis* strains grown at different temperatures in low-$Mg^{2+}$ (20 $\mu$M $MgCl_2$) TMH medium, and the *ugd* transcript levels were determined by quantitative reverse transcription-PCR (RT-qPCR). The results were normalized against 16S rRNA levels. An approximately 2-fold decrease in the *ugd* transcript level was observed in the *phoP-G215* strain compared to that in the wild type (*phoP-S215*), and even less transcript was detected in the Δ*phoP* strain (Fig. 3A and B). Similar results were obtained when *ugd* expression levels were determined in logarithmically growing cultures (data not shown). The expression levels of other PhoP targets such as *pmrH* and *mgtC* showed similar trends, although the results were not statistically significant (data not shown). The transcript levels of *phoP* itself were not significantly different between the wild-type and the *phoP-G215* strains (Fig. 3C), and the immunoblot of the bacterial cell lysates showed similar PhoP protein levels between these strains (Fig. 3D). Therefore, we concluded

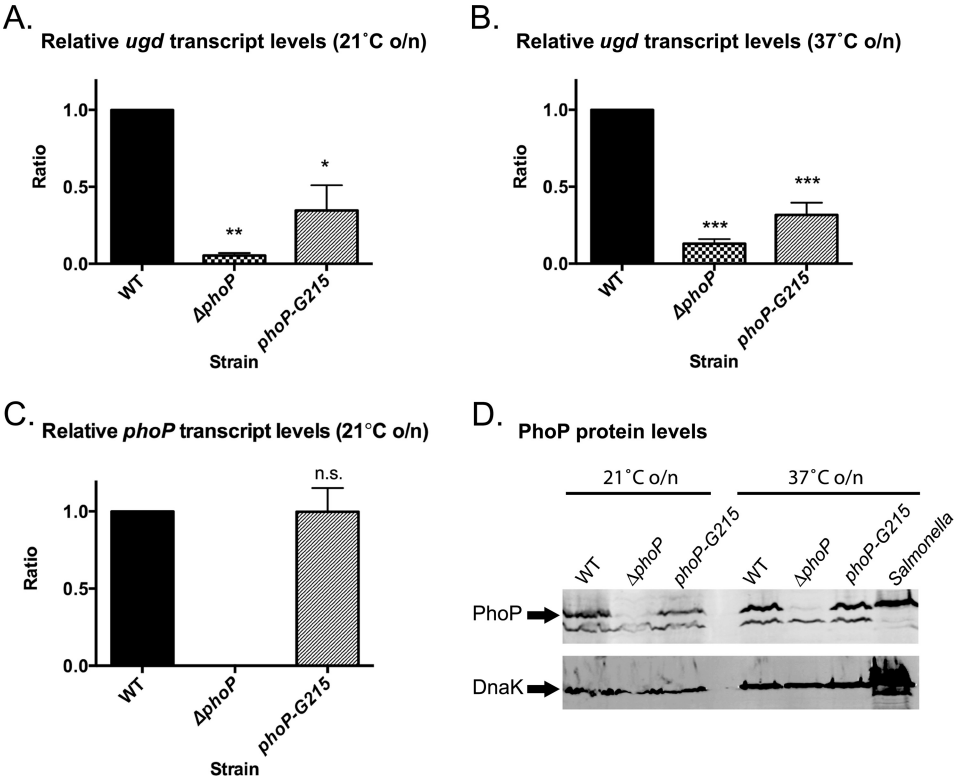


**FIG 3** *ugd* transcript levels are decreased in *Y. pestis* KIM6+ *phoP-G215*, while *phoP* transcript or PhoP protein levels are unaffected. (A to C) mRNA levels of the PhoP-regulated gene *ugd* at 21°C (A) and 37°C (B), as well as *phoP* at 21°C (C), were determined in the indicated *Y. pestis* strains by RT-qPCR. The strains were grown overnight (o/n) at indicated temperatures in low-$Mg^{2+}$ (20 $\mu$M $MgCl_2$) TMH medium. The results were normalized against the 16S rRNA expression levels. Relative levels of expression (with the wild-type levels set at 1) are shown. Data represent the averages from at least three biological replicates. Error bars represent standard errors of the means (SEMs). The asterisks indicate significant differences compared to KIM6+ WT (*, $P < 0.05$; ***, $P < 0.001$) and "n.s." denotes not significant statistically ($P > 0.05$) as determined by one-way ANOVA with Tukey's multiple-comparison tests. (D) PhoP protein levels of the indicated strains were compared by immunoblotting. Approximately $2 \times 10^8$ bacteria grown in low-$Mg^{2+}$ TMH medium were lysed in 1× Laemmli sample buffer and loaded in each lane. DnaK protein levels were used as a loading control, and *Salmonella* Typhimurium was used as a positive control for the anti-PhoP antibody. At least three independent experiments were performed and the images of a representative blot are shown.

that the S-to-G change does not alter the expression level or the stability of PhoP but decreases the ability of PhoP to induce the transcription of its target, *ugd*.

The decreased induction of the PhoP target gene *ugd* in the KIM6+ *phoP-G215* strain is consistent with the idea that the PhoP-G215 protein has a decreased activity as a transcriptional regulator compared to that of the wild-type PhoP. To test if PhoP-G215 has a reduced ability to bind the target DNA sequence, electrophoretic mobility shift assays (EMSAs) were performed. Increasing amounts of purified PhoP proteins (either PhoP-S215 or PhoP-G215) were incubated with biotin-labeled PCR-amplified DNA fragments spanning the promoter regions of *ugd* and *pmrH*, and the ability of the protein to cause a gel shift was determined. As shown in Fig. 4, similar amounts of PhoP-S215 and PhoP-G215 proteins were required to retard the mobility of the *ugd* and *pmrH* promoter fragments. Similar gel shift patterns were observed when the PhoP proteins were preincubated with acetyl phosphate to produce phosphorylated PhoP (data not shown).

The PhoP-regulated *ugd* and *pmrHFIJKLM* genes promote the resistance of *Y. pestis* to antimicrobial peptides by adding aminoarabinose to the lipid A portion of LPS (16). Since the S-to-G substitution in PhoP affects the transcription levels of *ugd*, we next determined the susceptibility of the *phoP* variant strains to the antimicrobial peptide polymyxin B. The strains were incubated in low-$Mg^{2+}$ (20 $\mu$M $MgCl_2$) TMH medium

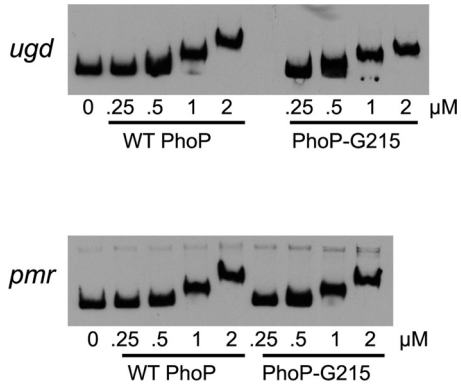


**FIG 4** WT PhoP and PhoP-G215 proteins have similar affinities to *ugd* and *pmr* promoters, as determined by electrophoretic mobility shift assays. Electrophoretic mobility shift assays (EMSAs) were performed using 1 nM biotin-labeled DNA fragments spanning the promoter regions of *ugd* (A) and *pmrH* (B) and increasing amounts (0 to 2 $\mu$M) of purified WT PhoP or PhoP-G215 proteins as indicated. The results shown are representative of three independent experiments.

containing various concentrations of polymyxin B, and the MICs were determined. At 37°C, both the wild-type and the *phoP-G215* strains were highly susceptible to polymyxin B. However, at 21°C and 28°C, the MICs of the *phoP-G215* strain were approximately 4-fold lower than those of the wild type, showing that the *phoP-G215* strain has an increased susceptibility to polymyxin B (Table 2). The control strain lacking *phoP* was completely susceptible to polymyxin B at all temperatures (Table 2).

The increased susceptibility to antimicrobial peptides could influence the ability of *Y. pestis* to survive inside insect or mammalians hosts. As mammalian macrophages are known to produce cationic antimicrobial peptides such as CRAMPs to limit the intracellular growth of bacterial pathogens (32), we first tested whether the bacterial survival inside macrophages is affected in the *phoP-G215* strain. We infected murine bone marrow-derived macrophages with the *phoP-G215* and control strains that were pregrown at 28°C in a rich heart infusion (HI) medium and enumerated the intracellular bacteria at various times postinfection. No significant difference was detected between the wild-type and *phoP-G215* strains in their ability to survive inside macrophages (data not shown).

We next compared the ability of the *phoP* variant strains to colonize the flea midgut in coinfection experiments. The flea vector *Xenopsylla cheopis* was fed with the 1:1 mixture of wild-type and the mutant bacteria in a blood meal, and the percentage of each strain colonizing the midgut was determined at various times postinfection. When the wild-type (*phoP-S215*) and the *phoP-G215* strains were coinfected, similar numbers of bacteria from each strain were recovered from the flea midgut at 7 days postinfection, showing that neither strain had a significant competitive advantage over the other (Fig. 5). In contrast, wild-type bacteria dominated when the wild-type and the Δ*phoP* strains were coinfected, suggesting that *phoP* function may be necessary for the successful survival and colonization of the bacteria in the flea midgut (Fig. 5).

**TABLE 2** *Y. pestis phoP* G215 mutant strain is more susceptible to antimicrobial peptide polymyxin B[a]

| Strain | Polymyxin B MIC ($\mu$M) at: | | |
|---|---|---|---|
| | 21°C | 28°C | 37°C |
| KIM6+ WT | 7.81 | 31.25 | 0.49 |
| KIM6+ Δ*phoP* | 0.49 | 0.98 | ND |
| KIM6+ *phoP-G215* | 0.98 | 3.91 | 0.49 |

[a]Bacteria were grown for 20 h in low-$Mg^{2+}$ TMH medium containing various concentrations of polymyxin B (0.12 to 125 $\mu$M) at the indicated temperatures, and the MICs were determined as the lowest concentration at which no bacterial growth was seen. At least three independent experiments were performed, and the results of one representative experiment are shown.

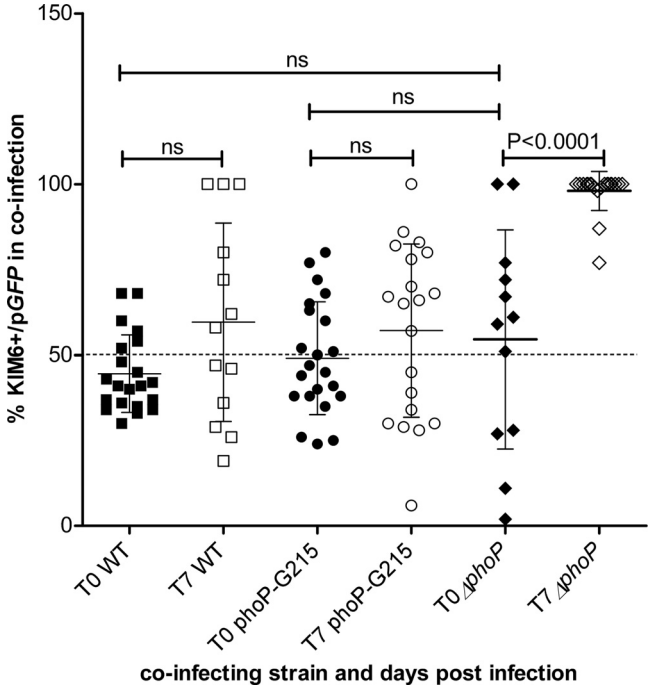


**FIG 5** KIM6+ Δ*phoP* mutant is outcompeted by the wild type during flea coinfection, while KIM6+ *phoP-G215* can colonize the flea equally well as the wild type. Cohorts of *Xenopsylla cheopis* fleas were fed on a blood meal containing a 1:1 mixture of wild-type (KIM6+ harboring an ampicillin/carbenicillin resistance cassette on plasmid pGFP [KIM6+/pGFP]) and the indicated *phoP* mutant or wild-type KIM6+ *Y. pestis* strains. At days 0 (T0) and 7 (T7) postinfection, *Y. pestis* cells colonizing the flea midgut were enumerated and the percentages of each strain colonizing the midgut were determined. For each coinfection experiment at a given time point, CFU was determined for 20 to 25 fleas that had fed on infected blood. Data points showing that 0 CFU were recovered from a flea were removed from the analysis (there was no apparent difference among three *phoP* variant strains in the number of fleas that gave 0 CFU.) Data were plotted as the percentage of the KIM6+/pGFP recovered from each coinfected flea. Error bars represent means ± standard deviations (SDs) from the percentage CFU data. Bars indicate the two groups that were assessed for statistical differences.

## DISCUSSION

The aim of this study was to compare two naturally occurring alleles of *phoP* in *Y. pestis* that result in the S-to-G single amino acid substitution at position 215 of the PhoP response regulator. We found that this amino acid substitution has small but reproducible effects on PhoP functions. Compared to the parental KIM6+ strain carrying the modern allele (*phoP-S215*), a derivative of KIM6+ carrying the basal allele (*phoP-G215*) exhibited slightly defective growth under a low-$Mg^{2+}$ condition and decreased transcription of a PhoP target gene, *ugd*, as well as an ~8-fold increase in the susceptibility to the antimicrobial peptide polymyxin B. The increased susceptibility to polymyxin B is likely due to the decreased aminoarabinose modification of lipid A, since *ugd* encodes an enzyme required for this process (16). Given the known roles of *Y. pestis* PhoP in growth under low-$Mg^{2+}$ conditions and resistance against antimicrobial peptides (9–12), our results suggest that the S-to-G amino acid substitution has led to the slight reduction in the activity of PhoP protein.

Residue 215 of the PhoP protein is predicted to be located within the DNA binding domain that is likely to contact the target DNA (10, 31). A similar single amino acid variation exists in the equivalent residue (amino acid 211) of a related response regulator, PmrA, in *Salmonella* (31). The PmrA protein from *Salmonella enterica* serovar Paratyphi B has a glutamate residue at position 211 (PmrA E211), whereas most other *S. enterica* strains have glycines at this position (PmrA G211). The purified PmrA E211 protein was shown to bind DNA less efficiently than PmrA G211 and have a decreased ability to activate PmrA-dependent genes, including the *pmrCAB* operon itself (31). It was proposed that this is partly because a negatively charged glutamate at position 211

does not bind strongly to DNA, which is also negatively charged (31). Similarly, the decreased *ugd* expression in KIM6+ *phoP-G215* may be caused by the lower binding affinity of PhoP-G215 to the *ugd* promoter, as the polar serine residue may have a stronger affinity for DNA than the nonpolar glycine. Our EMSAs did not reveal an observable difference in the abilities of PhoP-S215 and PhoP-G215 proteins to bind the promoters of *ugd* or *pmrH*. The change in the DNA affinity due to this single amino acid substitution may be too small to be detected by EMSA under the conditions tested. This possibility is consistent with our finding that the changes in the expression levels of PhoP target genes are also small between the *phoP-S215* and *phoP-G215* strains. Alternatively, the amino acid substitution could affect the transcription of PhoP-regulated genes more indirectly *in vivo*, involving other factors. For example, it could influence the phosphorylation status of PhoP, thereby affecting the activity of the transcription factor. In *Salmonella*, the G211E substitution in PmrA changes not only the DNA affinity but also the levels of phosphorylated PmrA *in vivo*, likely due to the altered efficiency of the PmrD protein to protect PmrA from dephosphorylation (31). The amino acid substitution in PhoP could also alter its interaction with RNA polymerase via a conformational change, potentially affecting the transcription of the downstream genes (33). More sensitive and quantitative assays for the DNA affinity and/or assays that can detect the PhoP/DNA interaction *in vivo* (e.g., chromatin coimmunoprecipitation) might reveal the mechanism for differential gene regulation by PhoP-S215 and PhoP-G215 proteins.

The reduced *ugd* expression in the *phoP-G215* strain suggests that the expression levels of other PhoP-regulated genes also may be altered. It would be interesting to see if the S-to-G amino acid substitution results in a uniform decrease in the expression of all the PhoP-induced genes or the alteration of the repertoire of genes activated by this response regulator. Interestingly, the levels of *phoP* RNA as well as PhoP protein were unchanged in the *phoP-G215* strain even though the autoregulation of PhoP has been reported in both *Salmonella* and *Y. pestis* (34, 35). Further studies should reveal the extent of the effects the S215G amino acid substitution causes on the PhoP regulon.

The resistance to cationic antimicrobial peptides is important for many pathogens, since both insect and mammalian hosts produce a variety of them to combat infectious agents (36). Our finding that the *phoP-G215* strain is more susceptible to polymyxin B than the *phoP-S215* strain raises a possibility that the acquisition of the *phoP-S215* allele contributed to the increased virulence of the modern *Y. pestis* strains through increased resistance to antimicrobial peptides. Consistent with this idea, Anisimov et al. reported previously that *Y. pestis* subsp. *microtus* bv. caucasica, a biovar that is virulent in mice but not in guinea pigs or humans, also carries the *phoP-G215* allele and shows increased susceptibility to polymyxin B at 25 to 28°C (37, 38). However, so far, we have been unable to establish that the strains carrying *phoP-S215* have increased fitness in the host environments. In our coinfection experiment using the flea host, the wild-type (S215) strain did not exhibit any competitive advantage over the *phoP-G215* strain in colonizing the flea midgut. We also did not detect a difference between the *S215* and *G215* strains in their ability to survive inside murine macrophages, although our experiment did not use the bacteria grown in low-$Mg^{2+}$ TMH medium (data not shown). Also unknown is the effect of this amino acid variation in the virulence of *Y. pestis* in mammalian hosts. Previous infection studies using mouse bubonic plague models showed that even the complete loss of PhoP function results in only mild attenuation (11, 13). Therefore, the single amino acid change in PhoP may not cause a dramatic attenuation in these models. Further studies are needed to determine if this allelic variation causes any changes in the ability of *Y. pestis* to infect and colonize mammalian hosts or to be transmitted between hosts.

In previous single-strain infection studies, *phoP* was shown to play roles in biofilm formation and the blocking of the proventriculus in fleas but not in the infectivity of the bacteria, since a Δ*phoP* strain exhibited bacterial loads and infection rates in the flea midgut similar to those of the wild type (14). However, our coinfection studies showed that a *phoP* deletion strain is outcompeted by the wild-type strain in the flea midgut.

To our knowledge, this is the first study that directly indicated a potential role of *phoP* in the survival of *Y. pestis* within the flea digestive tract. Our results are consistent with the finding in a *Drosophila melanogaster* model for the *Y. pestis* colonization of insect vectors that the loss of *phoP* results in decreased colonization of the midgut of fly larvae (39). Although the mechanism by which *phoP* promotes bacterial survival in the fleas is unclear, it may be a subtle role that is not easily detected in a single-strain infection assay. It has been demonstrated that the *Y. pestis* strain lacking *ugd* is not defective in either flea infection or blockage, suggesting that the lipid A modification is not involved in these processes (14). However, this was determined in single-strain infection assays and not coinfection assays. A recent study reported that *Y. pestis* lacking other genes in the lipid A modification pathway (*galU* and *arnB*) is defective in colonizing the flea midgut (40). In addition, the aforementioned *Drosophila* model of *Y. pestis* colonization showed that the decreased colonization of fly larvae by the *phoP* mutant strain can be rescued by the inactivation of the host *imd*, the gene that drives the production of antimicrobial peptides (39). Therefore, we cannot rule out the possibility that the lipid A modification and the resistance to antimicrobial peptide play some role in *Y. pestis* survival in the flea. Alternatively, the flea colonization by *Y. pestis* may depend on *phoP*-dependent pathways other than for lipid A modification, such as those for metabolism (19, 41) or the formation of a cohesive biofilm (14).

Recent studies have shown that several SNPs and other small genetic changes have played critical roles in the emergence of modern *Y. pestis* strains during the evolution of the pathogen (27, 28). However, it is difficult to determine whether a given genetic change/polymorphism has been fixed in the modern *Y. pestis* genomes because it confers a selective advantage (i.e., positive selection) or it is simply the result of neutral genetic drift. Indeed, it has been argued that most *Y. pestis* SNPs fall into the latter category (29). Accordingly, the presence of the *phoP-S215* allele in modern *Y. pestis* strains could have little to do with any putative selective advantage. Nevertheless, our observation that the *phoP-S215* allele renders the bacteria more resistant to polymyxin B is consistent with the idea that this allele confers a slight fitness advantage in hostile host environments and that the acquisition of this allele might have contributed to the increased virulence of the modern *Y. pestis* strains. The evolution of bacteria can be driven by changes in the gene regulatory network due to the altered functions of transcription regulators, because such changes can affect the ecological niche in which the pathogen can thrive (33). Therefore, it is possible that the subtle changes in PhoP can influence the adaptability or the specificity of *Y. pestis* to its hosts through the modification of its regulon.

In addition to *phoP*, a comparison of the genome sequences of *Y. pestis* subsp. *microtus* 91001 and *Y. pestis* ESS revealed a number of SNPs in genes implicated in virulence, such as *hmsT*, *iucD*, and *ail* (2). Interestingly, *Y. pestis* bv. caucasica, which carries the same *ail* allele as *Y. pestis* subsp. *microtus* 91001, is reported to be less resistant to human serum than strain KIM, suggesting that this SNP may also contribute to the increased virulence of modern *Y. pestis* to humans (37). Perhaps the SNP in *phoP* is one of many small genetic changes that yielded subtle effects on the pathogenic characteristics of *Y. pestis*, and it is the combinatorial effects of multiple mutations that have led to more noticeable differences in pathogen virulence.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The *Y. pestis* strains used in this study (Table 1) were derived from strain KIM (molecular group 2.MED [21]). All *Y. pestis* strains used did not contain the pCD1 plasmid, and are therefore avirulent and exempt from tier 1 select agent guidelines. Unless otherwise indicated, *Y. pestis* strains were cultivated at 28°C on heart infusion ([HI] Difco) plates, and cultures were grown in HI broth or the chemically defined TMH medium (42) containing 20 $\mu$M magnesium chloride (low-$Mg^{2+}$ TMH) with aeration at 21°C (the room temperature of the Bliska lab), 28°C, or 37°C. The growth medium for *Y. pestis* was supplemented with kanamycin at 25 $\mu$g/ml or chloramphenicol at 10 $\mu$g/ml when appropriate. *Y. pestis* cells from the 21°C cultures were collected by scraping off the biofilm that had formed on the surface of the glass tube at the air-liquid interface, as there were not enough bacteria in the liquid suspension. The *Escherichia coli* strain S17-1 used for plasmid and mutant construction was grown as described previously (10). The *Salmonella enterica* serovar Typhimurium strain ATCC 14028 was

**TABLE 3** List of primers used in this study

| Primer | Sequence (5′→3′) | Reference |
|---|---|---|
| *phoP*-F2[a] | TCTAGCGGCCGCGTGCTCAAGACGGTATAGCACC | |
| *phoP*-R[a] | TTAGGATCCAGAGGTCGCGGCGTAGGTATTG | |
| *phoP*-mut1[b] | AAGTCATTACGACTATTCGT**GG**C**C**AGGGATATCGTTTTGAC | |
| *phoP*-mut2[b] | GTCAAAACGATATCCCTG**GCC**ACGAATAGTCGTAATGACTT | |
| *phoP*-qRTF | TGTTGCGTCACCATCTGACA | |
| *phoP*-qRTR | TCACCGGGCAAACCAAGAT | |
| *ugd*-RTF | GCTCCGTTGGTCAAAGAAAA | |
| *ugd*-RTR | CCGTCTTCATCAGGAGGTGT | |
| yr005 (16S rRNA)_f | TGAACCCAGATGGGATTAGC | 51 |
| yr005 (16S rRNA)_r | CGCTTTACGCCCAGTAATTC | 51 |
| *mgtC*-RTF | GCACAGATTGTTTCGGGAAT | |
| *mgtC*-RTR | GACCACAAAGCACACCAATG | |
| *pmrH*-RTF | CATGCGATCGCTGTATGTTC | |
| *pmrH*-RTR | CACGTCTGTGATGGCGTAAT | |
| *ugd*-EMSA-F2 (CO92-YPO2174EMSA-F) | biotin(TEG)-GCTTAACAATGGTGTCCC | 17 |
| *ugd*-EMSA-R2 (CO92-YPO2174EMSA-R) | biotin(TEG)-ACTCCCAGTGATTATCGG | 17 |
| *pmrH*-EMSA-F1 | biotin(TEG)-GCGTTAAATCCAACTCATTG | |
| *pmrH*-EMSA-R1 | biotin(TEG)-GCGATCGCATGTTTGCAAC | |

[a]Underlined letters represent specific restriction sites added to the primer.
[b]Letters in boldface font represent specific nucleotide changes that were introduced by the site-directed mutagenesis, and the underlined letters show the MscI restriction site that has been introduced by the change.

a gift from M. Starnbach (Table 1). It was grown on Luria-Bertani (LB) agar plates at 37°C and cultured in LB or low-$Mg^{2+}$ TMH medium with aeration at 37°C. To isolate logarithmically growing bacteria, the overnight cultures were subcultured in fresh medium and bacteria were grown with aeration for 4 h at indicated temperatures.

**Construction of KIM6+ *phoP-G215* mutants.** The *phoP* open reading frame was amplified from KIM6+ by PCR using primers *phoP*-F2 and *phoP*-R (Table 3), digested with BamHI and NotI, and ligated into the pSB890 suicide plasmid (43) that also had been cut with BamHI and NotI. The resulting plasmid was subjected to site-directed mutagenesis to introduce a serine-to-glycine substitution at position 215 of PhoP protein, using primers *phoP*-mut1 and *phoP*-mut2 (Table 3) and a QuikChange site-directed mutagenesis kit (Stratagene/Agilent Technologies) according to the manufacturer's instructions. The mutagenesis also introduced a silent mutation that creates an additional MscI restriction site in the *phoP-G215* allele for easy recognition. The suicide plasmid carrying *phoP-G215* was mated into wild-type KIM6+, and the KIM6+ *phoP-G215* strain was created by allelic exchange as described previously (10).

**Growth curves.** Growth analysis was performed at ambient room temperature ~23°C in the Vadyvaloo lab) using TMH medium supplemented with 0.2% ribose as the carbon source. Ribose was added because a transcriptional analysis of *Y. pestis* from blocked fleas suggests that *Y. pestis* specifically takes up pentose sugars, including ribose, during development in the flea digestive tract (41). The strains were cultured successively over two nights, first in brain heart infusion (BHI) medium and then in TMH ribose containing 20 mM $Mg^{2+}$ (high $Mg^{2+}$). On day three, the strains were diluted 1:100 in high-$Mg^{2+}$ TMH ribose medium or TMH ribose containing 10 $\mu$M $Mg^{2+}$ (low $Mg^{2+}$), and growth without shaking was recorded on a Bioscreen C (Growth Curves, USA) for 48 h. Growth curves were plotted using GraphPad Prism 5.

**Polymyxin B MIC assay.** The MIC of polymyxin B for *Y. pestis* was determined as previously described (44), except for the following modifications. Polymyxin B (Sigma) was serially diluted 2-fold into low-$Mg^{2+}$ (20 $\mu$M $Mg^{2+}$) TMH medium at concentrations ranging from 250 to 0.24 $\mu$M, and 100 $\mu$l of the dilution was transferred to the wells of the 96-well plate. Overnight cultures of *Y. pestis* were grown at 21°C, 28°C, or 37°C in low-$Mg^{2+}$ TMH medium and diluted in fresh low-$Mg^{2+}$ TMH medium, and 100 $\mu$l of the dilution was added to the wells containing a TMH-polymyxin B solution at a final concentration of ~$1 \times 10^6$ CFU/ml. The plates were incubated at 21°C, 28°C, or 37°C, respectively, for 20 h with shaking in a C24 shaker (New Brunswick Scientific) at 200 rpm. The bacterial growth in each was examined visually and compared to that of a control grown in the medium with no polymyxin B.

**RT-qPCR.** Relative transcription levels of *phoP* and *ugd* and other PhoP target genes were determined by quantitative reverse transcription-PCR (RT-qPCR). The primers used for RT-qPCR were designed using the Primer3 Plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (45) and are listed in Table 3. Total bacterial RNA was stabilized and isolated from overnight cultures or logarithmically growing cultures of *Y. pestis* grown in low-$Mg^{2+}$ (20 $\mu$M $MgCl_2$) TMH medium at the indicated temperatures (21°C, 28°C, or 37°C). To obtain logarithmically growing cultures, the overnight cultures were diluted in low-$Mg^{2+}$ TMH medium to an optical density at 600 nm ($OD_{600}$) of ~0.1, and incubation was continued at the same temperatures for 4 h with aeration. For the stabilization of RNA, 2 to 5 ml of each culture (~$1 \times 10^9$ cells) was centrifuged, and the cell pellet was mixed immediately with 1 ml of RNAprotect Bacteria reagent (Qiagen) and incubated at room temperature for 5 min. The cells were then harvested by centrifugation at 4,000 $\times$ *g* for 10 min. The cell pellets were used immediately for RNA isolation or stored at −80°C until use. Total RNA was isolated using a Qiagen RNeasy Mini kit, and the on-column digestion of genomic DNA was performed using RNase-free DNase (Qiagen) as described previously (15).

cDNA synthesis was performed with 0.6 $\mu$g of total RNA and 300 ng of random hexamers (Invitrogen), using SuperScript III reverse transcriptase according to the manufacturer's instructions, except that the reaction was carried out for ~8 h at 42°C. Each of the 25-$\mu$l qPCR mixtures contained 2.0 $\mu$l of a 1:20 dilution of cDNA, 0.3 $\mu$M gene-specific primers, and 12.5 $\mu$l of 2× QuantiTect SYBR green master mix (Qiagen). The reaction was performed according to the manufacturer's instructions and monitored using an ABI Prism Applied Biosystems 7300 SDS real-time PCR machine. The expression level for each gene was calculated using standard curves, and the results were normalized to *Y. pestis* 16S rRNA. RT-qPCR was performed on at least three independent RNA samples.

**Detection of PhoP protein by immunoblotting.** Approximately $2 \times 10^8$ bacteria (as determined by the $OD_{600}$) grown in low-$Mg^{2+}$ (20 $\mu$M $MgCl_2$) TMH medium were lysed in 1× Laemmli sample buffer and loaded on a 12% SDS-PAGE gel. The blots were probed with a 1:2,000 dilution of a rabbit anti-*Salmonella* PhoP antibody (a gift from E. Groisman) in Tris-buffered saline–0.05% Tween 20 (TBST) containing 1% casein for 2 h at room temperature. The *Salmonella enterica* serovar Typhimurium strain ATCC 14028 (Table 1) was run as a positive control for the anti-PhoP antibody. As a loading control, a mouse anti-DnaK monoclonal antibody (Enzo) was used at a 1:500 dilution. Secondary antibodies, an IRDye680-conjugated anti-IgG rabbit antibody and an IRDye800-conjugated anti-IgG mouse antibody (LI-COR), were used at dilutions of 1:5,000 and 1:10,000, respectively. The infrared signal from each band was detected and quantified using an infrared imaging system (Odyssey; LI-COR).

**Purification of His-tagged PhoP and its derivative mutant.** His-tagged *Y. pestis* PhoP proteins were expressed in *E. coli* BL21(DE) containing either pT7.7-PhoP yersinia (33) (a gift from E. Groisman) or pT7.7-PhoP-G215 yersinia expression vectors. pT7.7-PhoP-G215 yersinia was created from pT7.7-PhoP yersinia carrying wild-type PhoP (PhoP-S215) by site-directed mutagenesis, using phoP-mut1/phoP-mut2 primers (Table 3) and a QuikChange site-directed mutagenesis kit (Stratagene/Agilent Technologies). Overnight cultures of these expression strains were diluted to an $OD_{600}$ of 0.1 in 50 to 150 ml LB medium supplemented with 100 $\mu$g/ml ampicillin and grown at 37°C until reaching an $OD_{600}$ of ~0.6. Then, isopropyl-1-thio-B-D-galacopyranoside (IPTG) was added to a final concentration of 100 $\mu$M and incubated at 30°C for an additional 4 h. The cells were harvested and suspended in lysis buffer (50 mM Tris [pH 8.0], 100 mM NaCl, 10 mM imidazole) and disrupted by sonication. The insoluble fraction was pelleted and removed by centrifugation at 14,000 rpm for 20 min. PhoP protein was purified using a His-Bind purification kit (Novagen) according to the manufacturer's instructions, except that the lysis buffer described above was used instead of the binding buffer provided by the kit. The purified protein was concentrated and the buffer was exchanged into the storage buffer (50 mM Tris [pH 7.6], 100 mM NaCl, 10% glycerol, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol [DTT]) using Amicon Ultra centrifugal filter units (molecular weight cutoff [MWCO] 10; Millipore).

**EMSAs.** Biotin-labeled DNA fragments spanning the promoter regions of PhoP target genes *ugd* and *pmrH* were generated by PCR using *Y. pestis* KIM6+ genomic DNA as a template. The upstream sequence of *ugd* containing the consensus PhoP box was amplified using primers *ugd*-EMSA-F2 (CO92-YPO2174EMSA-F) and *ugd*-EMSA-R2 (CO92-YPO2174EMSA-F) (17) (Table 3), and that of *pmrH* was amplified using primers *pmrH*-EMSA-F1 and *pmrH*-EMSA-R1 (Table 3), resulting in 405-bp and 386-bp fragments, respectively. The PCR products were gel purified with the QIAquick PCR purification kit (Qiagen). The biotin-labeled DNA fragment and the purified PhoP protein were mixed with binding buffer in a total volume of 20 $\mu$l and incubated at room temperature for 20 min. The binding reaction mixture contained 20 fmol (1 nM) of the labeled DNA target, various amounts (0 to 40 pmol or 0 to 2 $\mu$M) of PhoP protein, 25 mM Tris-HCl (pH 7.6), 50 mM NaCl, 50 $\mu$g/ml poly(dI-dC), 5 mM $MgCl_2$, 50 $\mu$g/ml bovine serum albumin [BSA], 0.05 mM EDTA, 0.5 mM DTT, and 5% glycerol. Samples were separated on a 6% nondenaturing polyacrylamide gel in 0.5× Tris-borate-EDTA (TBE) buffer at 4°C and transferred to a nitrocellulose membrane (Thermo Fisher). The biotin-labeled DNA fragments were detected using a LightShift chemiluminescent EMSA kit (Thermo Fisher) according to the manufacturer's instructions. To phosphorylate PhoP proteins, purified PhoP was incubated with 20 mM fresh acetyl phosphate (Sigma) for 2.5 h (33) at room temperature or 37°C prior to the EMSA.

**Flea coinfection experiments.** For flea coinfections, the KIM6+/pGFP strain carrying a carbenicillin/ampicillin antibiotic selective marker was coinfected with either the KIM6+ wild-type, *phoP* mutant, or *phoP-G215* strains. Bacteria were grown in BHI broth overnight, first at 28°C with aeration and then at 37°C without aeration. Bacteria were harvested by centrifugation. The two strains were mixed together in a 1:1 ratio and used to seed prewarmed mouse blood (Bioreclamation) to a concentration of ~$5 \times 10^8$ cells/ml per strain. A cohort of *Xenopsylla cheopis* fleas was allowed to feed on the infected blood using a previously described artificial feeding chamber (46). Fleas that ingested a blood meal were maintained at 21°C and 75% relative humidity and fed twice a week on uninfected mice. The total *Y. pestis* bacterial load per flea was determined from 20 to 25 infected fleas at 0 and 7 days postinfection by plating on *Yersinia* selective agar base (YSAB) to determine CFU. Simultaneously, plating was carried out on YSAB plus 100 $\mu$g/ml carbenicillin to select for the KIM6+/pGFP strain. The percentage of KIM6+/pGFP cells was calculated from the CFU counts. For each experiment, the CFU was determined only on the fleas that were microscopically confirmed to contain red blood in their midguts at time zero. The data points were removed from the calculation of the average CFU and the graph if 0 CFU were recovered. In situations where a slightly higher CFU count was obtained in the plate containing antibiotic than in the one without antibiotic (i.e., >100% KIM6+/pGFP values indicating that fleas contained KIM6+/pGFP only), the data points were plotted at 100%.

**Macrophage infection and analysis of bacterial survival.** Murine bone marrow-derived macrophages (BMMs) were cultured and infected with *Y. pestis* as described previously (10, 47). The bacterial survival inside macrophages was quantified by CFU assays at 0, 0.25, 1.5, 4, and 24 h postinfection as

described previously (47), with the following modifications. BMMs were seeded in 24-well tissue culture plates at a concentration of $1.5 \times 10^5$ cells/well and infected with *Y. pestis* at a multiplicity of infection (MOI) of 5. The infection was performed with *Y. pestis* grown overnight at 28°C in HI broth. For CFU assays, the results were log transformed prior to statistical analysis.

**Statistical analyses of the data.** When appropriate, statistical tests were performed using a one-way analysis of variance (ANOVA) with Tukey's multiple-comparison test (Prism version 4.0c for Macintosh; GraphPad Software, San Diego, CA). For flea coinfections, a one-way ANOVA with Bonferroni's multiple-comparison test was applied to the data (GraphPad Prism version 5).

**Ethics statement.** Animal work (the flea infection study) was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols (04001 and 04524) were approved by the Committee on the Ethics of Animal Experiments at Washington State University.

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## REFERENCES

1. Wagner DM, Klunk J, Harbeck M, Devault A, Waglechner N, Sahl JW, Enk J, Birdsell DN, Kuch M, Lumibao C, Poinar D, Pearson T, Fourment M, Golding B, Riehm JM, Earn DJ, Dewitte S, Rouillard JM, Grupe G, Wiechmann I, Bliska JB, Keim PS, Scholz HC, Holmes EC, Poinar H. 2014. *Yersinia pestis* and the plague of Justinian 541–543 AD: a genomic analysis. Lancet Infect Dis 14:319–326. https://doi.org/10.1016/S1473-3099(13)70323-2.
2. Bos KI, Schuenemann VJ, Golding GB, Burbano HA, Waglechner N, Coombes BK, McPhee JB, DeWitte SN, Meyer M, Schmedes S, Wood J, Earn DJ, Herring DA, Bauer P, Poinar HN, Krause J. 2011. A draft genome of *Yersinia pestis* from victims of the Black Death. Nature 478:506–510. https://doi.org/10.1038/nature10549.
3. Perry RD, Fetherston JD. 1997. *Yersinia pestis*—etiologic agent of plague. Clin Microbiol Rev 10:35–66.
4. Chouikha I, Hinnebusch BJ. 2012. Yersinia–flea interactions and the evolution of the arthropod-borne transmission route of plague. Curr Opin Microbiol 15:239–246. https://doi.org/10.1016/j.mib.2012.02.003.
5. Groisman EA. 2001. The pleiotropic two-component regulatory system PhoP-PhoQ. J Bacteriol 183:1835–1842. https://doi.org/10.1128/JB.183.6.1835-1842.2001.
6. García Véscovi E, Soncini FC, Groisman EA. 1996. $Mg^{2+}$ as an extracellular signal: environmental regulation of *Salmonella* virulence. Cell 84:165–174. https://doi.org/10.1016/S0092-8674(00)81003-X.
7. Prost LR, Daley ME, Le Sage V, Bader MW, Le Moual H, Klevit RE, Miller SI. 2007. Activation of the bacterial sensor kinase PhoQ by acidic pH. Mol Cell 26:165–174. https://doi.org/10.1016/j.molcel.2007.03.008.
8. Bader MW, Sanowar S, Daley ME, Schneider AR, Cho U, Xu W, Klevit RE, Le Moual H, Miller SI. 2005. Recognition of antimicrobial peptides by a bacterial sensor kinase. Cell 122:461–472. https://doi.org/10.1016/j.cell.2005.05.030.
9. Rebeil R, Ernst RK, Gowen BB, Miller SI, Hinnebusch BJ. 2004. Variation in lipid A structure in the pathogenic yersiniae. Mol Microbiol 52:1363–1373. https://doi.org/10.1111/j.1365-2958.2004.04059.x.
10. Grabenstein JP, Marceau M, Pujol C, Simonet M, Bliska JB. 2004. The response regulator PhoP of *Yersinia pseudotuberculosis* is important for replication in macrophages and for virulence. Infect Immun 72:4973–4984. https://doi.org/10.1128/IAI.72.9.4973-4984.2004.
11. Oyston PC, Dorrell N, Williams K, Li SR, Green M, Titball RW, Wren BW. 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. https://doi.org/10.1128/IAI.68.6.3419-3425.2000.
12. Hitchen PG, Prior JL, Oyston PC, Panico M, Wren BW, Titball RW, Morris HR, Dell A. 2002. Structural characterization of lipo-oligosaccharide (LOS) from *Yersinia pestis*: regulation of LOS structure by the PhoPQ system. Mol Microbiol 44:1637–1650. https://doi.org/10.1046/j.1365-2958.2002.02990.x.
13. Bozue J, Mou S, Moody KL, Cote CK, Trevino S, Fritz D, Worsham P. 2011. The role of the phoPQ operon in the pathogenesis of the fully virulent CO92 strain of *Yersinia pestis* and the IP32953 strain of *Yersinia pseudotuberculosis*. Microb Pathog 50:314–321. https://doi.org/10.1016/j.micpath.2011.02.005.
14. Rebeil R, Jarrett CO, Driver JD, Ernst RK, Oyston PC, Hinnebusch BJ. 2013. Induction of the *Yersinia pestis* PhoP-PhoQ regulatory system in the flea and its role in producing a transmissible infection. J Bacteriol 195:1920–1930. https://doi.org/10.1128/JB.02000-12.
15. Grabenstein JP, Fukuto HS, Palmer LE, Bliska JB. 2006. Characterization of phagosome trafficking and identification of PhoP-regulated genes important for survival of *Yersinia pestis* in macrophages. Infect Immun 74:3727–3741. https://doi.org/10.1128/IAI.00255-06.
16. Winfield MD, Latifi T, Groisman EA. 2005. Transcriptional regulation of the 4-amino-4-deoxy-L-arabinose biosynthetic genes in *Yersinia pestis*. J Biol Chem 280:14765–14772. https://doi.org/10.1074/jbc.M413900200.
17. Li Y, Gao H, Qin L, Li B, Han Y, Guo Z, Song Y, Zhai J, Du Z, Wang X, Zhou D, Yang R. 2008. Identification and characterization of PhoP regulon members in *Yersinia pestis* biovar Microtus. BMC Genomics 9:143. https://doi.org/10.1186/1471-2164-9-143.
18. Zhou D, Han Y, Qin L, Chen Z, Qiu J, Song Y, Li B, Wang J, Guo Z, Du Z, Wang X, Yang R. 2005. Transcriptome analysis of the $Mg^{2+}$-responsive PhoP regulator in *Yersinia pestis*. FEMS Microbiol Lett 250:85–95. https://doi.org/10.1016/j.femsle.2005.06.053.
19. Vadyvaloo V, Viall AK, Jarrett CO, Hinz AK, Sturdevant DE, Joseph Hinnebusch B. 2015. Role of the PhoP-PhoQ gene regulatory system in adaptation of *Yersinia pestis* to environmental stress in the flea digestive tract. Microbiology 161:1198–1210. https://doi.org/10.1099/mic.0.000082.
20. Knirel YA, Dentovskaya SV, Bystrova OV, Kocharova NA, Senchenkova SN, Shaikhutdinova RZ, Titareva GM, Bakhteeva IV, Lindner B, Pier GB, Anisimov AP. 2007. Relationship of the lipopolysaccharide structure of *Yersinia pestis* to resistance to antimicrobial factors. Adv Exp Med Biol 603:88–96. https://doi.org/10.1007/978-0-387-72124-8_7.
21. Achtman M, Morelli G, Zhu P, Wirth T, Diehl I, Kusecek B, Vogler AJ, Wagner DM, Allender CJ, Easterday WR, Chenal-Francisque V, Worsham P, Thomson NR, Parkhill J, Lindler LE, Carniel E, Keim P. 2004. Microevolution and history of the plague bacillus, *Yersinia pestis*. Proc Natl Acad Sci U S A 101:17837–17842. https://doi.org/10.1073/pnas.0408026101.
22. Morelli G, Song Y, Mazzoni CJ, Eppinger M, Roumagnac P, Wagner DM, Feldkamp M, Kusecek B, Vogler AJ, Li Y, Cui Y, Thomson NR, Jombart T, Leblois R, Lichtner P, Rahalison L, Petersen JM, Balloux F, Keim P, Wirth T, Ravel J, Yang R, Carniel E, Achtman M. 2010. *Yersinia pestis* genome sequencing identifies patterns of global phylogenetic diversity. Nat Genet 42:1140–1143. https://doi.org/10.1038/ng.705.
23. Rasmussen S, Allentoft ME, Nielsen K, Orlando L, Sikora M, Sjogren KG, Pedersen AG, Schubert M, Van Dam A, Kapel CM, Nielsen HB, Brunak S, Avetisyan P, Epimakhov A, Khalyapin MV, Gnuni A, Kriiska A, Lasak I, Metspalu M, Moiseyev V, Gromov A, Pokutta D, Saag L, Varul L, Yepiskoposyan L, Sicheritz-Ponten T, Foley RA, Lahr MM, Nielsen R, Kristiansen K, Willerslev E. 2015. Early divergent strains of *Yersinia pestis* in Eurasia

5,000 years ago. Cell 163:571–582. https://doi.org/10.1016/j.cell.2015.10.009.
24. Achtman M, Zurth K, Morelli G, Torrea G, Guiyoule A, Carniel E. 1999. *Yersinia pestis*, the cause of plague, is a recently emerged clone of *Yersinia pseudotuberculosis*. Proc Natl Acad Sci U S A 96:14043–14048. https://doi.org/10.1073/pnas.96.24.14043.
25. Wren BW. 2003. The yersiniae—a model genus to study the rapid evolution of bacterial pathogens. Nat Rev Microbiol 1:55–64. https://doi.org/10.1038/nrmicro730.
26. Chain PS, Carniel E, Larimer FW, Lamerdin J, Stoutland PO, Regala WM, Georgescu AM, Vergez LM, Land ML, Motin VL, Brubaker RR, Fowler J, Hinnebusch J, Marceau M, Medigue C, Simonet M, Chenal-Francisque V, Souza B, Dacheux D, Elliott JM, Derbise A, Hauser LJ, Garcia E. 2004. Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis*. Proc Natl Acad Sci U S A 101:13826–13831. https://doi.org/10.1073/pnas.0404012101.
27. Zimbler DL, Schroeder JA, Eddy JL, Lathem WW. 2015. Early emergence of *Yersinia pestis* as a severe respiratory pathogen. Nat Commun 6:7487. https://doi.org/10.1038/ncomms8487.
28. Sun YC, Jarrett CO, Bosio CF, Hinnebusch BJ. 2014. Retracing the evolutionary path that led to flea-borne transmission of *Yersinia pestis*. Cell Host Microbe 15:578–586. https://doi.org/10.1016/j.chom.2014.04.003.
29. Cui Y, Yu C, Yan Y, Li D, Li Y, Jombart T, Weinert LA, Wang Z, Guo Z, Xu L, Zhang Y, Zheng H, Qin N, Xiao X, Wu M, Wang X, Zhou D, Qi Z, Du Z, Wu H, Yang X, Cao H, Wang H, Wang J, Yao S, Rakin A, Li Y, Falush D, Balloux F, Achtman M, Song Y, Wang J, Yang R. 2013. Historical variations in mutation rate in an epidemic pathogen, *Yersinia pestis*. Proc Natl Acad Sci U S A 110:577–582. https://doi.org/10.1073/pnas.1205750110.
30. Song Y, Tong Z, Wang J, Wang L, Guo Z, Han Y, Zhang J, Pei D, Zhou D, Qin H, Pang X, Han Y, Zhai J, Li M, Cui B, Qi Z, Jin L, Dai R, Chen F, Li S, Ye C, Du Z, Lin W, Wang J, Yu J, Yang H, Wang J, Huang P, Yang R. 2004. Complete genome sequence of *Yersinia pestis* strain 91001, an isolate avirulent to humans. DNA Res 11:179–197. https://doi.org/10.1093/dnares/11.3.179.
31. Chen HD, Jewett MW, Groisman EA. 2012. An allele of an ancestral transcription factor dependent on a horizontally acquired gene product. PLoS Genet 8:e1003060. https://doi.org/10.1371/journal.pgen.1003060.
32. Rosenberger CM, Gallo RL, Finlay BB. 2004. Interplay between antibacterial effectors: a macrophage antimicrobial peptide impairs intracellular *Salmonella* replication. Proc Natl Acad Sci U S A 101:2422–2427. https://doi.org/10.1073/pnas.0304455101.
33. Perez JC, Groisman EA. 2009. Transcription factor function and promoter architecture govern the evolution of bacterial regulons. Proc Natl Acad Sci U S A 106:4319–4324. https://doi.org/10.1073/pnas.0810343106.
34. Soncini FC, Vescovi EG, Groisman EA. 1995. Transcriptional autoregulation of the *Salmonella typhimurium* phoPQ operon. J Bacteriol 177:4364–4371. https://doi.org/10.1128/jb.177.15.4364-4371.1995.
35. Zhang Y, Wang L, Han Y, Yan Y, Tan Y, Zhou L, Cui Y, Du Z, Wang X, Bi Y, Yang H, Song Y, Zhang P, Zhou D, Yang R. 2013. Autoregulation of PhoP/PhoQ and positive regulation of the cyclic AMP receptor protein-cyclic AMP complex by PhoP in *Yersinia pestis*. J Bacteriol 195:1022–1030. https://doi.org/10.1128/JB.01530-12.
36. Hancock RE, Lehrer R. 1998. Cationic peptides: a new source of antibiotics. Trends Biotechnol 16:82–88. https://doi.org/10.1016/S0167-7799(97)01156-6.
37. Anisimov AP, Dentovskaya SV, Titareva GM, Bakhteeva IV, Shaikhutdinova RZ, Balakhonov SV, Lindner B, Kocharova NA, Senchenkova SN, Holst O, Pier GB, Knirel YA. 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. https://doi.org/10.1128/IAI.73.11.7324-7331.2005.
38. Kislichkina AA, Bogun AG, Kadnikova LA, Maiskaya NV, Platonov ME, Anisimov NV, Galkina EV, Dentovskaya SV, Anisimov AP. 2015. Nineteen whole-genome assemblies of *Yersinia pestis* subsp. *microtus*, including representatives of biovars caucasica, talassica, hissarica, altaica, xilingolensis, and ulegeica. Genome Announc 3:e01342-15. https://doi.org/10.1128/genomeA.01342-15.
39. Earl SC, Rogers MT, Keen J, Bland DM, Houppert AS, Miller C, Temple I, Anderson DM, Marketon MM. 2015. Resistance to innate immunity contributes to colonization of the insect gut by *Yersinia pestis*. PLoS One 10:e0133318. https://doi.org/10.1371/journal.pone.0133318.
40. Aoyagi KL, Brooks BD, Bearden SW, Montenieri JA, Gage KL, Fisher MA. 2015. LPS modification promotes maintenance of *Yersinia pestis* in fleas. Microbiology 161:628–638. https://doi.org/10.1099/mic.0.000018.
41. Vadyvaloo V, Jarrett C, Sturdevant DE, Sebbane F, Hinnebusch BJ. 2010. Transit through the flea vector induces a pretransmission innate immunity resistance phenotype in *Yersinia pestis*. PLoS Pathog 6:e1000783. https://doi.org/10.1371/journal.ppat.1000783.
42. Straley SC, Bowmer WS. 1986. Virulence genes regulated at the transcriptional level by $Ca^{2+}$ in *Yersinia pestis* include structural genes for outer membrane proteins. Infect Immun 51:445–454.
43. Palmer LE, Hobbie S, Galan JE, Bliska JB. 1998. YopJ of *Yersinia pseudotuberculosis* is required for the inhibition of macrophage TNF-alpha production and downregulation of the MAP kinases p38 and JNK. Mol Microbiol 27:953–965. https://doi.org/10.1046/j.1365-2958.1998.00740.x.
44. Klein KA, Fukuto HS, Pelletier M, Romanov G, Grabenstein JP, Palmer LE, Ernst R, Bliska JB. 2012. A transposon site hybridization screen identifies galU and wecBC as important for survival of *Yersinia pestis* in murine macrophages. J Bacteriol 194:653–662. https://doi.org/10.1128/JB.06237-11.
45. Rozen S, Skaletsky H. 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132:365–386.
46. Hinnebusch BJ, Perry RD, Schwan TG. 1996. Role of the *Yersinia pestis* hemin storage (hms) locus in the transmission of plague by fleas. Science 273:367–370. https://doi.org/10.1126/science.273.5273.367.
47. Pujol C, Bliska JB. 2003. The ability to replicate in macrophages is conserved between *Yersinia pestis* and *Yersinia pseudotuberculosis*. Infect Immun 71:5892–5899. https://doi.org/10.1128/IAI.71.10.5892-5899.2003.
48. Une T, Brubaker RR. 1984. *In vivo* comparison of avirulent $Vwa^-$ and $Pgm^-$ or Pstr phenotypes of yersiniae. Infect Immun 43:895–900.
49. Pujol C, Grabenstein JP, Perry RD, Bliska JB. 2005. Replication of *Yersinia pestis* in interferon gamma-activated macrophages requires ripA, a gene encoded in the pigmentation locus. Proc Natl Acad Sci U S A 102:12909–12914. https://doi.org/10.1073/pnas.0502849102.
50. Jarvik T, Smillie C, Groisman EA, Ochman H. 2010. Short-term signatures of evolutionary change in the *Salmonella enterica* serovar Typhimurium 14028 genome. J Bacteriol 192:560–567. https://doi.org/10.1128/JB.01233-09.
51. Fukuto HS, Svetlanov A, Palmer LE, Karzai AW, Bliska JB. 2010. Global gene expression profiling of *Yersinia pestis* replicating inside macrophages reveals the roles of a putative stress-induced operon in regulating type III secretion and intracellular cell division. Infect Immun 78:3700–3715. https://doi.org/10.1128/IAI.00062-10.