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PhoP and OxyR transcriptional regulators contribute to *Yersinia pestis* virulence and survival within *Galleria mellonella*

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ABSTRACT

The virulence of *Yersinia pestis* KIM6+ was compared with multiple isolates of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* toward larvae of the greater wax moth *Galleria mellonella*. Although *Y. pestis* and *Y. pseudotuberculosis* were able to cause lethal infection in *G. mellonella*, these species appeared less virulent than the majority of *Y. enterocolitica* strains tested. *Y. pestis* survived primarily within hemocytes of *G. mellonella*, and induced a strong antibacterial peptide response that lasted for at least 3 days in surviving larvae. Immunization with dead bacteria to induce an antibacterial response led to increased survival of the larvae following infection. Mutant strains lacking the either *phoP* or *oxyR*, which were less resistant to antibacterial peptides and hydrogen peroxide respectively, were attenuated and restoration of the wild-type genes on plasmids restored virulence. Our results indicate that the *Y. pseudotuberculosis*-*Y. pestis* lineage is not as virulent toward *G. mellonella* as are the majority of *Y. enterocolitica* isolates. Further, we have shown that *G. mellonella* is a useful infection model for analyzing *Y. pestis* host-pathogen interactions, and antibacterial peptide resistance mediated by *phoP* and reactive oxygen defense mediated by *oxyR* are important for *Y. pestis* infection of this insect.

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1. Introduction

The genus *Yersinia* includes the mammalian pathogens *Yersinia enterocolitica*, *Yersinia pseudotuberculosis* and *Yersinia pestis*. *Y. pestis*, the cause of bubonic plague, is closely related to *Y. enterocolitica* and *Y. pseudotuberculosis* and descended from the latter. *Y. pestis* is unique in that it is capable of being transmitted by fleas to susceptible mammals, whereas *Y. pseudotuberculosis* and *Y. enterocolitica* are not. It is becoming increasingly apparent that *Y. pestis* is not the only member of this lineage that infects insects. Both *Y. enterocolitica* and *Y. pseudotuberculosis* are enteropathogens that infect mammalian and avian species via contaminated food and water. However, they have also been isolated from flies in the wild [1], have loci that encode predicted insect toxins [2–5], and are able to adhere to and invade cultured insect cells [4]. *Y. enterocolitica* and *Y. pseudotuberculosis* lysates are orally toxic toward caterpillars [3,4] and fleas [6], but *Y. pestis* is not, which presumably was important for adoption of a vector-borne lifestyle.

Both *Y. enterocolitica* and *Y. pseudotuberculosis* strains have been shown to cause lethal infections in the larvae of the greater wax moth *Galleria mellonella* following subcutaneous injection [7,8]. We were interested in determining whether *Y. pestis* is also able to infect this insect. Specifically, we sought to compare the virulence of a variety of *Y. enterocolitica* and *Y. pseudotuberculosis* strains with *Y. pestis* under identical conditions. We also sought to evaluate the utility of *G. mellonella* as a model for testing specific host and bacterial factors that affect *Y. pestis* infection. For instance, the two-component PhoP–PhoQ system is an important regulator of virulence in several gram-negative bacteria, and may aid survival in response to specific environmental challenges within insects such as acidic pH and antibacterial peptides [9]. Similarly, the transcriptional regulator OxyR enhances resistance of several bacteria to reactive oxygen species, which are thought to be another important component of insect immune defenses [10]. Thus, we tested the virulence of *Y. pestis* mutants lacking *phoP* or *oxyR* in the *G. mellonella* model.

2. Materials and methods

2.1. Bacterial strains and plasmids, generation of mutants

Y. enterocolitica, *Y. pseudotuberculosis* and *Y. pestis* strains used in this study are described in Table 1. All of the *Y. pseudotuberculosis*

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Table 1
Survival of *G. mellonella* larvae after infection with 10^5 cfu of each *Yersinia* strain at 24, 48 and 72 h at 21 °C.

	Source or reference	Serotype	% Survival ($n = 50$)		
			24 h	48 h	72 h
Y. enterocolitica					
Y2	[50]	O:3	90	36	28
Y3	[51]	O:3	92	54	38
ATCC 9610	[51]	O:8	78	42	26
8081	[52]	O:8	64	18	16
634-83	[53]	O:4,32	72	56	40
655-83	[53]	O:18	70	30	28
657-83	[53]	O:20	84	28	18
70-419-2F	[54]	O:21	88	64	56
CS080	[55]	unknown	60	24	12
Y. pseudotuberculosis					
IP32953	[2]	O:1b	98	88	86
PB1	[56]	O:1b	92	90	90
IP32951	[2]	O:2	96	94	90
ATCC 29910	ATCC	O:2	94	88	88
YP204	[57]	O:5a	100	96	94
YPR708ly	[57]	O:9	100	100	98
ATCC 29833	ATCC	ND	98	96	96
Y. pestis					
KIM6+	R.D. Perry	NA	94	90	88
KIM6+ Δ*phoP*	This study	NA	Not tested	Not tested	Not tested
KIM6+ Δ*phoP* (pCRphoPQ)	This study	NA	Not tested	Not tested	Not tested
KIM6+ Δ*oxyR*	This study	NA	Not tested	Not tested	Not tested
KIM6+ Δ*oxyR* (pJETоxyR)	This study	NA	Not tested	Not tested	Not tested

and *Y. enterocolitica* isolates contain the virulence plasmid whereas the *Y. pestis* KIM6+ strain does not. *Yersinia* strains were grown in Terrific Broth (TB) (ISC Bioexpress) at 30 °C. Where appropriate, ampicillin and kanamycin (Sigma) were added to the media at 100 μg ml^{-1} or 30 μg ml^{-1} in order to maintain plasmids and select for recombinant strains. *Yersinia* strains were labeled with green fluorescent protein by electroporation of plasmid pAKgfp1 [11] (Addgene plasmid 14076).

Generation of an isogenic Δ*phoP* mutant strain in the *Y. pestis* KIM6+ background was performed essentially as described [12] for the *Y. pestis* GB strain. The *phoP* region including upstream and downstream sequences was amplified from *Y. pestis* KIM6+ genomic DNA using primers 5′ TGG GTG CCA GCC GCC ATG CAT 3′ and 5′ GCG TCT AGA AGA GGT CGC GGC GTA GGT ATT 3′, and the PCR product was cloned into plasmid pCR2.1 (Invitrogen) to generate plasmid pCRphoP. Inverse PCR was used to create an internal 31 bp deletion using primers 5′ CCC ATA TGG GAT GGC TTA AGC CTT ATC and 5′ AAC ATA TGT ATC TGG GCC ATG TTC CTG 3′ (NdeI sites underlined), digesting with NdeI, and religating the PCR product. The *ΔphoP* allele was subcloned into the suicide vector pCVD442 using the XbaI and KpnI restriction sites. The resulting plasmid was transferred to *Y. pestis* through bi-parental mating with the donor strain *Escherichia coli* SM10λpir and an allelic exchange mutant in which the *phoP* mutant gene containing the 31 bp deletion had replaced the wild-type gene was selected by growing the transconjugants on plates containing ampicillin, followed by selection on plates containing 10% sucrose. The mutant strain was verified by PCR and complemented by amplifying the *phoPQ* operon using primers 5′ ATT TGA CTG TGT GGC CCG TTT CAA 3′ and 5′ TAC CAC CGC GAC CAT GCC ATA AGA 3′, and inserting the PCR product into pCR2.1 to produce plasmid pCRphoPQ. This plasmid was introduced into the *ΔphoP* mutant strain by electroporation.

To generate the isogenic Δ*oxyR* mutant, a kanamycin resistance cassette was inserted between the start and stop codons of *oxyR* via lambda red recombination as described [13]. Long-flanking regions (500 bp) upstream and downstream were amplified using primers 5′ GCG CTC TAG AAG CCC AGA TCT GCT TTC TCA ACC A 3′ and 5′ AAG CAG CTC CAG CCT ACA CCC ACC TTA TGA TAG CTA CTG ACG 3′ for the upstream, and 5′ GGC CGA CGG ATC CCC GGA ATG ATT TAA GCC ATT GAG TGC GGC CA 3′ and 5′ GCG CTC TAG AAG CCG TGG TTT GCT GAA AGT GAA C 3′ for the downstream. The kanamycin resistance gene was amplified from plasmid pKD13 using primers P1 and P4 [14]. The 3-way PCR product (upstream-kan^r-downstream) was digested with DpnI and electroporated into strain KIM6+ carrying pKOBEG-sacB. Mutants were selected by growth on kanamycin plates and verified by PCR, followed by curing of pKOBEG-sacB by growth on plates containing 10% (w/v) sucrose. The mutant was complemented by amplifying the *oxyR* gene using primers 5′ GGG CCG TTG CAT ATT GGG CTT 3′ and 5′ AAC AGC GTA ATA CCA CTA CCC 3′, and inserting the PCR product into pJET1.2 (Fermentas) to produce plasmid pJEToxyR. This plasmid was introduced into the *oxyR* mutant strain by electroporation.

2.2. Infection experiments

Larvae were purchased from Vanderhorst Wholesale Inc. (St. Mary's, Ohio), stored at 15 °C in the dark and used within ten days of delivery. *Yersinia* were incubated for 24 h at either 21 or 37 °C, centrifuged, and resuspended in phosphate buffered saline (PBS, 137 mM NaCl; 2.7 mM KCl, 10 mM phosphate buffer pH 7.3) to an absorbance of 1.0 at 600 nm and diluted in PBS to the desired concentration. The concentration of each strain was determined by serial dilution and colony counting after 48 h growth on TB agar plates. If the plate counts indicated that the inoculum was not within 0.5 log of the expected value, those larvae were discarded. Those larvae that were 2.0–2.5 cm in length and had a cream-colored cuticle were injected through the left hindmost proleg with 10 μl of bacterial suspension using a Hamilton syringe and a 30-gauge needle. Control larvae were injected with 10 μl of sterile PBS. If more than one of the control larvae per twenty that were injected died, then that group of larvae was not counted in the analysis. The larvae were then held in darkness in Petri dishes at either 21 or 37 °C. Survival was monitored at 12 h intervals and deaths noted by lack of movement when gently prodded.

2.3. Quantitation and visualization of bacteria during infection

Hemocoel samples were collected at specified intervals and the number of bacteria associated with the hemolymph was determined by serial dilution in PBS and spreading on TB agar plates. To determine the number of bacteria associated with the hemocytes, the hemocoel samples were centrifuged at low-speed (1000×*g*) to pellet the hemocytes. The supernatant was removed and the hemocytes resuspended in sterile water to lyse the cells [15], and spread on TB agar. Colonies were counted after 24 h incubation at 30 °C. Bacteria in hemocoel expressing green fluorescent protein were visualized using a Zeiss fluorescent microscope.

2.4. Antimicrobial peptide assay

Infected larvae were surface-sterilized with 70% ethanol, the bottom 2 mm were removed with a sterile scalpel and the hemocoel collected in microcentrifuge tubes. Twenty-five μl of hemocoel was immediately transferred to an equal volume of Insect Physiological Saline (150 mM NaCl and 5 mM KCl in 0.1 M Tris–HCl, final pH 6.9) containing 10 mM EDTA and 30 mM sodium citrate as anticoagulants on ice. Antibacterial activity in these samples was determined using a zone of inhibition assay [16]. The indicator *E. coli* strain D31 was prepared by adding 100 μl of overnight culture to 7 ml of molten 0.7% agarose in LB broth containing 0.5% (w/v)

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lysozyme and 100 μg streptomycin ml^{-1} at 42 °C. The suspension was immediately poured into a sterile Petri dish, allowed to set, and 2 mm holes drilled into the agarose using a 1000 μl pipet tip. Aliquots of the hemocoel samples (5 μl) were added to each well and the zone of inhibition was measured after overnight incubation at 37 °C.

2.5. Hydrogen peroxide sensitivity assay

Overnight broth cultures of the wild type *Y. pestis* KIM6+, the Δ*oxyR* mutant, and the complemented Δ*oxyR* mutant strains were spread as lawns on TB agar plates. The plates were allowed to dry and 5 mm filter disks were placed on the lawns, followed by the addition of various concentrations of hydrogen peroxide. The plates were incubated at 30 °C for 24 h, and the clear zone of inhibition around the disks was measured.

2.6. Immunization experiments

Groups of ten larvae were injected with 10 μl of either sterile PBS or 10^4 cfu *Y. pestis* KIM6+ that had been killed by incubation at 65 °C for 20 min. The larvae were injected in the right hindmost proleg, kept at room temperature for 24 h followed by infection with either 10^6 cfu *Y. pestis* KIM6+ or 10^5 cfu *Y. enterocolitica* 8081. The infected larvae were kept at 37 °C and monitored for survival as described above.

3. Results

3.1. Virulence of Yersinia strains in G. mellonella

We compared the ability of several *Y. enterocolitica* and *Y. pseudotuberculosis* isolates with that of *Y. pestis* KIM6+ to cause lethal infection in *G. mellonella* larvae (Table 1). At this initial dose and incubation temperature (10^5 cfu/larvae, 21 °C), all but one of the *Y. enterocolitica* strains killed greater than 50 percent of the larvae within 72 h (mean survival ±SD 29.1 ± 13.8%), whereas the *Y. pseudotuberculosis* strains and *Y. pestis* KIM6+ killed very few (mean survival ±SD 91.3 ± 4.3%). These results show that virulence toward *G. mellonella* is species and strain-specific, but that *Y. enterocolitica* exhibits significantly ($p < 0.0001$ via unpaired *t*-test) greater virulence than *Y. pseudotuberculosis* and *Y. pestis* in this model. We then infected larvae with higher numbers of *Y. pseudotuberculosis* and *Y. pestis* bacteria. Mortality was evident for *Y. pseudotuberculosis* strains and *Y. pestis* KIM6+ when the infectious dose was increased to 10^6 cfu/larvae, and the *Y. pseudotuberculosis* strains were not uniform in virulence toward *G. mellonella* (Fig. 1a). Our results for *Y. pseudotuberculosis* strain IP32953 were very similar to those of Champion et al. [7]. When larvae infected with *Y. pseudotuberculosis* IP32953 or *Y. pestis* KIM6+ were incubated at 21 or 37 °C, similar numbers of larvae died at both temperatures. Although the larvae appeared to die more quickly when incubated at 37 °C compared to 21 °C, the survival curves for larvae infected with either *Y. pestis* or *Y. pseudotuberculosis* at the two temperatures were not significantly different from each other when analyzed via logrank test (Fig. 1b).

Previous reports indicated that *Y. pseudotuberculosis* survived primarily within hemocytes 24 h after infection [7]. We used fluorescence microscopy and culture of hemocoel samples to determine the survival and location of *Y. pestis* in *G. mellonella* larvae. In larvae that remained alive, the bacterial load remained at or near the initial 10^6–10^7 cfu ml^{-1} hemocoel for 12 h, but decreased 10–100 fold by 24 h. Low-speed centrifugation of the hemocoel samples followed by plating of the collected hemocytes was used to estimate the proportion of the bacteria that were associated with hemocytes vs. those that were extracellular. By 4 h after infection, the cellular fraction of the hemocoel contained the majority of the bacteria, suggesting that the hemocytes quickly attached to or phagocytosed the bacteria, and that *Y. pestis* is able to survive in association with the hemocytes (Fig. 2a). At 24-h post-infection, GFP-labeled *Y. pestis* were clearly visible associated with hemocytes but not extracellularly (Fig. 2b).

3.2. Yersinia induces protective antibacterial activity in G. mellonella hemolymph

The presence of bacteria in the insect circulatory system induces production of antibacterial immune effectors, particularly cationic antimicrobial peptides. We measured the antibacterial activity in *G. mellonella* hemocoel samples following *Y. pestis* and *Y. pseudotuberculosis* using a zone of inhibition assay with *E. coli* strain D31, which is hypersensitive to antibacterial peptides, as the indicator (Fig. 3a). Hemocoel samples from mock (PBS)-infected larvae did not cause any inhibition, whereas the antibacterial activity in hemocoel from larvae infected with *Y. pestis* and *Y. pseudotuberculosis* was evident within 2 h after infection, and remained high for at least 3 days in surviving larvae.

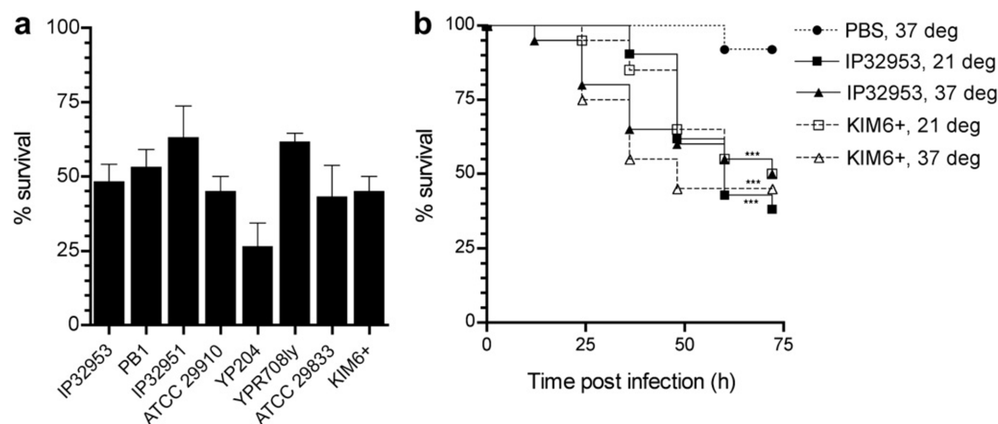

Fig. 1. Survival of *G. mellonella* infected with *Y. pseudotuberculosis* or *Y. pestis* KIM6+. a) Three groups of 20 larvae were infected with 10^6 cfu of the indicated strain and kept at 37 °C, and survival scored after 24 h b) Three groups of 20 larvae were infected with 10^6 cfu of either *Y. pseudotuberculosis* IP32953 or *Y. pestis* KIM6+ cultured at 21 or 37 °C and kept at the indicated temperature. ***$p < 0.001$ compared with PBS injected control larvae by logrank test. The survival curves of *Y. pestis* or *Y. pseudotuberculosis* infected larvae at 21 or 37 °C were not significantly different from each other.

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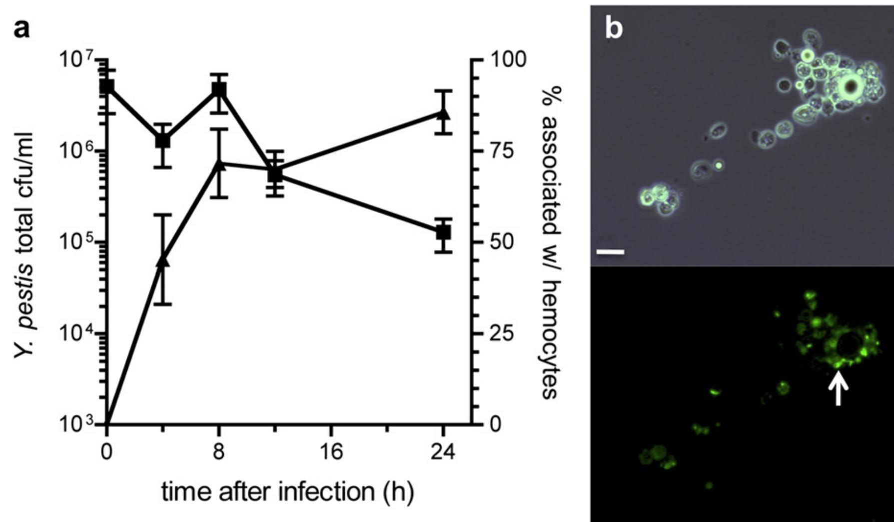

Fig. 2. Intracellular survival of *Y. pestis* within *G. mellonella* hemocytes. a) Total numbers of bacteria (expressed as mean ± s.d. cfu ml^{-1}) in five hemocoel samples from larvae infected with 10^6 cfu *Y. pestis* KIM6+ collected at the indicated time points (left *Y*-axis, squares), and the proportion of the bacteria that were associated with hemocyte fraction (right *Y*-axis, triangles). b) Hemocoel collected 24 h following infection with *Y. pestis* expressing green fluorescent protein visualized using phase-contrast (top) or fluorescence microscopy. Individual bacteria were visible within hemocytes (arrow) but not extracellularly in five hemocoel samples, 10 fields of view each. Scale bar = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

We next wanted to determine whether pre-induction of the antibacterial response could protect larvae from lethal *Yersinia* infections. When *G. mellonella* were immunized with heat-killed *Y. pestis* KIM6+ prior to infection with *Y. pestis* KIM6+, more of the larvae survived compared with sham-immunized larvae. Similarly, immunization with heat-killed *Y. pestis* followed by infection with *Y. enterocolitica* resulted in significantly higher survival as compared to those larvae that were sham-immunized with sterile PBS (Fig. 3b).

3.3. The PhoP and OxyR transcriptional regulators are required for Y. pestis virulence in G. mellonella

Since pre-induction of antimicrobial defenses limited the virulence of *Y. pestis* and Y. *pseudotuberculosis*, we hypothesized that mutants that are more susceptible to antibacterial peptides might be less virulent in these insects. To test this, we created a *Y. pestis* KIM6+ mutant lacking a functional PhoP response regulator, which is known to control the expression of genes that modify lipopolysaccharide in *Yersinia* and several other Gram-negative bacteria, thereby increasing resistance to antibacterial peptides [17,18]. The Δ*phoP* mutant was significantly reduced in its virulence toward *G. mellonella*, and virulence was restored by adding back a functional *phoPQ* allele on a plasmid (Fig. 4a). The reduction in virulence in the Δ*phoP* mutant strain was also correlated with reduced bacterial loads in the hemocoel from infected larvae (Fig. 4b).

G. mellonella hemocytes also produce reactive oxygen species to kill bacteria [19], and *Y. pseudotuberculosis* requires the superoxide dismutase SodC for full virulence [7]. The redox-sensing transcriptional regulator OxyR was recently shown to regulate the expression of the catalases KatY and KatA in *Y. pestis* in response to hydrogen peroxide and temperature [20]. To examine the contribution of this regulatory pathway to *G. mellonella* infection, we

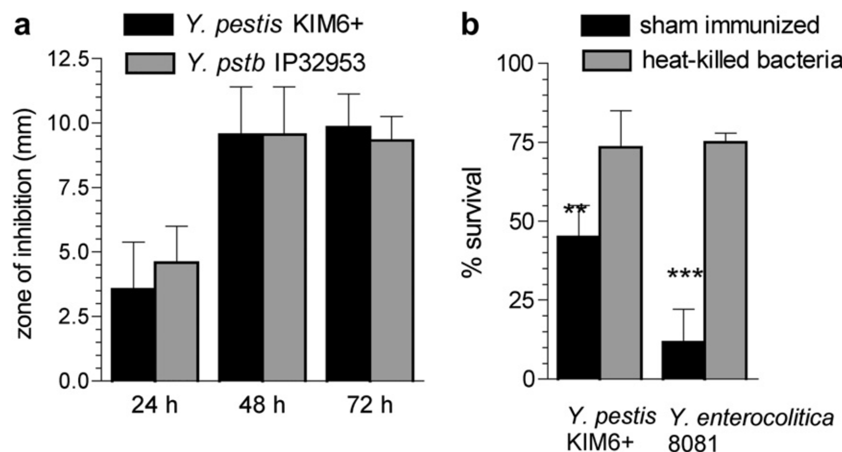

Fig. 3. a) Antimicrobial peptide production during *Yersinia* infection measured using a zone of inhibition assay with *E. coli* D31 as the indicator. Hemocoel samples were collected from 10 larvae at 24, 48 and 72 h following infection with either *Y. pestis* KIM6+ or *Y. pseudotuberculosis* IP32953. Hemocoel from control larvae inoculated with sterile PBS showed no inhibition. b) Survival of larvae (mean ± s.d., 3 groups of 20) immunized with heat-killed *Y. pestis* or sham (PBS)-immunized, and infected with 10^6 *Y. pestis* or 10^5 *Y. enterocolitica* 8081 24 h following immunization. Survival was measured 48 h following infection. $^{**}p < 0.005$, $^{***}p < 0.001$ by Fisher's exact test.

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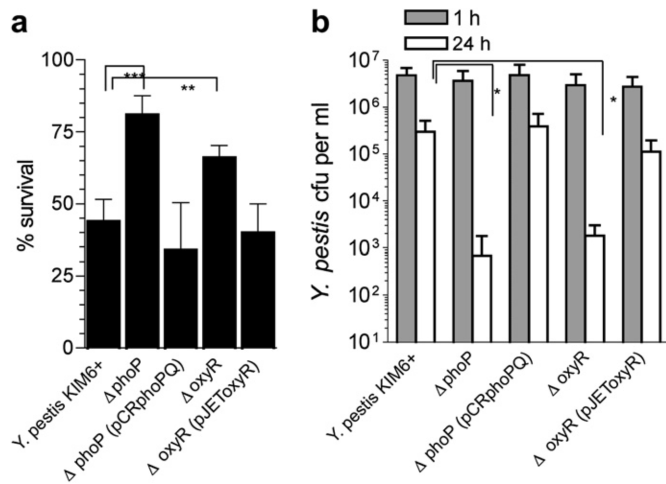

Fig. 4. a) Survival of larvae (mean ± s.d., 5 groups of 20) over 48 h at 37 °C following infection with wild-type *Y. pestis* KIM6+, KIM6+ Δ*phoP*, KIM6+ Δ*phoP* pCRphoPQ), KIM6+ Δ*oxyR*, or KIM6+ Δ*oxyR* (pJEToxyR) $^{**}p < 0.005$, $^{***}p < 0.001$ by Fisher's exact test. b) Numbers of bacteria (expressed as mean ± s.d. cfu ml^{-1} hemocoel) that were present in five surviving larvae at the indicated times post-infection. $^{*}p < 0.05$ by unpaired *T*-test.

created a mutant strain lacking the *oxyR* gene. As expected, this mutant was hypersensitive to hydrogen peroxide (data not shown). The Δ*oxyR* mutant was attenuated in *G. mellonella*; it appeared slightly less attenuated than was the Δ*phoP* mutant strain but this difference was not statistically significant (Fig. 4a). Bacterial survival within the hemocoel was also lower for the Δ*oxyR* mutant strain (Fig. 4b). Complementation by adding a functional *oxyR* gene on a plasmid restored virulence and bacterial survival to near wild-type levels.

4. Discussion

G. mellonella have been used extensively to study virulence of several bacterial pathogens [7,8,15,21–24]. This insect model is attractive because it is inexpensive, easy to handle, and can be incubated at mammalian body temperature. It also has an innate immune response that in many ways is very similar to mammals [25]. Pathogens are contained via coagulation of the hemolymph, followed by activation of multiple types of hemocytes, which act to phagocytose invading microbes. Similar mechanisms are employed by hemocytes to kill ingested microbes as mammalian neutrophils and macrophages, including an oxidative burst, the production of lysozyme and numerous antimicrobial peptides. In addition, *Galleria* can recognize microbe-associated molecular patterns by receptors such as Toll and peptidoglycan recognition proteins [26]. Thus, bacterial factors that enable survival in this insect host are likely to be directly relevant to human infection [27–29].

We have shown here that the plague bacterium *Y. pestis* is virulent toward *G. mellonella* when injected into the hemocoel of these insects (Fig. 1). We observed that similar numbers of larvae died over a three-day period when infected and incubated at 21 °C as at 37 °C. The *Yersinia* virulence plasmid pYV/pCD1 encodes a type III secretion system expressed at 37 °C that plays a major role in resistance to mammalian phagocytes. However, our data show that *Y. pestis* KIM6+, which lacks this plasmid, is as virulent as the majority of the plasmid-containing *Y. pseudotuberculosis* strains we tested at either temperature. This suggests that other factors determine bacterial survival during interaction with *Galleria* hemocytes. Infection with *Listeria* and *Acinetobacter* at 37 °C results in more rapid death than 30 °C [23,30], while other species including *Y. enterocolitica* [3,31] are more virulent at low temperatures (10–15 °C). In contrast to *G. mellonella* infections, *Y. pestis* inflicts very little harm when it infects the digestive tract of its natural flea host, and even when *Y. pestis* or *Y. pseudotuberculosis* are injected directly into the body cavity of fleas, the bacteria can persist for at least one week without causing excess mortality [6]. In *G. mellonella*, the majority of *Yersinia* are associated with hemocytes at 24 h, but a significant portion are able to survive (Fig. 2), similar to what has been reported previously for *Y. pseudotuberculosis* [7]. Conversely, in bubonic and pneumonic plague infections it is believed that although *Y. pestis* can survive at least transiently within phagocytes, the majority of the bacteria are extracellular once infection is established [12,32,33].

Galleria possesses inducible immune defense molecules that result in protection against repeated infections, as demonstrated by the enhanced immunity resulting from pre-challenge with heat-killed *Y. pestis* (Fig. 3). We observed that pre-challenging larvae prior to *Y. pestis* infection did not make as great a difference to survival as it did prior to *Y. enterocolitica* infection. *Y. pestis* is more resistant to cecropin A and polymyxin than is *Y. enterocolitica* [18]. If *Y. pestis* is also more resistant to the antibacterial peptides of *G. mellonella*, then the inducible response may have less protective effect. Mukherjee et al. [24] also observed that expression of antimicrobial peptides, lysozyme, and inhibitors of host and bacterial metalloproteinases is enhanced following infection, and pre-activation of this response either with whole bacteria or LPS could protect against subsequent challenge. The recent characterization of the immune-inducible transcriptome of *G. mellonella* [34] will aid in identification of the relevant *Yersinia*-induced immune responses.

The reduced survival of the *phoP* and *oxyR* mutant strains we observed 24 h after infection likely reflects impaired intracellular survival following phagocytosis, although it is also possible that these strains are also more sensitive to extracellular killing. The PhoP regulatory protein is part of a two-component system present in many Gram-negative bacteria that activates numerous genes in response to low magnesium conditions, low pH, or membrane perturbations induced by antibacterial peptides [35–37]. Prominent among PhoP-regulated genes of *Y. pestis* are those involved with remodeling the outer leaflet, including addition of aminoarabinose to the lipid A moiety [18], thus reducing the overall negative charge and interaction with cationic antibacterial peptides. However, PhoP also induces expression of a range of genes not necessarily related to antibacterial peptide resistance. For instance, microarray analysis of the *Y. pestis* PhoP regulon indicates that it also activates catalases and superoxide dismutases, in addition to other regulatory genes [38]. Therefore, the decreased virulence of the *phoP* mutant may be due to altered expression of multiple pathogenesis-related genes, including altered resistance to antibacterial peptides and reactive oxygen species.

The *Y. pestis phoP* gene is required for resistance to human neutrophils [39] and growth in macrophages [12]. Our results show that *phoP* is required for full virulence in *G. mellonella* and *phoP* mutants do not survive as well in this host, which is consistent with a primarily intracellular niche for the bacteria in *G. mellonella*. It is believed that *Y. pestis* has a transient intracellular phase during bubonic plague infection after being taken up by macrophages or other phagocytic cells in the dermis and transported within these cells to the draining lymph node. If so, then the *G. mellonella* model might be useful in identifying *Y. pestis* factors relevant to early events in plague infection. Significantly, *phoP* expression is upregulated during chronic midgut infection of fleas [40], and mice infected subcutaneously with a *phoP* mutant strain of *Y. pestis* survive much longer than mice infected with wild-type strain,

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although the LD_{50} is not significantly different [32]. Upregulation of *phoP* in fleas may prepare *Y. pestis* for encounters with phagocytic cells in the dermis, at a time when the type III secretion apparatus would not yet been fully active [41]. Thus, future studies of the significance of *phoP* in the transmission of *Y. pestis* via intradermal inoculation or fleabite challenge might be warranted.

We found that *oxyR* was required for full virulence in *G. mellonella*. OxyR is a LysR-type regulator that, in addition to controlling expression of genes required for reactive oxygen defense, is also involved in the regulation of other virulence factors in several species [10,42–45]. OxyR also affects biofilm formation in a wide range of bacteria, including *Pseudomonas aeruginosa* [46], *Klebsiella pneumoniae* [44], *Neisseria gonorrhoeae* [47], and *Serratia marcescens* [48]. The ability of *Y. pestis* to produce biofilm within the digestive tract of fleas is important for transmission to new hosts [49]. Reactive oxygen species are also present in flea tissues and may limit the proliferation of *Y. pestis* (unpublished data). Studies to address the role of *oxyR* in the ability of *Y. pestis* to establish flea infections and form biofilms are ongoing.

In conclusion, we have shown that the plague bacterium *Y. pestis* is able to cause infection in the greater wax moth *G. mellonella*. *Y. pestis* and *Y. pseudotuberculosis* are generally not as virulent toward this insect as are *Y. enterocolitica* isolates. While infecting *G. mellonella*, *Y. pestis* is able to survive intracellularly within hemocytes, and the presence of the bacteria in the hemocoel induces an antibacterial response that limits the growth of the bacteria. Within this environment, the PhoP and OxyR regulators contribute to bacterial survival and virulence, likely through increasing resistance to antibacterial peptides and reactive oxygen species.

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