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Induction of the *Yersinia pestis* PhoP-PhoQ Regulatory System in the Flea and Its Role in Producing a Transmissible Infection

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Transmission of *Yersinia pestis* is greatly enhanced after it forms a bacterial biofilm in the foregut of the flea vector that interferes with normal blood feeding. Here we report that the ability to produce a normal foregut-blocking infection depends on induction of the *Y. pestis* PhoP-PhoQ two-component regulatory system in the flea. *Y. pestis phoP*-negative mutants achieved normal infection rates and bacterial loads in the flea midgut but produced a less cohesive biofilm both *in vitro* and in the flea and had a greatly reduced ability to localize to and block the flea foregut. Thus, not only is the PhoP-PhoQ system induced in the flea gut environment, but also this induction is required to produce a normal transmissible infection. The altered biofilm phenotype in the flea was not due to lack of PhoPQ-dependent or PmrAB-dependent addition of aminoarabinose to the *Y. pestis* lipid A, because an aminoarabinose-deficient mutant that is highly sensitive to cationic antimicrobial peptides had a normal phenotype in the flea digestive tract. In addition to enhancing transmissibility, induction of the PhoP-PhoQ system in the arthropod vector prior to transmission may preadapt *Y. pestis* to resist the initial encounter with the mammalian innate immune response.

Yersinia pestis, the causative agent of bubonic and pneumonic plague, is unique among the enteric group of Gram-negative bacteria in having adopted an arthropod-borne route of transmission. During its life cycle, *Y. pestis* alternates between two eukaryotic hosts: a mammal (usually a rodent) and an insect (a flea). *Y. pestis* faces quite different physiological challenges in these disparate host environments. Prokaryotes have evolved sophisticated systems to detect changes in their environment and to respond appropriately by selective synthesis of adaptive gene products. One archetypal environmental sensing and response mechanism in bacteria is the two-component regulatory system, in which an inner membrane sensor kinase protein detects the presence or absence of an environmental stimulus and transduces a signal by phosphotransfer to a cytoplasmic transcriptional regulator (1). The activated transcription factor then coordinately regulates the expression of genes under its control to adapt to the new environmental condition.

One such two-component signal transduction system, PhoP-PhoQ, has a proven role in adaptation of Gram-negative bacteria to vertebrate, invertebrate, and plant host environments (2–8). PhoP and PhoQ homologs are widely distributed among both pathogenic and nonpathogenic Gram-negative bacteria, and the system is considered to constitute a general stress response (4). The prototypical function of the PhoP-PhoQ system appears to be adaptation to low-Mg^{2+} environments, which stimulate the system to upregulate genes involved in Mg^{2+} transport and homeostasis and in the modification of outer membrane components such as lipopolysaccharide (LPS) (4, 9–12). In addition to low Mg^{2+}, other environmental stresses such as low pH or cationic antimicrobial peptide (CAMP) binding to the bacterial surface can induce the PhoP-PhoQ system (13, 14). Loss of functional *phoP* in *Salmonella* and other pathogens results in attenuated virulence, because PhoP-activated genes include virulence factors that confer resistance to components of the innate immune response such as antimicrobial peptides and macrophages (2, 15–18). PhoP is considered to be a central element in a complex regulatory hierarchy because it regulates other transcription factors and two-component systems; the expression of approximately 3% of *Salmonella* genes is directly or indirectly affected by PhoP (19, 20).

The *Y. pestis* PhoP and PhoQ proteins have 90% and 77% amino acid similarity, respectively, to their *S. enterica* homologs (21). A *Y. pestis phoP* mutant was more sensitive to low pH, high osmolarity, and oxidative stress than the wild-type parent strain and was also more susceptible to killing by J774 macrophages *in vitro* (8, 22, 23). Microarray analyses indicate that the expression of as many as 400 *Y. pestis* genes is influenced by PhoP under low-Mg^{2+} conditions *in vitro* (12, 23–25). These genes include *Y. pestis* homologs of LPS-modifying and other PhoP-regulated genes of *S. enterica* that are known to be important for survival within macrophages. In general, however, only limited overlap of PhoP-regulated genes has been observed among the *Enterobacteriaceae* (11, 12, 26, 27). Closely related species have evolved distinct regulatory pathways in some cases. An example pertinent to this study is the covalent attachment of 4-amino-4-deoxy-L-arabinose (4-aminoarabinose) to the phosphate residues of the lipid A component of LPS, which is required for bacterial resistance to CAMPs that are produced by both insects and mammals (16, 28, 29). Aminoarabinose addition in *Salmonella* requires upregula-

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TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Description[a]	Reference or source
Y. pestis strains		
KIM6+	pCD1-negative, Pgm$^+$ biovar Medievalis strain	83
KIM6	Pgm- and pCD1-negative	84
KIM6+ Δ*phoP*	In-frame internal deletion of amino acids 25–75 of PhoP	29
KIM6+ Δ*phoP*(pLG338)	Empty vector control (Kan, Tet)	This study
KIM6+ Δ*phoP*(pLG*phoP*)	Complemented *phoP* mutant (Kan)	This study
KIM6+ Δ*phoP*(pGFP)	GFP expressing (Amp)	This study
KIM6+ Δ*pmrA*	In-frame internal deletion of amino acids 28–113 of PmrA	This study
KIM6+ Δ*phoP* Δ*pmrA*	*pmrA* deletion introduced into the *phoP* mutant	This study
KIM6+ Δ*pbgP* Δ*ugd*	Deletion of aminoarabinose synthesis genes (Kan, Amp)	This study
KIM6+ Δ*ugd*	Deletion of aminoarabinose synthesis gene (Kan)	This study
KIM6+ Δ*ugd*(pLG*ugd*)	Complemented *ugd* mutant (Kan, Amp)	This study
GB	Wild-type biovar Orientalis strain	22
GB SAI2.2	*Y. pestis* GB *phoP* mutant	22
E. coli strains		
S17-1λpir	Host and conjugation donor strain for pCVD442 and derivatives	85
TOP-10	pCR 2.1 TOPO plasmid host strain	Invitrogen
D31	Rough LPS mutant (Str)	86
Plasmids		
pLG338	Low-copy-no. plasmid vector (Kan, Tet)	87
pLG*phoP*	Complementation plasmid; wild-type *phoP* gene and promoter cloned into pLG338 (Kan)	This study
pLG338-30	Low-copy-no. plasmid vector (Amp)	88
pLG*ugd*	Complementation plasmid; wild-type *ugd* gene and promoter cloned into pLG338-30 (Amp)	This study
pCR4Blunt-TOPO, pCR-2.1 TOPO	Cloning vectors (Amp)	Invitrogen
pGFP	GFP expression plasmid (Amp)	Clontech
pCVD442	Suicide vector for allelic exchange mutagenesis (Amp)	38
pKOBEG::*sacB*	λRed and *sacB* plasmid for mutagenesis (Cam)	40

[a] Antibiotic resistance is noted in parentheses (Kan, kanamycin; Tet, tetracycline; Amp, ampicillin; Str, streptomycin; Cam, chloramphenicol). GFP, green fluorescent protein.

tion of the PmrA-PmrB two-component system via PhoP-PhoQ, whereas in *Y. pestis* it can be independently regulated by either PhoP-PhoQ or PmrA-PmrB alone (30). In addition, the type and magnitude of the inducing stress can result in differential regulation of subsets of PhoP-regulated genes (12, 31).

After being taken up in a blood meal, *Y. pestis* multiplies extracellularly in the lumen of the flea midgut and grows in the form of a biofilm, a dense bacterial aggregate that is enclosed in an extracellular matrix (32). In some fleas, the biofilm adheres to the cuticle-covered spines that line the interior of the proventriculus, a valve in the foregut that connects the midgut to the esophagus. The bacterial growth can interfere with the normal valvular action of the proventriculus during feeding attempts and eventually block the flow of blood into the midgut (33). Partial or complete blockage of the proventriculus by the *Y. pestis* biofilm greatly enhances transmissibility to a new host (33–35).

In a previous study, we reported that the *Y. pestis* PhoP-PhoQ system was upregulated during infection of the flea at the stage when blockage-dependent transmission occurs (36). In this study, we investigated the role of the PhoP-PhoQ system in the ability of *Y. pestis* to produce a transmissible infection in the flea. Because certain PhoP-regulated genes are cooperatively or independently regulated by PmrB in *Y. pestis* (30), we also examined the role of the PmrA-PmrB two-component system in fleas.

MATERIALS AND METHODS

Bacterial strains and mutagenesis. Bacterial strains used in this study are listed in Table 1. The wild-type *Y. pestis* GB strain and the isogenic mutant *Y. pestis* GB SAI2.2, which has a 31-bp internal deletion in the *phoP* gene, have been described (22). A 153-bp in-frame deletion of the *phoP* gene was made in *Y. pestis* KIM6+ (which lacks the 70-kb *Yersinia* virulence plasmid) by using a megaprimer mutagenesis strategy and allelic exchange (29, 37, 38). The 353-bp megaprimer was generated by PCR of *Y. pestis* genomic DNA using an upstream primer and a 43-nt mutagenic primer that consisted of the 24 nt upstream and 19 nt downstream of the desired 153-bp deletion and thus incorporated the exact deletion junction. The megaprimer was used in a second PCR with a downstream primer to generate the mutated *phoP* allele. This second PCR product was ligated into the pCR4Blunt-TOPO vector (Invitrogen, Carlsbad, CA). Making use of the SacI and XbaI sites in the primers, the mutated *phoP* allele was removed from the pCR4 vector and ligated into SacI- and XbaI-digested pCVD442. The ligation mixture was used to transform *Escherichia coli* S17-1 by electroporation. The pCVD442 suicide vector containing the mutated *phoP* gene was introduced into *Y. pestis* KIM6+ by conjugation with the transformed *E. coli* S17-1 clone. An allelic exchange mutant, designated *Y. pestis* KIM6+ Δ*phoP*, in which the mutant *phoP* containing the 153-bp deletion had replaced the wild-type *phoP* gene, was selected using Congo red agar plates containing 7% sucrose (38). *Y. pestis* KIM6+ Δ*phoP* and KIM6+ transformants containing pGFP (Clontech, Palo Alto, CA) were obtained by electroporation.

To complement *Y. pestis* KIM6+ Δ*phoP*, a recombinant plasmid containing the full-length wild-type *Y. pestis phoP* gene was constructed. The entire 673-bp *phoP* gene flanked by 564 bp of upstream sequence and 769 bp of downstream sequence was PCR amplified from *Y. pestis* genomic DNA using primers that contained EcoRI or BamHI restriction sites. The PCR product was digested with EcoRI and BamHI and ligated with pLG338 that had been linearized with the same two enzymes. The ligation mix was used to transform *E. coli* TOP10 cells (Invitrogen), and a clone

TABLE 2 Primers and probes used in this study

Target gene	Use	Sequence[a] (5′ to 3′)
phoP (y1794)	Deletion	CGAGCTCTGACCAAGCGATGCAAGTCG (U primer)
		GCTTTCCCGTGCGGTCAGGACCAAGCCCATTTCACGCATTTGC (M primer)
		GCTCTAGATTCACCCTGATGTTCTGCCAGCAG (D primer)
	Complementation	GCGCGAATTCAGATGCCGTTCTTGGATTAGG
		GCGCGGATCCAGCAAGATGTTGAGGTTACG
pmrA (y0677)	Cloning	GCGCTCTAGATGGTCATCAGCGTTTTGGG
		GCGCGAGCTCAAGGGGGAAAGGTTATCTGCGGAG
	Deletion	GCGCATCGATGCAGACATAGCCTTCACTGGTCAGC
		GCGCATCGATGCTCTCATCCGCCGTTATCAGG
pbgP (y1917)	Deletion	CCTGGTGATGAGGTTATTACGCCATCACAGACGTGGGTTTCTACGAT-GATCTTTTCTACGGGGTCTGACG
		TGTAGCCCACTGCCAATACCCATATCTTTCAAACACGCCATCAGTTGAT-CTTTTCGGGGAAATGTGCG
ugd (y2147)	Deletion	CACTGGGAGTAAGTCTGGTATTGAATTTCAACTCGGAGATCGAGCGAATG-GAGGTCTGCCTCGTGAAGAAGG
		TTAACTGACCGATGTCATCACCGTTGAAATGTCCACTGTACAGCCGCTG-GGGAAAGCCACGTTGTGTCTC
	Complementation	CTGATGCTTGCTGCTGAAGAATAG and CGAACTGAAAACTTGGACAGGC
phoQ (y1793)	TaqMan primers	CCTGCACCGCGCAAGT and CGCGGGAACCGAATGA
	TaqMan probe[b]	TGCGTTCCGAACATAATGTTCTAGGACGTG
caf1	TaqMan primers	CACCACTGCAACGGCAACT and TTGGAGCGCCTTCCTTATATGT
	TaqMan probe[b]	TTGTTGAACCAGCCCGCATCACTCT
proS	TaqMan primers	ACGCGCACCGGCTACA and CTCGGCGATGGTTTTTGC
	TaqMan probe[b]	AGAGCTGCGAATCGTTGACACCCC

[a] Underlined 6-nt sequences are restriction enzyme sites added to facilitate cloning; underlined 19- to 22-nt sequences are the portions of the primers specific for the Bla or Kan resistance cassette. U, upstream; M, mutagenic; D, downstream.
[b] TaqMan probes contain the reporter 5′-6-carboxyfluorescein (5′-FAM) and the quencher 3′-6-carboxy-tetramethyl-rhodamine (3′-TAMRA).

containing the recombinant plasmid (designated pLG*phoP*) was isolated. *Y. pestis* KIM6+ Δ*phoP* was transformed with the pLG338 empty vector, pLG*phoP*, and pGFP (Clontech, Palo Alto, CA) by electroporation. The presence of both full-length and deletion-containing *phoP* alleles in the pLG*phoP* transformants was confirmed by PCR.

A *Y. pestis* KIM6+ strain with a 258-bp in-frame deletion of the *pmrA* gene (y0677) was produced by allelic exchange mutagenesis (38). The complete *pmrA* gene and upstream and downstream flanking sequences was first PCR amplified and cloned into the pCR2.1 TOPO vector. Inverse PCR of this recombinant plasmid was performed to delete a 258-bp internal fragment (corresponding to amino acids 28 to 113 of PmrA) and the PCR product was circularized by ligation (39). The mutated *pmrA* allele was subcloned into pCVD442, which was introduced by electroporation into *E. coli* S17-1. The suicide vector construct was transferred to *Y. pestis* KIM6+ and KIM6+ Δ*phoP* strains by conjugation, and *Y. pestis* transconjugants in which allelic exchange had occurred were selected.

Y. pestis KIM6+ mutants in which bp 261 to 919 of the *pbgP* gene (y1917) were deleted and replaced with an ampicillin resistance gene and/or 1,675 bp of the *ugd* locus extending from bp 31 of the *ugd* gene (y2147) to 314 bp downstream of it were deleted and replaced with a kanamycin resistance gene were generated by using the pKOBEG-*sacB* lambda red-recombinase mutagenesis system (40). PCR fragments composed of the *bla* gene from pUC19 or the *aph* gene from pUC4K flanked by 47 to 50 bp of *Y. pestis* sequence upstream and downstream of the targeted deletion sites of *pbgP* and *ugd*, respectively, were made and purified. The DNA fragments were sequentially introduced into *Y. pestis* KIM6+ (pKOBEG-*sacB*) by electroporation, and antibiotic-resistant transformants were isolated. pKOBEG-*sacB*-cured derivatives of the Δ*pbgP* Δ*ugd* double mutant and the Δ*ugd* single mutant were obtained by selection on LB agar plates containing 7% sucrose. The Δ*ugd* strain was complemented by transformation with pLG*ugd*, containing the PCR-amplified *ugd* gene plus 303 bp and 531 bp of upstream and downstream sequence, respectively. The sequences of the primers described above are in Table 2. PCR and DNA sequencing were performed to verify that the expected mutations were present.

Biofilm analyses. *Y. pestis* was incubated for 48 h at 21°C in N-minimal medium (9) containing 0.1% Casamino Acids, 38 mM glycerol, and 1 mM $MgCl_2$, quantitated in a Petroff-Hausser counting chamber, and diluted to 1×10^7 cells/ml with fresh medium. A 0.4-ml portion of the bacterial suspension was injected into a 4-mm by 40-mm by 1-mm-deep flow cell (Stovall, Greensboro, NC) that was connected to a reservoir of sterile medium via a peristaltic pump at the influent end and to a discard reservoir at the effluent end. After a 20-min period to allow bacteria to attach to the glass surface (designated time zero), sterile medium was pumped through the flow cell at 1 ml/min. After 48 h, the medium flow was stopped, and 0.4 ml of 5 μM Syto 9 stain (Molecular Probes, Eugene, OR) was injected into the flow cell. After a 20-min staining period, the medium flow was resumed for 5 min to remove unbound dye. Biofilm attached to a representative 921-μm^2 area of the borosilicate glass surface of the flow cell was visualized by scanning confocal laser microscopy using a Zeiss LSM 510 system.

Congo red binding to the biofilm extracellular matrix of *Y. pestis* cells grown for 48 h on heart infusion agar (Difco) containing 0.2% (wt/vol) galactose at 21, 25, or 37°C was quantified as described previously (41).

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). RNA was isolated from *Y. pestis* grown in N-minimal medium supplemented with 0.1% Casamino Acids, 38 mM glycerol, and 8 μM or 1 mM $MgCl_2$ (9). Fresh medium was inoculated to an optical density at 600 nm (OD_{600}) of 0.025 with an overnight culture and incubated at 21°C and 200 rpm until logarithmic phase (OD_{600} = 0.1) or early stationary phase (OD_{600} = 0.6). At each time point, 8 ml of the 50-ml cultures was centrifuged, and the resulting cell pellet was resuspended in 1.0 ml ice-cold RLT buffer (RNeasy minikit; Qiagen, Valencia, CA) and then transferred to prechilled 2-ml lysing matrix B tubes (Qbiogene, Carlsbad, CA). Bacteria were lysed by using a FastPrep FP120 instrument (Qbiogene) for

30 s at setting 6.0. Lysates were mixed with 0.4 ml ethanol, and total RNA was isolated using RNeasy minicolumns (Qiagen) and purified using a DNase-free kit (Ambion, Austin, TX). The purified RNA was evaluated electrophoretically with a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA) and spectrophotometrically at A_{260} and A_{280}.

To obtain *in vivo Y. pestis* RNA samples at 3 h and 14 days after infection, samples of 50 infected fleas were placed in 2-ml lysing matrix B tubes, flash-frozen in liquid nitrogen, and stored at −80°C. RLT buffer mix was added to the tubes and the FastPrep instrument used to triturate the fleas and lyse the bacteria. The lysates were then passed through QIAshredder columns (Qiagen) to further fragment the DNA and to remove particulate matter derived from the flea exoskeleton. Total RNA was then purified and quantified as described above.

Triplicate samples were obtained for each condition tested, and RNA samples were diluted to 1 μg/ml before analysis. TaqMan RT-qPCR quantification of *Y. pestis phoQ* and *caf* expression relative to that of the reference gene *proS* was performed using primers and probes listed in Table 2 and the TaqMan one-step RT-PCR master mix kit and an ABI 7700 thermocycler from Applied Biosystems (Foster City, CA) as described previously (42). A standard curve was prepared for each primer-probe set using threshold cycle (C_T) values obtained from amplification of a dilution series of a total *Y. pestis* RNA standard sample. The standard curve was used to transform experimental C_T values to relative numbers of cDNA molecules in the samples. The quantity of *phoQ*- and *caf1*-derived cDNA was normalized to the quantity of reference gene *proS* cDNA to determine relative expression of the genes.

Flea infections. *Xenopsylla cheopis* fleas were infected by allowing them to feed on fresh heparinized mouse blood containing ~5×10^8 *Y. pestis* organisms/ml, using previously reported bacterial preparation and artificial feeding protocols (43, 44). Fleas (~50 males and 50 females) that took an infectious blood meal were kept at 21°C and 75% relative humidity, fed twice weekly thereafter on uninfected mice, and monitored for 4 weeks for proventricular blockage (43). Blockage was diagnosed by direct microscopic examinations of each flea immediately following their twice-weekly maintenance feeds. Fleas were considered blocked when they contained fresh red blood only in the esophagus and none in the midgut, indicative of physical blockage of the proventriculus by a *Y. pestis* biofilm (32, 33, 43). The infection rate and average bacterial load at 1 h and 28 days after the infectious blood meal were determined by CFU counts from additional samples of 20 female fleas that were individually triturated and plated on brain heart infusion (BHI) agar containing 1 μg/ml triclosan (43).

Flea physiology and immunity. Calcium, magnesium, and iron concentrations in *X. cheopis* midgut contents were determined by atomic absorption spectrophotometry. The digestive tract was dissected intact from 5 uninfected female fleas 1 or 6 days after their last blood meal, the midgut epithelium was pierced, and the midgut contents were carefully expressed. A 1:200 dilution of the pooled midgut contents in phosphate-buffered saline (PBS) was filter sterilized, and ion concentrations were measured using a model 405 graphite furnace atomic absorption spectrophotometer (GFAAS; PerkinElmer, Downers Grove, IL) with an HGA-2000 controller. Calibration curves were prepared from triplicate measurements of a dilution series from 10 ppm to 1 ppb of Ca^{2+}, Mg^{2+}, and Fe^{3+} standard solutions (Aldrich, St. Louis, MO), using sterile water as a blank. Ion concentrations in the flea samples were determined by triplicate measurements of 1:2 and 1:4 dilutions of the original samples and extrapolation from the standard curve, using PBS as a blank. GFAAS settings were as follows: drying for 30 s at 100°C, charring for 35 s at 1,000°C, and atomizing for 9 s at 2,700°C, followed by measurements at wavelengths of 422.7 (Ca), 285.2 (Mg), and 248.3 (Fe) nm.

To demonstrate the flea immune response, two groups of 60 female fleas each were challenged by piercing the integument between posterior dorsal scleral plates with a fine-tipped glass capillary that had been either sterilized or dipped into a paste composed of a mixture of *E. coli* D31 and *Micrococcus luteus* colonies. Fleas were kept at 21°C for 24 h, and then each group, plus a third group of 60 unchallenged female fleas, was placed in separate Eppendorf tubes and triturated in 100 μl of 10% acetic acid containing 10 μg/ml aprotinin. Triturates were centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatant was removed, and the flea debris was reextracted and recentrifuged. The pooled supernatants from each extraction were centrifuged at 18,500 × *g* for 20 min at 4°C to remove fine debris. Supernatants were then heat treated at 95°C for 5 min and recentrifuged at 18,500 × *g* for 5 min. These supernatants were lyophilized and resuspended in 20 μl of 0.2 M sodium acetate, pH 5.2. The antibacterial activity of flea extracts was determined in a zone-of-inhibition bioassay (45). A lawn of the indicator strain *E. coli* D31 was prepared by adding 0.01 ml of overnight culture to 7 ml of molten LB–0.7% SeaKem LE agarose containing 0.5% (wt/vol) lysozyme and 100 μg/ml streptomycin. The mixture was poured into a sterile petri dish and allowed to harden, and then 5 μl of each flea extract was added to a 2-mm-diameter well drilled in the L-agarose layer. The zone of inhibition around each well was measured after overnight incubation at 37°C.

Antimicrobial peptide susceptibility assay. Susceptibility to the cationic antimicrobial peptides polymyxin B and cecropin A (both from Sigma-Aldrich; St. Louis, MO) was determined by using a microdilution MIC assay in LB (29). Stationary-phase LB cultures used to prepare the inocula, and the MIC plates were incubated at 21°C. Two independent experiments gave identical MICs.

RESULTS

***Y. pestis phoP* is required for normal proventricular blockage of fleas but is not required for infectivity.** We compared a *Y. pestis* KIM6+ clone containing a 153-bp internal in-frame deletion in *phoP* and its isogenic parent strain for their ability to produce a transmissible infection in the rat flea *Xenopsylla cheopis*. The deletion eliminated sequence encoding amino acids 25 through 75 of the wild-type PhoP, including the conserved aspartate residue that is predicted to be phosphorylated by PhoQ (4, 22, 46). The fleas were monitored for 4 weeks for the development of proventricular blockage, indicative of a transmissible infection. In three independent experiments, 28 to 48% of fleas infected with the KIM6+ parent strain developed proventricular blockage (Fig. 1A), consistent with previous reports (43, 47). In contrast, the *Y. pestis* KIM6+ Δ*phoP* strain blocked only 6 to 11% of fleas. Blockage in fleas infected with the mutant also appeared later (mean = 23 days after infection) than in fleas infected with wild-type bacteria (mean = 17 days). When the *Y. pestis* mutant was complemented with a plasmid containing a wild-type copy of *phoP*, normal rates of proventricular blockage were restored. In contrast, when the *phoP* mutant was transformed with the empty pLG338 complementation vector, blockage remained low (10%). Experiments using the *Y. pestis* GB strain and a previously described GB *phoP* mutant (22) gave similar results (Fig. 1A).

To compare the infectivity of the different *Y. pestis* strains for *X. cheopis*, samples of 20 female fleas were collected at 0 and 28 days after the infectious blood meal and used to determine *Y. pestis* CFU per flea. Neither the infection rate nor the average bacterial load differed significantly for fleas infected with the *Y. pestis* KIM6+, KIM6+ Δ*phoP*, or the complemented KIM6+ Δ*phoP* strains (Fig. 1B and C). Thus, the limited ability of the *Y. pestis* KIM6+ Δ*phoP* strain to block fleas was not due to decreased ability to establish a stable infection of the flea digestive tract. However, microscopic examination of the midguts dissected from infected fleas revealed a difference in infection phenotype (Fig. 2). Both *phoP*$^+$ and *phoP* strains grew as a biofilm in the flea gut, i.e., they formed large bacterial aggregates that were surrounded by a brown extracellular matrix, as described previously (32, 33, 44).

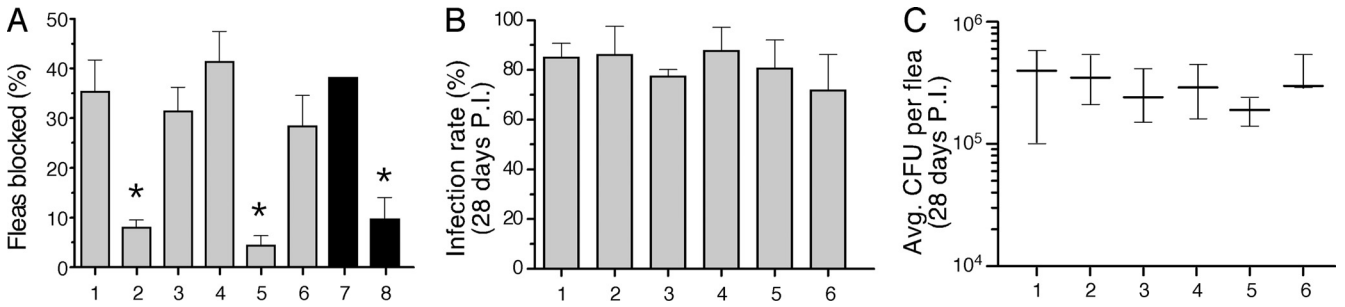

FIG 1 *Y. pestis phoP* mutants are defective for flea blockage but are able to stably infect the flea digestive tract. (A) Percentages of fleas that developed proventricular blockage during the 4-week period after feeding on blood containing the *Y. pestis* strain indicated. (B) Percentages of fleas still infected 4 weeks after the infectious blood meal. (C) Average bacterial load per infected flea 4 weeks after the infectious blood meal. In panels A and B, the means and standard errors of the means (SEM) from two (GB Δ*phoP*) or three (KIM6+ strains) independent experiments are shown; the wild-type GB infection was performed only once. In panel C, the means and ranges from three independent experiments are given. *, $P < 0.001$ compared to wild-type parent strain by Fisher's exact test. Bars: 1, KIM6+; 2, KIM6+ Δ*phoP*; 3, KIM6+ Δ*phoP* (pLG*phoP*); 4, KIM6+ Δ*pmrA*; 5, KIM6+ Δ*phoP* Δ*pmrA*; 6, KIM6+ Δ*pbgP* Δ*ugd*; 7, wild-type GB; 8, GB Δ*phoP*.

However, the *Y. pestis* Δ*phoP* aggregates were less cohesive and more easily fragmented than the aggregates formed by the *phoP*$^+$ parent strain, and they appeared to adhere to and colonize the proventricular spines to a much lesser extent.

PhoPQ- or PmrAB-regulated aminoarabinose modification of *Y. pestis* lipid A is not required to infect or block fleas. Modification of the lipid A moiety of LPS is regulated by PhoP and has important effects on bacterium-host interactions. For example, loss of *phoP* does not change the acylation pattern of lipid A from *Y. pestis* grown *in vitro* (29, 48) but greatly reduces aminoarabinose addition (29). Aminoarabinose modification makes the LPS less negatively charged, decreasing the binding affinity to CAMPs and thereby conferring resistance to them. Since changes in the electrostatic properties of the bacterial surface also affect adherence and biofilm formation (49, 50), we hypothesized that the blockage-deficient phenotype of the *Y. pestis phoP* mutant in fleas was attributable to a lack of aminoarabinose modification. In *Y. pestis*, aminoarabinose modification can be regulated independently by both the PhoP-PhoQ and PmrA-PmrB two-component systems (30). Therefore, we constructed additional *Y. pestis* mutant strains, one with *pmrA* deleted and one with *pbgP* and *ugd*, which are required for the biosynthesis of aminoarabinose (51), deleted. As shown in Fig. 1, the *Y. pestis* Δ*pmrA* and Δ*pbgP* Δ*ugd* strains were no different than wild-type KIM6+ in their ability to infect and block fleas. *Y. pestis* Δ*phoP* Δ*pmrA*, like the Δ*phoP* single mutant, had a greatly reduced ability to block fleas but not

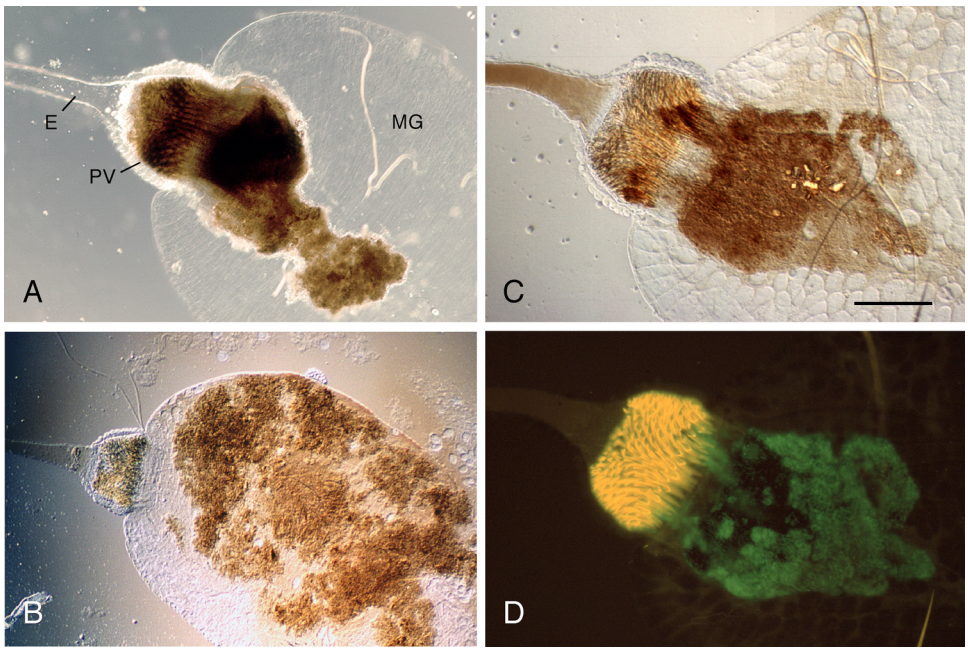

FIG 2 Fragile biofilm produced by PhoP$^-$ *Y. pestis* in the flea gut. Digestive tracts of *X. cheopis* fleas infected with *Y. pestis* KIM6+ (A), KIM6+ Δ*phoP* (B), or KIM6+ Δ*phoP* (pGFP) (C and D) were dissected and examined by light (A to C) and fluorescent (D) microscopy. The proventriculus (PV) of the flea (A) is filled and blocked with a dense cohesive biofilm that extends into the midgut (MG). The biofilm produced by the *phoP* mutant is less cohesive and is usually confined to the MG (B) or attached only peripherally to the posterior ends of the autofluorescent spines of the PV (C and D). The examples shown are representative of several flea dissections. Bar = 0.1 mm. E, esophagus.

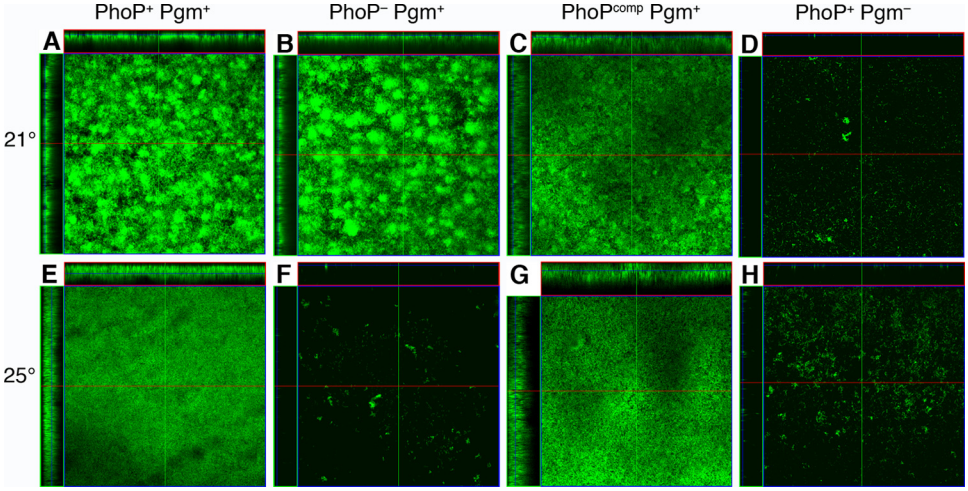

FIG 3 Effect of PhoP on Pgm-dependent biofilm formation in *Y. pestis*. *In vitro* biofilms produced by *Y. pestis* KIM6+ (PhoP$^+$ Pgm$^+$) (A and E), KIM6+ Δ*phoP* (pLG338) (PhoP$^-$ Pgm$^+$) (B and F), KIM6+ Δ*phoP* (pLG*phoP*) (PhoP^{comp} Pgm$^+$) (C and G), and KIM6 (PhoP$^+$ Pgm$^-$) (D and H) at 21°C (A to D) and 25°C (E to H). The results are representative of at least three independent experiments.

to infect them. Thus, loss of PhoP-mediated aminoarabinose addition to *Y. pestis* lipid A is not responsible for the altered biofilm phenotype of the *Y. pestis phoP* mutant in the flea. Lastly, because fleas that become blocked are unable to feed, they starve to death within a few days, and excess mortality is thus a surrogate indicator of blockage (43). Fleas infected with the PhoP$^-$ strains experienced significantly lower mortality during the 4-week experiments than PhoP$^+$ strains (data not shown); the mortality rates mirrored the blockage rates shown in Fig. 1A.

Effect of *phoP* mutation on *in vitro* biofilm formation on a glass surface. Colonization and blockage of the flea proventriculus by *Y. pestis* is essentially a biofilm phenomenon, and the ability of *Y. pestis* to block *X. cheopis* fleas correlates with the ability to produce a biofilm on the surface of a glass flow cell at low temperatures (32). Therefore, we examined the phenotype of *in vitro* biofilms produced by the *Y. pestis phoP* mutant. Both *Y. pestis* KIM6+ and KIM6+ Δ*phoP* produced confluent biofilm in flow cells at 21°C (Fig. 3A and B). At 25°C, the PhoP$^+$ parent strain still produced a dense, confluent biofilm, but the *phoP* mutant strain did not (Fig. 3E, F). The flow-cell biofilm phenotypes of the wild-type and complemented *phoP* mutant strains were identical at 25°C (Fig. 3C and G).

The *Y. pestis hms* gene products, including the *hmsHFRS* operon within the chromosomal Pgm locus, regulate and catalyze the synthesis of a polysaccharide extracellular matrix (ECM) that is essential for biofilm formation both *in vitro* (e.g., Fig. 3A versus D) and in the flea and for an *in vitro* pigmentation (Pgm) phenotype based on binding of hemin or Congo red (32, 41, 52–55). As there is some evidence that one of the *hms* genes, *hmsT*, is PhoP regulated in *Yersinia pseudotuberculosis* (56), we quantitated Congo red binding to *Y. pestis* at 21, 25, and 37°C to determine if the Pgm phenotype is influenced by PhoP (Fig. 4). Deletion of *phoP* did not affect temperature-dependent binding of the dye, indicating that production of the ECM is independent of the PhoP-PhoQ system. In addition, the pigmentation phenotype of *Y. pestis* KIM6+ Δ*phoP*, Δ*pmrA*, Δ*phoP* Δ*pmrA*, and Δ*pbgP* Δ*ugd* colonies on Congo red agar was identical to that of the *Y. pestis* KIM6+ parent strain (data not shown).

Induction of the PhoP-PhoQ system in the flea. The preceding results indicated that the *Y. pestis* PhoP-PhoQ signal transduction system regulates genes that affect the ability to produce an adherent proventriculus-blocking biofilm in the flea. This finding is consistent with a microarray study in which transcription of the *Y. pestis phoQ* gene was reported to be upregulated ~2-fold in the flea (36). Since the *phoPQ* operon is autogenously upregulated by PhoP, we quantified relative amounts of *phoQ* mRNA to assess activation of the *Y. pestis* system both *in vitro* and in the flea at different times after infection (Fig. 5). As a control, relative expression of the *Y. pestis caf1* gene, which encodes the extracellular F1 capsular protein whose expression is known to be downregulated at 21°C and in the flea (57, 58), was also quantitated. As for other Gram-negative bacteria, growth in low Mg^{2+} activated the PhoP-PhoQ system of *Y. pestis* (Fig. 5A). However, PhoP-PhoQ induction was even higher in the flea gut 2 weeks after infection, a time corresponding to the peak incidence of proventricular blockage (Fig. 5B). The greater *in vivo* activation of the *Y. pestis* PhoP-PhoQ system occurred in spite of the fact that the concentration of

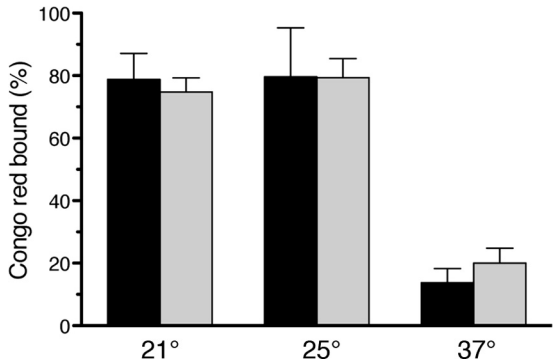

FIG 4 Loss of PhoP does not affect the Hms-dependent *in vitro* pigmentation phenotype of *Y. pestis*. The percentage of Congo red dye bound by *Y. pestis* KIM6+ (black bars) and KIM6+ Δ*phoP* (gray bars) after growth at different temperatures is shown (means and standard deviations [SD] from three experiments).

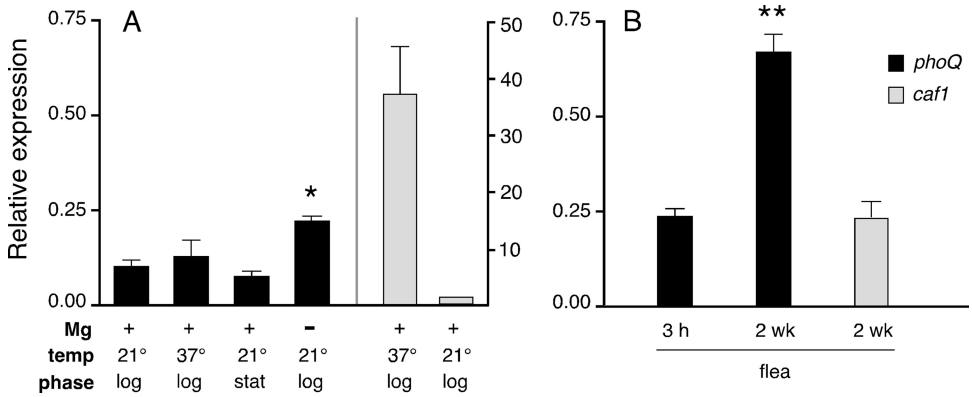

FIG 5 Induction of the *Y. pestis* PhoP-PhoQ regulatory system during infection of *X. cheopis* fleas. Relative amounts of *phoQ* mRNA expressed by *Y. pestis* KIM6+ in logarithmic-phase (log) and stationary-phase (stat) cultures grown at 21 or 37°C in media containing high (+) or low (−) Mg^{2+} (A) and in fleas 3 h and 2 weeks after infection (B) were determined by quantitative RT-PCR. As a control, relative expression of *caf1*, a highly expressed gene which is known to be downregulated at 21°C and in the flea, was also determined. The means and SEM from three experiments performed in triplicate are shown. *, $P < 0.05$ compared to Mg^{+} cultures by one-way analysis of variance and Tukey's multiple comparison test; **, $P = 0.0005$ compared to the 3-h sample by unpaired *t* test (two tailed).

Mg^{2+} in flea gut contents was 0.1 mM (Table 3), about 10-fold higher than in the low-Mg^{2+} medium used.

Role of PhoP in resistance to flea innate immunity. A major biological role of the *Salmonella* PhoP-PhoQ system is to upregulate genes required for bacterial survival in macrophages and for resistance to host CAMPs. The PhoP-PhoQ system is likewise responsible for resistance to polymyxin B and cecropin, an insect-derived CAMP, in *Y. pestis* (29, 30, 48) and *Y. pseudotuberculosis* (28). To demonstrate that fleas, like other insects, mount an antibacterial immune response, a mixture of Gram-positive and -negative bacteria was introduced into the hemocoel (body cavity) of fleas. Six hours after challenge, flea extracts were screened in a bioassay for antibacterial activity. The results showed that bacterial challenge induced the expression of antibacterial components in the flea (Fig. 6).

We determined the susceptibility of $PhoP^{+}$ and $PhoP^{-}$ *Y. pestis* to CAMPs, a major component of the insect antibacterial response (Table 4). As expected, the $PhoP^{-}$ strains as well as the Δ*ugd* and Δ*pbgP* Δ*ugd* mutants that are unable to synthesize or add aminoarabinose to lipid A were highly susceptible. In contrast, the $PhoP^{+}$ and the complemented Δ*phoP* and Δ*ugd Y. pestis* strains were highly resistant. Because the *Y. pestis* Δ*pbgP* Δ*ugd* mutant had a wild-type phenotype in the flea (Fig. 1), these results suggest that any CAMP response of the flea to oral infection is not sufficient to control CAMP-sensitive, aminoarabinose-negative *Y. pestis* in the flea gut.

DISCUSSION

Y. pestis colonizes its flea vector by growing as a biofilm in the digestive tract (32). The bacteria enter the flea midgut in a blood meal as individual cells but then reproduce and grow in the form of dense multicellular aggregates that are embedded within an extracellular matrix. Adherent biofilm in the flea proventriculus is crucial for the regurgitative transmission mechanism. When blocked or partially blocked fleas attempt to feed, the bacterial biofilm attached to the spines of the proventricular valve impedes the normal flow of blood into the midgut. This potentiates transmission, which occurs when blood containing *Y. pestis* released from the periphery of the biofilm is regurgitated into the bite site (33, 34).

The ability of *Y. pestis* to form a biofilm, both in the flea and *in vitro*, depends on the *hms* gene products encoded in the Pgm locus, which synthesize the β-1,6 *N*-acetyl-D-glucosamine polysaccharide component of the extracellular matrix of the biofilm (32, 41, 43, 54, 59, 60). The Pgm-dependent biofilm is produced only

TABLE 3 Cation concentrations in the midgut of *X. cheopis* fleas at different times after a blood meal

Cation	Concentration (mM) at:	
	1 day	6 days
Mg	0.14	0.10
Ca	0.14	0.30
Fe	3.6	2.0

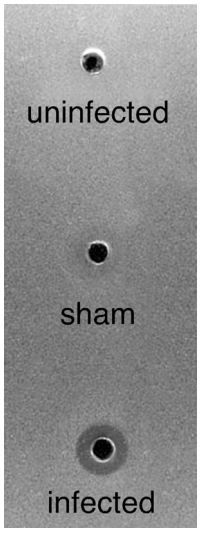

FIG 6 Antibacterial immune response of *X. cheopis* fleas induced by bacterial challenge. Zone of inhibition assay of extracts collected from fleas 6 h after challenge by piercing the exoskeleton with either a needle contaminated with *E. coli* and *M. luteus* (infected) or a sterile needle (sham) and from unchallenged fleas (uninfected).

TABLE 4 MICs of cationic antimicrobial peptides to *Y. pestis* KIM6+ strains

Y. pestis strain or genotype	MIC (μg/ml) of:	
	Polymyxin B	Cecropin A
KIM6+ (wild type)	50	>100
Δ*phoP* (pLG338)	<0.1	1.6
Δ*phoP* (pLG*phoP*)	25	>100
Δ*pmrA*	50	>100
Δ*phoP* Δ*pmrA*	0.8	1.6
Δ*pbgP* Δ*ugd*	<0.1	1.6
Δ*ugd* (pLG338-30)	<0.1	1.6
Δ*ugd* (pLG*ugd*)	50	>100

at temperatures below 28°C. In this study, we found that the *Y. pestis* PhoP-PhoQ system influences biofilm development and localization in the flea.

The biofilms formed by the Pgm^+ $PhoP^-$ *Y. pestis* strains in the flea were less cohesive and less adherent to the proventricular spines than those formed by wild-type strains (Fig. 2). This phenotype resulted in a greatly decreased incidence of proventricular blockage in fleas infected with $PhoP^-$ *Y. pestis* (Fig. 1). It is likely that the biofilm produced by $PhoP^-$ *Y. pestis* is less adherent to the acellular, hydrophobic surface of the proventricular spines and too fragile to withstand the threshing action generated by the rapid contractions of the proventricular valve that occur when a flea feeds and is swept back into the midgut along with the incoming blood (Fig. 2C and D). The effects of *phoP* mutation on *in vitro* biofilm formation are consistent with this interpretation. Sun et al. reported that, although a *Y. pestis phoP* mutant produced thicker biofilms than the wild type in the microtiter plate assay, the biofilms made by the *phoP* mutant were more loosely adherent, and special care had to be taken to prevent dislodging them during washing and staining steps (56). In our flow cell system, an effect of *phoP* mutation on biofilm formation was evident at the intermediate temperature of 25°C (Fig. 3). At this temperature, the *phoP* mutant usually failed to produce an adherent biofilm, whereas the wild-type parent strain always produced one. In *Y. pseudotuberculosis*, loss of *phoP* correlates with increased biofilm formation on the surface of *Caenorhabditis elegans* nematodes, but neither $PhoP^+$ nor $PhoP^-$ *Y. pseudotuberculosis* strains are able to block fleas (56, 61, 62). Thus, PhoP affects the biofilm phenotype of the two species differently under different environmental conditions. This is not surprising, because biofilm development depends on complex regulatory pathways and other physiologic factors that are sensitive to environmental conditions, as well as on the surface characteristics of the bacteria and the substrate to which they adhere (49, 63).

The results indicate that, in addition to the *hms* genes, PhoP-regulated genes of *Y. pestis* are required for the production of a stable, adherent biofilm in the flea proventriculus. We do not know which PhoP-regulated genes are responsible for this, but obvious candidates are those that affect cell surface characteristics. Changes in LPS structure, cell surface charge, and adhesion expression affect the biofilm phenotype of other bacteria (49, 64–67). *Y. pestis* undergoes temperature-dependent phase variation in the acylation pattern of its lipid A—during growth at 21°C (the temperature typical of the flea gut environment), the lipid A is hexa-acylated, whereas at 37°C the lipid A is primarily tetra-acylated (29, 68). We have previously shown that a *Y. pestis* mutant lacking *msbB* and *lpxP*, which encode the two acyltransferases required to produce hexa-acylated lipid A, constitutively makes the tetra-acylated form at both low and high temperatures but that this mutant is still able to infect and block fleas normally (69). The *Y. pestis* PhoP-PhoQ system does not appear to regulate lipid A acylation (29, 48) but greatly reduces aminoarabinose addition (29). We ruled out our hypothesis that PhoP-dependent addition of aminoarabinose to lipid A was required for normal biofilm formation in the flea; however, PhoP has been implicated in another modification of LPS—the addition of galactose to the oligosaccharide core (48). Alteration of LPS has previously been shown to affect biofilm formation in *Y. pestis*: *gmhA*, which is responsible for synthesis of the heptose component of the oligosaccharide core, is required for proventricular blockage of fleas (70), and *yrbH* and *waaA*, which are involved in the addition of Kdo (3-deoxy-D-manno-octulosonic acid) monosaccharide to the inner core of LPS, are required for normal biofilm formation *in vitro* (71). The *Y. pestis hms* genes themselves do not appear to be PhoP regulated, at least *in vitro*, because the prototypical Hms-dependent phenotype (pigmentation due to Congo red binding) was not affected by *phoP* mutation (Fig. 4).

The environmental factors in the flea gut that induce the *Y. pestis* PhoP-PhoQ system are also unknown. In Gram-negative bacteria, low Mg^{2+} and Ca^{2+} stimulate PhoQ, and high Fe^{3+} can activate a subset of PhoP-regulated genes by stimulating the PmrA-PmrB two-component regulatory system (9, 30, 72). As in *Salmonella*, the *Y. pestis* PhoP-PhoQ system was induced by low Mg^{2+} concentration *in vitro*, but induction was even higher during infection of the flea digestive tract (Fig. 5). Mg^{2+} and Ca^{2+} levels in the flea midgut would be expected to be high immediately after a blood meal, reflecting mammalian plasma levels (0.8 to 1.0 mM and 2.0 to 2.5 mM, respectively). However, the concentration of these cations is much higher in insect hemolymph, so it is likely that they are actively transported by the midgut epithelium (73). We detected 0.1 to 0.3 mM Mg^{2+} and Ca^{2+} in flea midgut contents (Table 3). These levels are intermediate between the micromolar concentrations that maximally stimulate *Salmonella* PhoP activation and the millimolar concentrations that do not stimulate (9); nevertheless, *Y. pestis* PhoP activation was greater in fleas than in low-Mg^{2+} medium (Fig. 5). One possible explanation is that bacteria within the dense biofilm are not exposed to the level of Mg^{2+} present in the lumen of the flea gut. In contrast, *pmrA* expression was lower in the flea than in N-minimal medium containing 10 μM $MgCl_2$ with or without 0.1 mM $FeSO_4$ (data not shown). These results are consistent with a recent microarray study in which *pmrA* expression was lower in the flea than *in vitro* and no *pmrB* expression was detected in the flea (36) as well as with the lack of a phenotype of the *Y. pestis pmrA* mutant in the flea (Fig. 1). Although *Y. pestis* has a Fe^{3+}-responsive PmrA-PmrB system (30) and the Fe concentration in the flea midgut was high (Table 3), as might be expected following digestion of red blood cells and hemoglobin), the ionic and chemical state (e.g., Fe^{2+} or Fe^{3+}; free or bound to heme) of this metal in the flea gut is unknown. The microarray study indicated that the *pbgP* and *ugd* genes were not upregulated in the flea compared to *in vitro* growth conditions (36).

Other environmental signals in the flea gut may contribute to PhoQ stimulation. For example, the *Salmonella* PhoP-PhoQ system responds to mildly acidic pH and to CAMPs (13, 14, 74). The

flea midgut pH is reportedly acidic (75), and the presence of bacteria in the blood meal induces the production and secretion of CAMPs into the midgut in other blood-feeding insects (76, 77). Fleas, like other insects, mount an immune response against bacterial challenge (Fig. 6), and PhoP mutation in *Y. pestis* and in the insect pathogen *Photorhabdus luminescens* results in an inability to infect the hemocoel and loss of virulence to insect (order Lepidoptera) larvae (7, 78). The PhoP-PhoQ system of *Sodalis glossinidius*, a Gram-negative endosymbiont of the tsetse fly, is also required for infectivity in the insect host (79). Consistent with previous results (29, 48) and the importance of aminoarabinose-modified lipid A for resistance to CAMPs (16), the *Y. pestis phoP*, *ugd*, and *pbgP* mutants were highly sensitive to both polymyxin and cecropin, a type of antibacterial peptide commonly produced by insects (Table 4). In spite of this, however, the *Y. pestis* Δ*phoP* and Δ*pbgP* Δ*ugd* strains multiplied normally in the flea midgut (Fig. 1). This suggests that the presence of *Y. pestis* in the blood meal does not induce a strong antimicrobial response into the midgut. Alternatively, since bacteria in a biofilm are more resistant to many antimicrobials (80), the biofilm ECM or other PhoP-independent, midgut-specific phenotype may have protected the *Y. pestis* Δ*phoP* and Δ*ugd* strains from flea CAMPs in the midgut.

Our results provide independent proof that the *Y. pestis* PhoP-PhoQ regulatory system is induced in the flea and that this induction is actually required to produce a normal transmissible infection. The specific inducing stress that stimulates PhoQ can result in the activation of different subsets of the PhoP-regulated genes (12, 31), and we are currently identifying the *Y. pestis* genes that are PhoP regulated in the flea gut environment. Certain known PhoP-regulated virulence factors have been shown to be upregulated in the flea, however, and their induction prior to transmission may enhance infectivity in the mammal by conferring resistance to the initial innate immune response encountered at the flea bite site (36). In addition to resistance to CAMPs, the PhoP-PhoQ two-component regulatory system is required for *Y. pestis* resistance to killing by neutrophils (81), which the bacteria encounter very soon after transmission. In this regard, upregulation of the PhoP-PhoQ system prior to transmission in the flea may be especially important because the major antiphagocytic virulence factors encoded by the type III secretion system are not expressed at a low temperature in the flea and are not fully functional until a few hours after a shift to 37°C (57, 75, 82).

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