IAI Journals.ASM.org

Modeling *Klebsiella pneumoniae* Pathogenesis by Infection of the Wax Moth *Galleria mellonella*

José Luis Insua,[a,b] Enrique Llobet,[a,b] David Moranta,[a,b] Camino Pérez-Gutiérrez,[a,b] Anna Tomás,[a,b] Junkal Garmendia,[b,c] José A. Bengoechea[a,b,d]

Laboratory of Microbial Pathogenesis, Fundació d'Investigació Sanitària de les Illes Balears (FISIB), Bunyola, Spain[a]; Program in Host-Pathogen Interactions, Centro de Investigación Biomédica en Red Enfermedades Respiratorias (CIBERES), Bunyola, Spain[b]; Instituto de Agrobiotecnología, CSIC-Universidad Pública de Navarra-Gobierno de Navarra, Mutilva, Spain[c]; Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain[d]

The implementation of infection models that approximate human disease is essential for understanding pathogenesis at the molecular level and for testing new therapies before they are entered into clinical stages. Insects are increasingly being used as surrogate hosts because they share, with mammals, essential aspects of the innate immune response to infections. We examined whether the larva of the wax moth *Galleria mellonella* could be used as a host model to conceptually approximate *Klebsiella pneumoniae*-triggered pneumonia. We report that the *G. mellonella* model is capable of distinguishing between pathogenic and nonpathogenic *Klebsiella* strains. Moreover, *K. pneumoniae* infection of *G. mellonella* models some of the known features of *Klebsiella*-induced pneumonia, i.e., cell death associated with bacterial replication, avoidance of phagocytosis by phagocytes, and the attenuation of host defense responses, chiefly the production of antimicrobial factors. Similar to the case for the mouse pneumonia model, activation of innate responses improved *G. mellonella* survival against subsequent *Klebsiella* challenge. Virulence factors necessary in the mouse pneumonia model were also implicated in the *Galleria* model. We found that mutants lacking capsule polysaccharide, lipid A decorations, or the outer membrane proteins OmpA and OmpK36 were attenuated in *Galleria*. All mutants activated *G. mellonella* defensive responses. The *Galleria* model also allowed us to monitor *Klebsiella* gene expression. The expression levels of *cps* and the loci implicated in lipid A remodeling peaked during the first hours postinfection, in a PhoPQ- and PmrAB-governed process. Taken together, these results support the utility of *G. mellonella* as a surrogate host for assessing infections with *K. pneumoniae*.

In 1890, Robert Koch formulated Koch's postulates as general guidelines for identifying disease-causing organisms. One century later, Stanley Falkow established the molecular version of Koch's postulates, this time to guide the identification of microbial genes encoding virulence factors. One of the key points of the molecular postulates is to test the virulence of a microorganism with an inactivated candidate virulence gene in an appropriate animal model. Therefore, the use of animal models to identify the virulence factors of human pathogens is indispensable. Currently, identification and characterization of novel virulence factors rely largely on assessing mutant bacteria for growth in the organs of infected mice. The dependence on mouse infection models limits large-scale analysis of virulence due to the large number of animals needed to obtain statistically significant results.

To circumvent these issues, the search for alternative host models is ongoing. Ideally, these alternative models should be easy to maintain and infect, should be amenable to genetic manipulation, and should model aspects of vertebrate defenses upon infection, chiefly the immune response. The immune defense consists of two main parts, an innate and an adaptive response, with the latter being delayed but highly specific and long lasting. However, because adaptive immune responses have appeared in the ancestors of cartilaginous fish, most alternative host models reflect only features of the interplay between the innate immune system and the pathogen. Although this is certainly a limitation, it should be noted that the innate immune system is responsible for the early clearance of most infections and also shapes adaptive immune responses (1). In any case, these models will be useful only if the pathogens employ the same virulence factors in the infection process regardless of host.

Drosophila melanogaster, *Caenorhabditis elegans*, and the fish *Danio rerio* have been used widely to model host-pathogen interactions. In only a few cases, however, have findings obtained in these models been validated using vertebrate hosts (mice) (2–10). A common caveat of these models is that the optimal temperature for maintaining them is below 28°C, whereas the optimum temperature for most human pathogens is 37°C. Since virulence gene expression is frequently regulated by temperature, it is likely that temperature requirements bias gene expression and affect the pathogen response to these hosts. The larva of the wax moth *Galleria mellonella* is emerging as a reliable model host for studying the pathogenesis of many human pathogens because, among other advantages, *G. mellonella* can grow at 37°C (11–18). However, there is a paucity of information on the extent to which the infection of *G. mellonella* can mimic the interplay between the innate immune system and human pathogens.

The present study was initiated to determine whether *G. mellonella* could be used as a host model to conceptually approximate

Received 27 March 2013 Returned for modification 30 April 2013
Accepted 2 July 2013
Published ahead of print 8 July 2013
Editor: J. B. Bliska
Address correspondence to José A. Bengoechea, bengoechea@caubet-cimera.es.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/IAI.00391-13.

doi:10.1128/IAI.00391-13

TABLE 1 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Genotype or comments[a]	Source or reference(s)
Strains		
Escherichia coli strains		
DH10B	F$^-$ *endA1 recA1 galE15 galK16 nupG rpsL* Δ*lacX74* ϕ80d*lacZ*ΔM15 *araD139* Δ(*ara,leu*)*7697 mcrA* Δ(*mrr-hsdRMS-mcrBC*) λ$^-$	Invitrogen
MG1655	F$^-$ λ$^-$ *ilvG rfb-50 rph-1*	Laboratory collection
Klebsiella aerogenes		Laboratory collection
Klebsiella pneumoniae strains		
MGH78578	Clinical isolate (serotype O?:K52)	Laboratory collection
43816	Clinical isolate (serotype O1:K2)	ATCC
NTUH-K2044	Clinical isolate (serotype O1:K1)	64, 65
52145	Clinical isolate (serotype O1:K2); Rif^r	51, 66
USA1850	Clinical isolate	47, 48
2330	Clinical isolate	47, 48
2073	Clinical isolate	47, 48
43816 Δ*manC*	43816 Δ*manC*; Km^r; the *manC* gene of the *wca* gene cluster was inactivated; no CPS expression	Unpublished data
52145 Δwca_{K2}	52145 Δ*manC*; Rif^r; the *manC* gene of the *wca* gene cluster was inactivated; no CPS expression	44
52145 Δ*pmrF*	52145 Δ*pmrF*; Rif^r; the *pmrF* gene was inactivated	56
52145 Δ*lpxO*	52145 Δ*lpxO*; Rif^r; the *lpxO* gene was inactivated	Unpublished data
52145 Δ*pagP*GB	52145 Δ*pagP*::Km-GenBlock; Rif^r Km^r; the *pagP* gene was inactivated	56
52145 Δ*pagP*GB Δ*pmrF*	52145 Δ*pmrF* Δ*pagP*::Km-GenBlock; Rif^r Km^r; the *pagP* and *pmrF* genes were inactivated	36
52OmpA2	52145 Δ*ompA*; Rif^r Cm^r; the *ompA* gene was inactivated	56
52OmpK36	52145 Δ*ompK36*; Rif^r Cm^r; the *ompK36* gene was inactivated	56
52145 Δ*phoQ*GB	52145 Δ*phoQ*::Km-GenBlock; Rif^r Km^r; the *phoQ* gene was inactivated	56
52145 Δ*pmrAB*	52145 Δ*pmrAB*; Rif^r; the *pmrAB* genes were inactivated	56
52145 Δ*pmrAB* Δ*phoQ*GB	52145 Δ*pmrAB* Δ*phoQ*::Km-GenBlock; Rif^r Km^r; the *phoQ* and *pmrAB* genes were inactivated	56
52145 Δ*rcsB*	52145 Δ*rcsB*; Rif^r; the *rcsB* gene was inactivated	56
Plasmids		
pGPL01	Firefly luciferase transcriptional fusion suicide vector; Amp^r	46
pGPLKpnCps	pGPL01 containing *cps* promoter region; Amp^r	56
pGPLKpnPmrH	pGPL01 containing *pmrH* promoter region; Amp^r	56
pGPLKpnPagP	pGPL01 containing *pagP* promoter region; Amp^r	56
pGPLKpnLpxO	pGPL01 containing *lpxO* promoter region; Amp^r	Unpublished data
pGPLKpnPhoP	pGPL01 containing *phoP* promoter region; Amp^r	56
pGPLKpnYojN	pGPL01 containing *yojN* promoter region; Amp^r	56
pGPLKpnRcsC	pGPL01 containing *rcsC* promoter region; Amp^r	56
pGPLKpnPmrC	pGPL01 containing *pmrC* promoter region; Amp^r	This study
pFPV 25.1	*rpsM*::*gfpmut3*; Amp^r	41

[a] Cm^r, chloramphenicol resistance; Rif^r, rifampin resistance; Km^r, kanamycin resistance; Amp^r, ampicillin resistance. The wca_{K2} gene is *manC*.

a human disease, *Klebsiella pneumoniae*-triggered pneumonia. *K. pneumoniae* is an important cause of community-acquired pneumonia in individuals with impaired pulmonary defenses and is a major pathogen in nosocomial pneumonia (19, 20). Results obtained using the mouse model of *Klebsiella* pneumonia highlight the fact that the clearance of the pathogen is primarily dependent upon a vigorous innate immune response (21–28). Furthermore, stimulation of this response enhances bacterial clearance and prolongs animal survival (29–32). Conversely, this suggests that *K. pneumoniae* tries to counteract the induction of these host defense responses. Indeed, we and others have shown that, in sharp contrast to wild-type strains, attenuated mutant strains activate an inflammatory program, ultimately favoring their clearance (33–37).

Here we present data indicating that the *G. mellonella* model replicates features of *K. pneumoniae* infection biology, including the protection induced by stimulation of host immunity. We also report that the *G. mellonella* model is useful for assessing the pathogenic potential of *K. pneumoniae*, as we observed a strong correlation with the virulence previously determined in the mouse pneumonia model of infection.

MATERIALS AND METHODS

Ethics statement. Mice were treated in accordance with the Directive of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes (directive 2010/63/EU) and in agreement with the Bioethical Committee of the University of the Balearic Islands. This study was approved by the Bioethical Committee of the University of the Balearic Islands, under authorization number 1748.

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown in lysogeny broth (LB) at 37°C on an orbital shaker (180 rpm). When appropriate, antibiotics were added to the growth medium at the following concentrations: rifampin (Rif), 50 μg/ml; ampicillin (Amp), 100 μg/ml; and kanamycin (Km), 100 μg/ml.

***G. mellonella* larvae.** *G. mellonella* larvae were acquired from Alcotán Valencia SLU and kept at 21°C in darkness with a nonrestricted diet. It has been reported that food deprivation of *G. mellonella* larvae leads to reductions in cellular and immune responses (38). Larvae were used within 12

days of receipt. Larvae of approximately 250 to 350 mg were selected for the experiments.

Infection of *G. mellonella* larvae. Bacteria were grown in 5 ml LB, harvested during exponential phase (2,500 × *g*, 20 min, 24°C), and washed once with 10 mM phosphate-buffered saline (PBS; pH 6.5). Bacteria were diluted in PBS to an optical density at 600 nm (OD_{600}) of 1, which corresponds to approximately 1×10^9 CFU/ml. After surface disinfection using ethanol (70% [vol/vol]), larvae were injected with 10 μl of bacterial suspension, containing approximately 1×10^8 CFU/ml, into the last right proleg by use of a Hamilton syringe with a 30-gauge needle. A group of 10 larvae were injected with 10 μl of PBS in parallel to ensure that death was not due to injection trauma. Larvae were placed in 9.2-cm petri dishes with food and kept at 37°C in the dark. Insects were considered dead when they did not respond to physical stimuli. Larvae were examined for pigmentation, and time of death was recorded. Assays were allowed to proceed for only 3 days, as pupa formation could occasionally be seen by day 4. At least three independent experiments were performed.

For 50% lethal dose (LD_{50}) experiments, a series of 10-fold serial dilutions containing 10^4 to 10^7 CFU in PBS were injected into *G. mellonella* larvae. Ten larvae were injected at each dilution. For each strain, data from three independent experiments were combined, and LD_{50}s were calculated according to the formula of Reed and Muench (39). Results are expressed as log LD_{50}.

Determination of *in vivo* bacterial loads. Insects were infected with approximately 1×10^6 CFU per larva of *K. pneumoniae* 52145 or 52145 Δwca_{K2}. Groups of three insects were collected at different postinfection time points (5, 10, and 24 h), and their surfaces were disinfected with ethanol. The three larvae were homogenized in 2 ml of PBS by use of an Ultra-Turrax T10 Basic homogenizer (IKA). Serial dilutions of the homogenate in PBS were plated on LB agar supplemented with rifampin, and colonies were counted after incubation at 37°C for 24 h. At least three independent experiments were performed. No CFU were recovered from noninfected larvae in LB agar supplemented with rifampin.

PO activity assay. Insects were infected with approximately 1×10^6 CFU of *K. pneumoniae* 52145 or 52145 Δwca_{K2} per larva. At different time points postinfection (5, 10, and 24 h), insects were surface sterilized with ethanol, immobilized in a sterile petri dish, and dissected at the last metamere. From each larva, 15 μl of the outflowing hemolymph was immediately collected in a sterile ice-cold microcentrifuge tube containing 10 μl of a saturated solution of *N*-phenylthiourea (Sigma) in distilled water to prevent melanization. Hemolymph samples from three larvae were pooled. Cells and debris were removed by centrifugation at 4,000 × *g* for 10 min at 4°C, and supernatants were diluted at a 3:1 (vol/vol) ratio with 50 mM PBS (pH 6.5). The phenoloxidase (PO) activity in the plasma was quantified using a microplate enzyme assay as described previously (40). Briefly, a reaction mixture containing 115 μl 50 mM PBS (pH 6.5), 10 μl diluted hemolymph plasma, and 2 μl of *Escherichia coli* lipopolysaccharide (LPS) (5 mg/ml; Sigma) was left for 1 h at room temperature to allow the activation of the enzyme. Twenty-five microliters of 20 mM 4-methyl catechol (Sigma) was then added to initiate the reaction, and the final volume was made up to 200 μl with sterile distilled water. Changes in absorbance at 490 nm were monitored at room temperature, with a reading taken every 5 min for 1 h, using a PowerWave HT microplate spectrophotometer (Biotek). The experiment was performed in triplicate and independently repeated at least three times. Phenoloxidase activity is expressed as the mean OD_{490}/min.

Hemocyte quantification and viability staining. Larvae were infected with 1×10^6 CFU of *K. pneumoniae* 52145 or 52145 Δwca_{K2}, and hemolymph was collected at 5, 12, and 24 h postinfection. Hemolymph samples from three larvae were pooled in a microcentrifuge tube containing 10 μl of a saturated solution of *N*-phenylthiourea (Sigma) in distilled water, and hemocytes were recovered by centrifugation (1,500 × *g* for 3 min). Hemocytes were resuspended in 50 μl of trypan blue (0.02% [vol/vol] in PBS). Samples were incubated at room temperature for 10 min, and viable hemocytes were enumerated using a Neubauer hemocytometer. Each sample was analyzed in triplicate. The averages for three independent experiments were plotted graphically.

Immunofluorescence assay with extracted hemocytes. Insects were infected with approximately 1×10^6 CFU per larva of *K. pneumoniae* 52145 or 52145 Δwca_{K2} carrying pFPV25.1. This plasmid expresses *gfp-mut3* under the control of the *Salmonella rpsM* promoter. This fusion has been reported to be expressed at similar levels in various environments, including growth media and mammalian cells (41, 42). At different time points postinfection (5, 12, and 24 h), the hemolymph samples from three infected larvae were collected, pooled in a microcentrifuge tube containing 10 μl of a saturated solution of *N*-phenylthiourea (Sigma) in distilled water, and diluted with 150 μl of PBS. Samples were seeded on poly-L-lysine-coated 12-mm circular coverslips in 24-well tissue culture plates. Plates were centrifuged at 200 × *g* for 5 min and incubated at 37°C for 2 h to allow attachment of hemocytes. Coverslips were washed twice with PBS, fixed with 400 μl of 4% paraformaldehyde for 20 min at room temperature, washed three times with PBS, and quenched with 14 mM ammonium chloride. Extracellular bacteria were stained with rabbit anti-*K. pneumoniae* polyclonal serum (1:5,000) followed by donkey anti-rabbit rhodamine red X-conjugated antibody (1:200) (Jackson ImmunoResearch Laboratories, Inc.). After permeabilization of the cells with 0.1% saponin in PBS, DNA was stained with Hoechst dye (1:2,500). Coverslips were mounted on Aqua Poly/Mount medium (Polysciences), and immunofluorescence was analyzed with a Leica CTR6000 fluorescence microscope.

Radial diffusion bioassay. To detect the activity of antimicrobial factors in the hemolymph of infected larvae, we used a previously described bioassay of growth inhibition zones (43, 44), with minor modifications. Briefly, an underlay gel that contained 1% (wt/vol) agarose of low electroendosmosis (agarose D1 low EEO; Pronadisa), 2 mM HEPES (pH 7.2), and 0.3 mg tryptone soy broth (TSB; Oxoid) powder per ml was equilibrated at 50°C and then inoculated with *E. coli* DH10B to a final concentration of 5×10^5 CFU per ml of molten gel. This gel was poured into standard square petri dishes (10 × 10 × 1.5 cm), and after solidification, small wells with a 15-μl capacity were carved out. Hemolymph samples from three larvae infected with 1×10^6 CFU of *Klebsiella* or heat-killed *E. coli* MG1655 were collected and pooled in an ice-cold microcentrifuge tube containing 10 μl of a saturated solution of *N*-phenylthiourea (Sigma). Wells were filled with 10-μl aliquots of these samples, and the agar plates were incubated for 3 h at 37°C. After that, a 30-ml overlay gel composed of 1% agarose and 6% TSB powder in water was poured on top of the previous gel, and the plates were incubated at 37°C. After 18 h, the diameters of the inhibition halos were measured to the nearest 1 mm and, after subtraction of the diameter of the well, were expressed in inhibition units (10 units = 1 mm). PBS and 32 μg/ml polymyxin B (Sigma) were used as negative and positive controls, respectively, in the bioassay. All measurements were done in duplicate on at least five separate occasions.

Antimicrobial peptide resistance assay. Insects were infected with approximately 1×10^6 heat-killed (65°C for 15 min) *E. coli* MG1655 cells per larva to increase the levels of antimicrobial factors in the hemolymph (45). After 24 h, hemolymph samples from three larvae were collected and pooled in an ice-cold microcentrifuge tube containing 10 μl of a saturated solution of *N*-phenylthiourea (Sigma). Fifty microliters of PBS was added to each mixture, and then 25 μl of diluted sample was mixed with 5 μl of a *Klebsiella* suspension containing 10^5 CFU per ml, prepared in 10 mM PBS (pH 6.5), 1% TSB, and 100 mM NaCl. The mixtures were incubated at 37°C for 3 h. Serial dilutions of the samples in PBS were plated on LB agar supplemented with rifampin, and colonies were counted after incubation at 37°C for 24 h. Results are expressed as percentages of the number of bacteria not exposed to antibacterial agents. All measurements were done in triplicate on three separate occasions.

RNA extraction and RT-PCR. Larvae were infected with approximately 1×10^6 CFU, and after 8 h, individual insects were homogenized on ice with 1 ml of Tri reagent (Ambion), using an Ultra-Turrax T10 Basic homogenizer (IKA). Total RNA was purified first by using a standard

chloroform-isopropyl alcohol protocol, and the obtained RNA was further purified using a Nucleospin RNAII kit (Macherey-Nagel) that included one step of on-column DNase treatment, following the manufacturer's instructions. cDNA was obtained by retrotranscription of 1.5 to 2 μg total RNA by use of a commercial Moloney murine leukemia virus (M-MLV) reverse transcriptase (Sigma). A mix (1:1) of an oligo(dT)$_{18}$ primer and random hexamer primers (Thermo Scientific) was used. Real-time PCR (RT-PCR) analyses were performed with a Smart Cycler real-time PCR instrument (Cepheid, Sunnyvale, CA).

Fifty nanograms of cDNA was used as the template in a 25-μl reaction mixture containing KapaSYBR Fast qPCR mix (Kapa Biosystems) and primer mix. Actin and 18S rRNA genes were amplified as housekeeping genes. The primers used are listed in Table S1 in the supplemental material. The thermocycling protocol was as follows: 95°C for 3 min for hot-start polymerase activation, followed by 45 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 30 s. SYBR green dye fluorescence was measured at 521 nm during the annealing phase. Relative quantities of mRNAs were obtained using the comparative threshold cycle ($\Delta\Delta C_T$) method, with normalization to the 18S rRNA and actin genes.

Construction of *pmrC*::*lucFF* reporter fusion. A 445-bp DNA fragment containing the promoter region of the *pmrCAB* operon was amplified by PCR using Vent polymerase, digested with EcoRI, gel purified, and cloned into EcoRI-SmaI-digested pGPL01 suicide vector (46). This vector contains a promoterless firefly luciferase gene (*lucFF*) and an R6K origin of replication. A plasmid in which *lucFF* was under the control of the *pmrCAB* promoter region was identified by restriction digestion analysis and named pGPLKpnPmrC. This plasmid was introduced into *E. coli* SY327-λ*pir*, from which it was mobilized into *K. pneumoniae* 52145 by triparental conjugation using the helper strain *E. coli* HB101/pRK2013. One strain in which the suicide vector was integrated into the genome by homologous recombination was selected. This was confirmed by Southern blotting and a PCR using primers CheckProkpnpmrCF and ChecklucFFR (data not shown).

***In vivo* monitoring of gene expression during *G. mellonella* infection.** Insects were infected with approximately 1×10^6 CFU *K. pneumoniae* carrying a *lucFF*-based transcriptional fusion per larva (Table 1). At the indicated time points, hemolymph samples from individual larvae (approximately 20 μl) were collected in microcentrifuge tubes containing 10 μl of a saturated solution of *N*-phenylthiourea (Sigma) and kept at room temperature. Five larvae were infected per time point. Ten microliters of sample was serially diluted in PBS and then plated on LB agar with rifampin to determine the number of CFU present in the hemolymph. Twenty microliters of mixture was diluted with 100 μl of PBS and mixed with 100 μl of luciferase assay reagent (1 mM D-luciferin [Synchem] in 100 mM citrate buffer, pH 5), and luminescence was immediately measured with a model LB9507 luminometer (Berthold) and expressed as relative light units (RLU). Control experiments showed that hemolymph did not quench luminescence. The luminescence levels of *lucFF*-expressing *Klebsiella* cells from culture did not change after mixing the bacteria with hemolymph extracted from infected larvae (at 18 h postinfection). Luminescence was also determined from the inoculum by mixing a 100-μl aliquot of the bacterial suspension with 100 μl of luciferase assay reagent.

Intranasal infection model. Five- to 7-week-old female C57BL/6JOlaHsd mice (Harlan) were anesthetized by intraperitoneal (i.p.) injection with a mixture containing ketamine (50 mg/kg of body weight) and xylazine (5 mg/kg). Overnight bacterial cultures were centrifuged (2,500 × *g*, 20 min, 22°C), resuspended in PBS, and adjusted to 5×10^4 CFU/ml for determination of bacterial loads. Twenty microliters of the bacterial suspension was inoculated intranasally in four 5-μl aliquots. Noninfected mice were inoculated intranasally with 20 μl of PBS in four 5-μl aliquots. To facilitate consistent inoculations, mice were held vertically during inoculation and placed on a 45° incline while recovering from anesthesia. At the indicated times after infection, mice were euthanized by cervical dislocation, and lungs were rapidly dissected for bacterial load determination.

Dissected lungs were homogenized on ice in 500 μl of PBS by use of an Ultra-Turrax TIO Basic homogenizer (IKA). Bacteria from the homogenates and serial dilutions thereof were recovered on LB agar plates containing rifampin. Results are reported as log CFU per gram of tissue.

Statistical methods. Statistical analyses were performed using one-way analysis of variance (ANOVA) with Bonferroni contrasts or the one-tailed *t* test or, when the requirements were not met, by the Mann-Whitney U test. *P* values of <0.05 were considered statistically significant. The analyses were performed using Prism4 for PC (GraphPad Software).

RESULTS

***K. pneumoniae* infection causes death of *G. mellonella* larvae.** We examined the susceptibility of *G. mellonella* to *K. pneumoniae* strain 52145 (O1:K2 serotype) (Table 1), which is used in molecular pathogenesis studies. Larvae were injected with 10^6 CFU, and their survival was monitored (Fig. 1A). *K. pneumoniae* 52145 caused a time-dependent death of all larvae; at 24 h postinfection, 75% of the larvae were dead, and the remaining survivors were dead at 72 h postinfection. No mortality was observed in the PBS-injected *G. mellonella* larvae (Fig. 1A). Three additional *K. pneumoniae* strains were tested (Table 1). Strains ATCC 43816 (serotype O1:K2) and NTUH-K2044 (serotype O1:K1) are also commonly used for *in vivo* and *in vitro* infection biology studies. Larvae were injected with 10^6 CFU of these strains. While both strains killed *G. mellonella*, strain 43816 killed 95% of the larvae after 24 h (Fig. 1B). Finally, we infected *G. mellonella* with 10^6 CFU of strain MGH78578, which is a multidrug-resistant isolate (serotype K52) (Table 1). MGH78578 also killed 100% of infected larvae at 72 h postinfection (Fig. 1B).

To determine whether *K. pneumoniae*-induced lethality was dependent on the number of injected bacteria, larvae were injected with 10^4, 10^5, 10^6, or 10^7 CFU, and mortality was recorded for up to 72 h postinfection (Fig. 1C). While infection with 10^7 CFU of all strains resulted in 100% *G. mellonella* mortality within 24 h of infection, no mortality was observed when *G. mellonella* larvae were infected with 10^4 CFU of *K. pneumoniae* strain 52145, 43816, or MGH78578 (Fig. 1C). NTUH-K2044 killed 25% of the larvae at this dose (Fig. 1C).

We next determined the LD$_{50}$s of the four strains. As shown in Table 2, NTUH-K2044 had the lowest LD$_{50}$, followed by strain 43816 and then strains 52145 and MGH78578. The LD$_{50}$s of *K. pneumoniae* 52145 and MGH78578 were not significantly different. Notably, three *K. pneumoniae* strains with reduced virulence in mammalian models (47, 48) had LD$_{50}$s significantly higher than those of *K. pneumoniae* 52145 and MGH78578 ($P < 0.05$ by the one-sample *t* test) (Table 2). Moreover, a nonpathogenic laboratory-adapted strain of *Klebsiella* had one of the highest LD$_{50}$s. This strain is used as a control strain in infections of nonmammalian hosts (49).

Collectively, these data show that *G. mellonella* is susceptible to *K. pneumoniae* infection, in a dose-dependent manner.

***K. pneumoniae* CPS is important for *G. mellonella* infection.** The capsule polysaccharide (CPS) is a well-characterized virulence factor of *K. pneumoniae*. CPS mutant strains are avirulent, as they are unable to cause pneumonia or urinary tract infections (50–52). We sought to determine the contribution of CPS to *K. pneumoniae* pathogenesis in *G. mellonella*. Infection of larvae with 10^6 CFU of strain 52145 Δ*wca*$_{K2}$, the isogenic *cps* mutant of *K. pneumoniae* 52145 (Table 1), did not cause any mortality of the

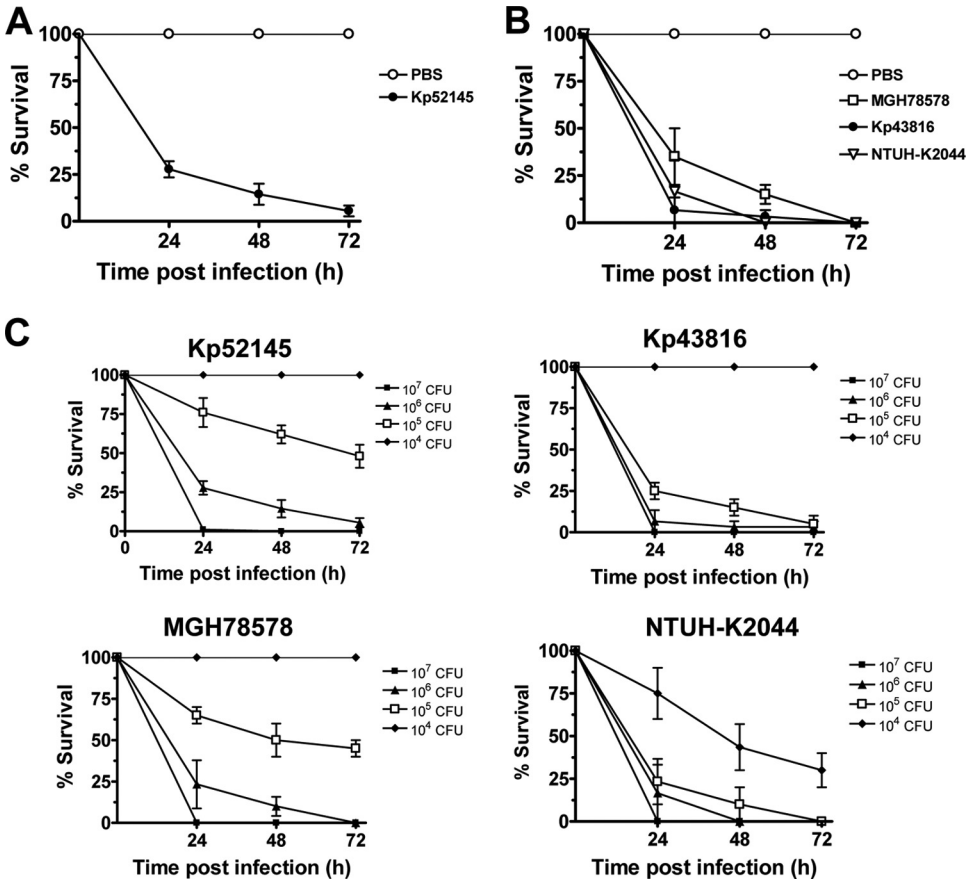

FIG 1 *K. pneumoniae* infection of *G. mellonella* induces dose-dependent lethality. (A) Larvae were injected with PBS or with 10^6 CFU of *K. pneumoniae* 52145 (Kp52145), and survival was monitored over 72 h postinfection. (B) Larvae were injected with PBS or with 10^6 CFU of *K. pneumoniae* 43816 (Kp43816), NTUH-K2044, or MGH78578, and survival was monitored over 72 h postinfection. (C) Mortality of larvae infected with *K. pneumoniae* 52145, 43816, NTUH-K2044, or MGH78578 was dose dependent.

larvae over the 72 h of the experiment, whereas the wild-type strain killed all larvae within this time (Fig. 2A). An inoculum of 10^6 CFU of *K. pneumoniae* 48136 killed 100% of *G. mellonella* larvae within 24 h. In contrast, the isogenic *cps* mutant, strain 43816 Δ*manC*, killed 50% of *G. mellonella* larvae within this time (Fig. 2A). The LD_{50}s of the *cps* mutants, i.e., strains 52145 Δwca_{K2} and 43816 Δ*manC* (6.97 ± 0.24 and 5.67 ± 0.03, respectively), were significantly higher than those of the isogenic wild-type strains, *K. pneumoniae* 52145 and 43816 (4.94 ± 0.11 and 4.49 ±

TABLE 2 LD_{50}s of *K. pneumoniae* strains in *G. mellonella* at 72 postinfection

Strain	Log LD_{50}
K. pneumoniae strains	
MGH78578	4.82 ± 0.16
43816	4.49 ± 0.03
NTUH-K2044	4.14 ± 0.26
52145	4.94 ± 0.11
USA1850	5.55 ± 0.05
2330	6.56 ± 0.06
2073	5.55 ± 0.17
K. aerogenes	6.00 ± 0.01

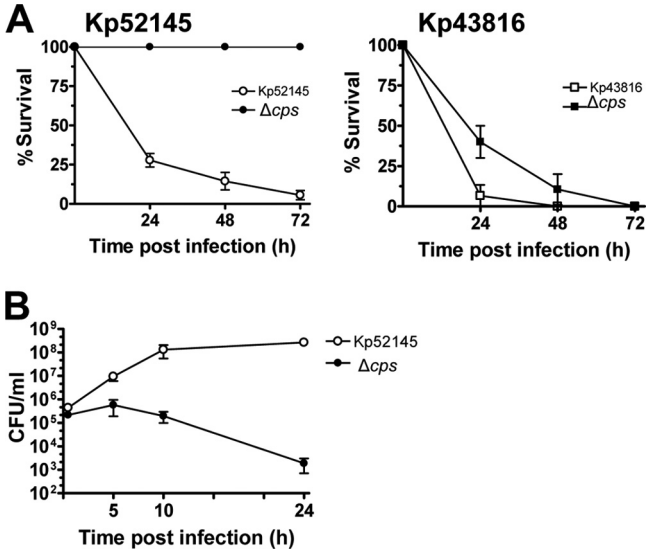

FIG 2 CPS is important for *K. pneumoniae*-induced *G. mellonella* lethality. (A) Larvae were injected with 10^6 CFU of *K. pneumoniae* 52145 or 43816 or the *cps* mutant 52145 Δwca_{K2} or 43816 Δ*manC*, and survival was monitored over 72 h postinfection. (B) Three *Klebsiella*-infected larvae were pooled and homogenized, and numbers of CFU were determined by plating.

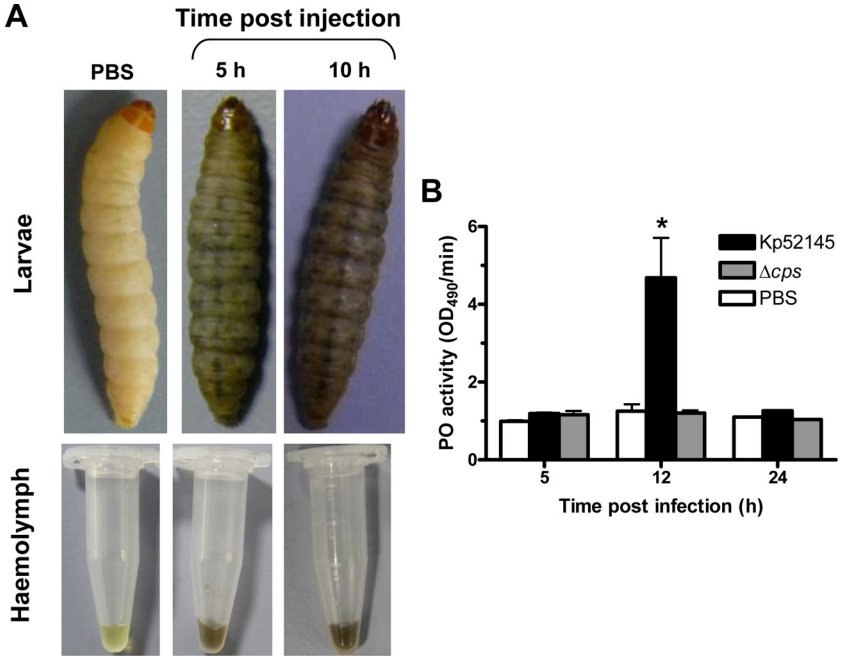

FIG 3 Characterization of *G. mellonella* innate responses to *K. pneumoniae* infection. (A) Larvae and extracted hemolymph became pigmented progressively darker over the course of the infection, indicative of melanin production by PO. (B) PO activity in the plasmas of insects injected with PBS or infected with *K. pneumoniae* 52145 or the *cps* mutant 52145 Δwca_{K2} (Δcps) was quantified at 5, 12, and 24 h postinfection. Results represent the means and standard deviations for three independent experiments. *, $P < 0.05$ (results are significantly different from the results for PBS-injected larvae by the one-tailed *t* test).

0.03, respectively) ($P < 0.05$ for comparison with the corresponding wild type by the one-sample *t* test). The LD_{50} of strain 43816 Δ*manC* was significantly lower than that of 52145 Δwca_{K2} ($P < 0.05$ by the one-sample *t* test). Altogether, these findings indicated that *K. pneumoniae*-induced mortality is dependent on the presence of CPS.

We sought to determine whether *G. mellonella* mortality is associated with the growth of *Klebsiella* in infected larvae. Larvae were infected with 10^6 CFU of *K. pneumoniae* 52145 or the *cps* mutant strain 52145 Δwca_{K2}. At selected time points, three larvae were pooled and homogenized, and the number of CFU per ml was determined. As shown in Fig. 2B, infection of *G. mellonella* with the *cps* mutant resulted in an initial 3-fold increase of CFU at 5 h postinfection. However, this was followed by a decrease in bacterial numbers and 99% clearance of the inoculum from the larvae at 24 h postinfection. In contrast, infection of *G. mellonella* with *K. pneumoniae* 52145 resulted in an increase in bacterial numbers over time (Fig. 2B), hence demonstrating that wild-type *K. pneumoniae* replicates in *G. mellonella*.

Interplay between *G. mellonella* innate immune system and *K. pneumoniae*. The progress of infection of *G. mellonella* by strains causing death of larvae, such as *K. pneumoniae* 52145, was accompanied by an increase in pigmentation (Fig. 3A). This is indicative of PO activity in the hemolymph (40, 53). The activity of this enzyme results in the deposition of melanin around invading bacteria by the insect hemocytes, which wrap around the bacteria to form nodules, thereby isolating the pathogens (53). To evaluate this immune response, we determined the levels of PO activity in the hemolymph of larvae infected with *K. pneumoniae* 52145 (Fig. 3B). The levels of PO in PBS-infected insects remained constant (Fig. 3B). In contrast, PO activity in insects infected with *K. pneumoniae* 52145 peaked at 12 h postinfection, whereas at 5 and 24 h postinfection, PO levels in larvae infected with *K. pneumoniae* 52145 were not significantly different from those in PBS-challenged insects (Fig. 3B). Interestingly, larvae infected with the *cps* mutant presented levels of PO similar to those in PBS-injected insects at all time points analyzed (Fig. 3B).

G. mellonella hemolymph contains hemocytes, which function in a manner similar to that of phagocytes of mammals (45). To further analyze the pathogen-host interaction in the insect model, we followed the interaction between insect hemocytes and *K. pneumoniae* wild-type and *cps* mutant strains throughout the course of infection. To assess whether *G. mellonella* hemocytes are able to engulf *K. pneumoniae*, hemocytes were isolated from the hemolymph of larvae infected with green fluorescent protein (GFP)-expressing *K. pneumoniae* 52145 at 5, 12, and 24 h postinfection and differentially immunostained to detect intra- and extracellular bacteria (Fig. 4). We were unable to detect intracellular bacteria at 5, 12 (Fig. 4A), or 24 h postinfection (data not shown). In contrast, at 5 h postinfection, the GFP-expressing *cps* mutant was found inside and associated with hemocytes (Fig. 4B), and by 12 h postinfection, the mutant was observed only inside hemocytes (Fig. 4B). At 24 h postinfection, we could not detect any bacteria in the samples (data not shown). Altogether, these findings indicate that *G. mellonella* hemocytes do not engulf wild-type *K. pneumoniae*.

Examination of the preparations from larvae infected with *K. pneumoniae* 52145 by immunofluorescence indicated a decrease in the overall number of hemocyte nuclei which was more evident at 24 h postinfection (data not shown). This observation prompted us to study whether *K. pneumoniae* 52145 may trigger hemocyte depletion. Insects were infected with *K. pneumoniae* 52145 or injected with PBS, and hemocytes purified from the hemolymph were counted by light microscopy at 5, 12, and 24 h

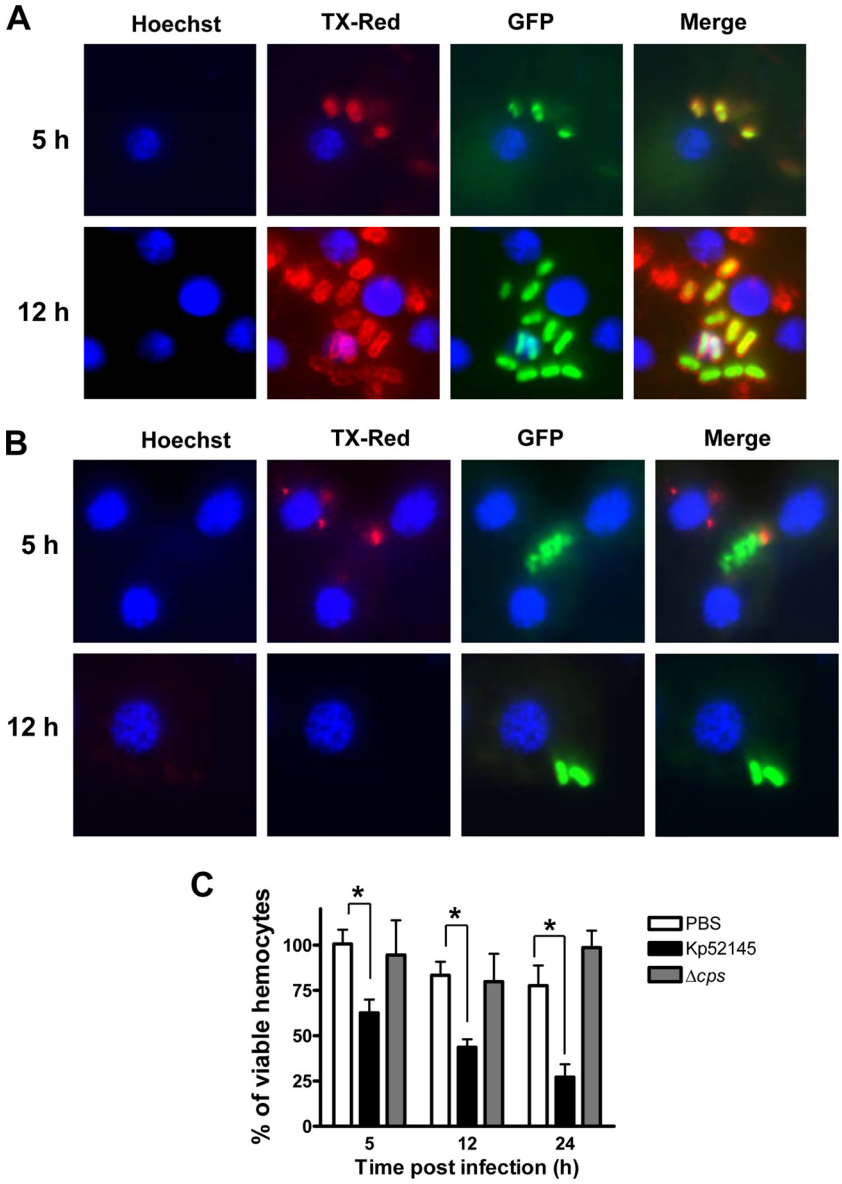

FIG 4 Wild-type *K. pneumoniae* is not engulfed by *G. mellonella* hemocytes. (A) *K. pneumoniae* 52145 expressing GFP is not engulfed by hemocytes and replicates extracellularly. External bacteria were immunostained red (TX-Red). (B) Strain 52145 Δwca_{K2} expressing GFP is engulfed by hemocytes. External bacteria were immunostained red. (C) Viable hemocyte concentrations were recorded at 5, 12, and 24 h postinfection. Larvae were injected with PBS or infected with *K. pneumoniae* 52145 or the *cps* mutant 52145 Δwca_{K2} (Δ*cps*). Results (means and standard deviations for three independent experiments) are expressed as percentages of the number of hemocytes recorded for PBS-injected larvae at 5 h postinfection. *, $P < 0.05$ (results are significantly different for the indicated comparisons by the one-tailed *t* test).

postinfection (Fig. 4C). The number of hemocytes decreased over time in larvae infected with *K. pneumoniae* 52145, hence suggesting that wild-type *K. pneumoniae* does indeed induce cell destruction (Fig. 4C). In contrast, the number of hemocytes was not significantly different between the *cps* mutant-infected and PBS-injected groups at all time points (Fig. 4C).

The innate immune cellular response in insects is accompanied by secretion of antimicrobial peptides in response to either injury or invasion by a pathogen (45). As anticipated, the hemolymph of larvae infected with a heat-killed laboratory-adapted *E. coli* strain contained antimicrobial factors which could be detected by a radial diffusion bioassay (Fig. 5A). This bioassay is typically used to evaluate the presence and action of antimicrobial peptides in biological samples (43). The levels of antimicrobial factors in the hemolymph of *E. coli*-infected larvae were higher at 24 than at 5 h postinfection (Fig. 5A). We tested the susceptibility of *K. pneumoniae* to these *E. coli*-elicited antimicrobial factors. Indeed, survival assays showed that *K. pneumoniae* 52145 and the *cps* mutant were susceptible to the antimicrobial factors present in the hemolymph of *G. mellonella* larvae infected with heat-killed *E. coli* for 24 h (53% ± 12% and 39% ± 12% survival, respectively; $P > 0.05$ by the one-sample *t* test).

The radial diffusion bioassay was used to determine whether *K. pneumoniae* infection triggers the production of antimicrobial

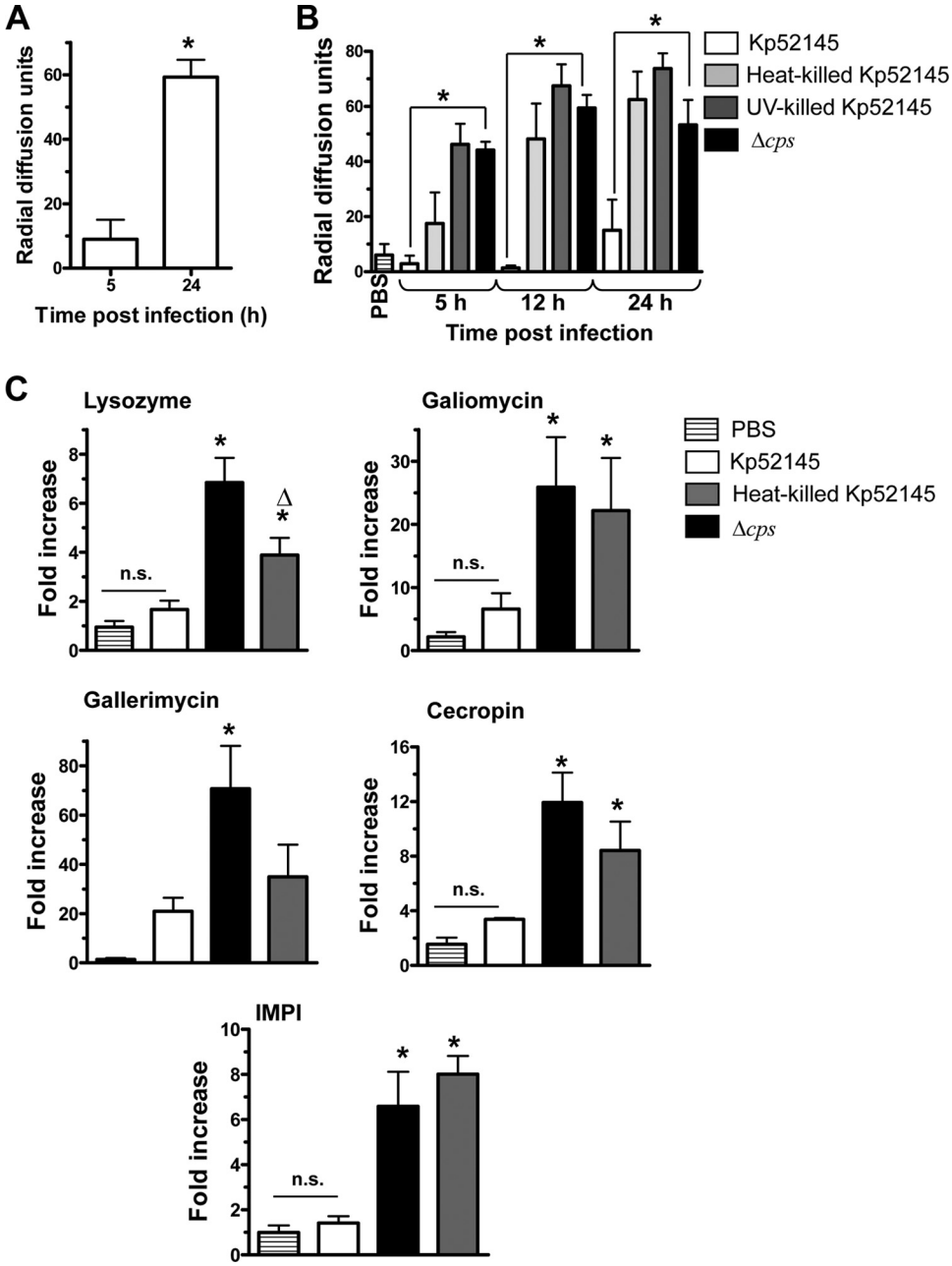

FIG 5 Wild-type *K. pneumoniae* does not upregulate the expression of *G. mellonella* antimicrobial peptides. (A) Larvae were infected with 10^6 heat-killed (65°C, 15 min) *E. coli* MG1655 cells, and at 5 and 24 h postinfection, hemolymph was extracted and the presence of antimicrobial factors assessed by radial diffusion bioassay. The results, expressed as radial diffusion units (10 units = 1 mm), represent means and standard deviations for at least four independent determinations. *, $P < 0.05$ (results are significantly different from the 5-h postinfection results by the one-tailed *t* test). (B) Presence of antimicrobial factors in the hemolymph of larvae at different time points postinfection, determined by radial diffusion bioassay. Larvae were injected with PBS or infected with *K. pneumoniae* 52415, heat-killed (65°C, 15 min) *K. pneumoniae* 52145, UV-killed (UV irradiated at 1 J for 10 min in a Bio-Link BLX cross-linker [Vilber Lourmat]) *K. pneumoniae* 52145, or the *cps* mutant strain 52145 Δwca_{K2} (Δcps). The results, expressed as radial diffusion units (10 units = 1 mm), represent means and standard deviations for five independent determinations. *, $P < 0.05$ (results are significantly different for the indicated comparisons by the one-tailed *t* test). (C) Transcriptional activation of immune-responsive genes following infection. The transcription levels of lysozyme, galiomycin, gallerimycin, cecropin, and IMPI were determined by RT-qPCR and are shown relative to the expression levels in PBS-injected animals. Larvae (five per group) were injected with PBS or infected with *K. pneumoniae* 52415, heat-killed (65°C, 15 min) *K. pneumoniae* 52145, or the *cps* mutant 52145 Δwca_{K2} (Δcps), and after 8 h, total RNA was purified. Results represent means and standard deviations. *, $P < 0.05$ (results are significantly different from the results for *K. pneumoniae* 52145-injected larvae by one-way ANOVA); △, results are significantly different from the results for 52145 Δwca_{K2}-injected larvae by one-way ANOVA; n.s., $P > 0.05$ for the indicated comparison.

factors. As shown in Fig. 5B, the levels of antimicrobials in the hemolymph of larvae infected with *K. pneumoniae* 52145 were similar to those found in PBS-infected larvae. Moreover, these levels were not significantly different over time ($P > 0.05$ for any comparison between hemolymph from *K. pneumoniae* 52145-infected larvae by one-way ANOVA). The lack of induction of antimicrobial factors by *K. pneumoniae* 52145 was dependent on the expression of CPS, since hemolymph from insects infected with

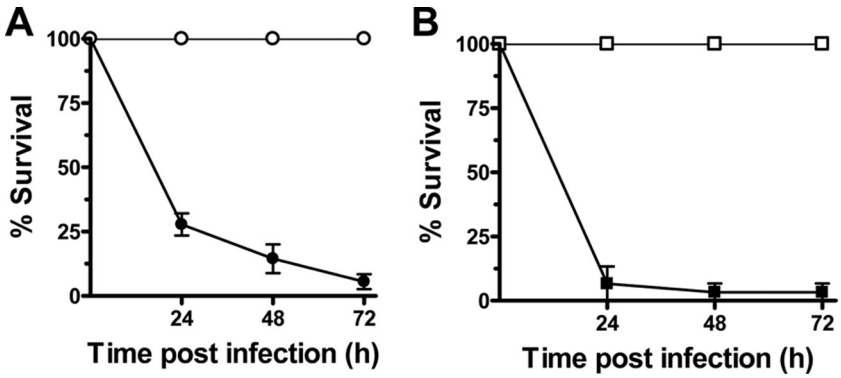

FIG 6 Effects of *G. mellonella* preimmune activation on subsequent infection with *K. pneumoniae*. Larvae were infected with 10^6 heat-killed (65°C, 15 min) *E. coli* MG1655 cells and, after 24 h, infected with 10 times the LD_{50} of *K. pneumoniae* 52145 (A) or *K. pneumoniae* 43816 (B). Survival was monitored over 72 h postinfection. ○, preimmunized larvae infected with *K. pneumoniae* 52145; ●, nonpreimmunized larvae infected with *K. pneumoniae* 52145; □, preimmunized larvae infected with *K. pneumoniae* 43816; ■, nonpreimmunized larvae infected with *K. pneumoniae* 43816. Twenty larvae were infected in each experimental group.

the *cps* mutant presented significantly higher levels of antimicrobial factors than those obtained from *K. pneumoniae* 52145-infected larvae (Fig. 5B). This was true at all time points analyzed postinfection (Fig. 5B). However, the mere presence of CPS was not enough to prevent the induction of antimicrobial factors, because the levels of factors found in the hemolymph of heat-killed *K. pneumoniae* 52145- or UV-killed *K. pneumoniae* 52145-challenged larvae were not significantly different from those obtained from insects infected with the *cps* mutant (Fig. 5B).

We sought to determine whether there is a correlation between the levels of antimicrobial effectors found in the hemolymph and the expression of antimicrobial peptides. Indeed, analysis by real-time quantitative PCR (RT-qPCR) showed that the levels of lysozyme, galiomycin, gallerimycin, cecropin, and insect metalloproteinase inhibitor (IMPI) were higher in insects infected with the *cps* mutant than in those infected with *K. pneumoniae* 52145 (Fig. 5C). Only the levels of gallerimycin were significantly different between *G. mellonella* larvae infected with *K. pneumoniae* 52145 and larvae mock infected with PBS (Fig. 5C). Notably, levels of lysozyme, galiomycin, cecropin, and IMPI were also higher in insects challenged with heat-killed *K. pneumoniae* 52145 than in larvae infected with *K. pneumoniae* 52145 (Fig. 5C). Except for lysozyme, no significant differences were found between the levels of the peptides induced by the *cps* mutant and heat-killed *K. pneumoniae* 52145 (Fig. 5C). Collectively, these findings support the notion that wild-type *K. pneumoniae* does not induce the expression of antimicrobial peptides.

Activation of immunity in *G. mellonella* enhances the host defense against *K. pneumoniae* infection. The fact that *K. pneumoniae* 52145 was susceptible to the antimicrobial factors present in the hemolymph of *E. coli*-infected insects led us to analyze whether prior induction of immune responses in *G. mellonella* would protect against a subsequent infection by *K. pneumoniae*. Larvae were inoculated with 10^6 heat-killed *E. coli* cells and, after 24 h, challenged with 10 times the LD_{50} of *K. pneumoniae* 52145. *E. coli*-mediated induction of immune responses provided protection against subsequent infection by a lethal dose of *K. pneumoniae* 52145 (Fig. 6A). This was also true when insects were challenged with the more virulent strain *K. pneumoniae* 43816 (Fig. 6B). In preimmunized larvae, the LD_{50}s of *K. pneumoniae* 52145 and 43816 were 7.00 ± 0.01 and 6.80 ± 0.02, respectively, which are 100-fold higher than those obtained with nonpreimmunized insects.

Analysis of virulence factors necessary for *K. pneumoniae* pathogenesis in *G. mellonella*. Having established that CPS is necessary for *K. pneumoniae* virulence in *G. mellonella*, we sought to determine whether other *K. pneumoniae* virulence factors necessary for virulence in the mouse pneumonia model are also important for pathogenesis in *G. mellonella*. We recently showed that *K. pneumoniae* LPS lipid A is decorated with palmitate and aminoarabinose (54). The gene encoding the acyltransferase (*pagP*) is required for the addition of palmitate to lipid A (54), whereas the *pmrHFIJKLM* (*arnBCADTEF*; referred to here as the *pmrF* operon) loci are required for the synthesis and addition of aminoarabinose to lipid A (54). Strains 52145 Δ*pmrF*, 52145 Δ*pagP*GB, and 52145 Δ*pmrF* Δ*pagP*GB are mutant strains lacking lipid A species containing aminoarabinose, palmitate, and both, respectively (54). These mutants express the same levels of CPS as the wild type and are attenuated in the mouse pneumonia model (54). To evaluate whether these modifications are necessary for *K. pneumoniae* pathogenesis in *G. mellonella*, we determined the LD_{50}s of the three lipid A mutants (Table 3). The LD_{50}s of the

TABLE 3 LD_{50}s of *K. pneumoniae* mutants in *G. mellonella* at 72 postinfection

Strain	Log LD_{50}[a]
52145	4.94 ± 0.11
52145 Δwca_{K2}	6.97 ± 0.24*
52145 Δ*pmrF*	5.74 ± 0.12*
52145 Δ*pagP*GB	5.53 ± 0.06*
52145 Δ*pagP*GB Δ*pmrF*	5.50 ± 0.06*
52145 Δ*lpxO*	5.61 ± 0.06*
52OmpA2	5.84 ± 0.08*
52OmpK36	6.06 ± 0.17*
52145 Δ*phoQ*GB	5.76 ± 0.41*
52145 Δ*pmrAB*	5.80 ± 0.36*
52145 Δ*pmrAB* Δ*phoQ*GB	5.75 ± 0.04*
52145 Δ*rcsB*	5.57 ± 0.01*

[a] *, results are significantly different ($P < 0.05$; one-tailed *t* test) from the results for strain 52145.

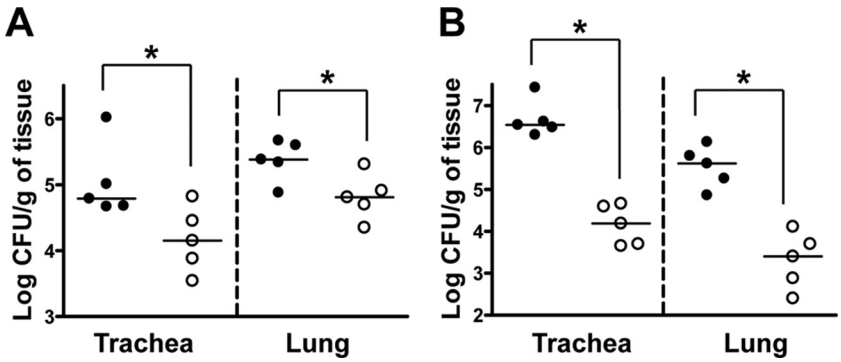

FIG 7 Virulence of *lpxO* mutant in mice. Bacterial counts in mouse organs at 24 h postinfection (A) or 96 h postinfection (B) are shown. Mice were infected intranasally with a bacterial mixture containing 5×10^4 bacteria of the wild-type (*K. pneumoniae* 52145; ●) or *lpxO* mutant (52145 Δ*lpxO*; ○) strain. Results are reported as log CFU per gram of tissue. *, results are significantly different ($P < 0.05$; one-tailed *t* test) from the results for *K. pneumoniae* 52145.

three mutants were higher than that of *K. pneumoniae* 52145 but lower than that of the *cps* mutant (Table 3). No significant differences were found between the three lipid A mutants.

OmpA and OmpK36 are the most abundant outer membrane proteins (OMPs) on the *K. pneumoniae* outer membrane (55). Previously, we showed that *ompA* and *ompK36* mutants express levels of CPS similar to that in *K. pneumoniae* 52145 and that they are attenuated in the mouse pneumonia model (33, 36, 56). Like those of the lipid A mutants, the LD_{50}s of the OMP mutants were higher than that of the wild type (Table 3).

In summary, these results demonstrate a strong correlation between findings obtained using the mouse model of pneumonia and the *G. mellonella* model. To add further evidence to this notion, we determined the virulence of an *lpxO* mutant. LpxO is the enzyme responsible for the 2-hydroxyacyl-modified lipid A found in *K. pneumoniae* (unpublished data). The virulence of this factor has not been assessed previously. The LD_{50} of the *lpxO* mutant was 5-fold higher than that of *K. pneumoniae* 52145 (Table 3). To evaluate the ability of this mutant to cause pneumonia, C57BL/6JOlaHsd mice were infected intranasally, and at 24 and 96 h postinfection, bacterial loads in trachea and lung homogenates were determined (Fig. 7). At 24 h postinfection, *K. pneumoniae* 52145 and the *lpxO* mutant colonized the trachea and lungs, although bacterial loads of the mutant strain were lower than those of the wild type in both organs (Fig. 7A). Similar results were observed at 96 h postinfection (Fig. 7B). Together, these results further highlight that the *G. mellonella* model is useful for assessing the pathogenic potential of *K. pneumoniae*.

To determine whether attenuated mutants activate *Galleria* defense responses, we quantified the levels of antimicrobial factors in the hemolymph of infected larvae. The levels of antimicrobials in the hemolymph of larvae infected with the mutants were significantly higher than those found in *K. pneumoniae* 52145-infected larvae (see Fig. S1 in the supplemental material).

***In vivo* monitoring of *K. pneumoniae* virulence gene expression during *G. mellonella* infection.** The finding that the *cps* and lipid A mutants were attenuated in *G. mellonella* led us to analyze whether the environment encountered by *K. pneumoniae* within the hemolymph affects the expression of the *cps* operon, the *pmrF* operon, *pagP*, or *lpxO*. We utilized chromosomally integrated promoter fusions to a *lucFF* reporter system to monitor expression from gene promoters in *K. pneumoniae* 52145 during infection of *G. mellonella* at different time points. Data are presented as fold increases over the results for the inocula (Fig. 8). The expression of *cps*::*lucFF* was higher in the hemolymph of larvae than in culture medium (Fig. 8A). However, expression decreased over time in infected larvae and was 2-fold lower at 12 h postinfection than at 5 h postinfection (Fig. 8A). The expression of *pmrH*::*lucFF* peaked at 5 h postinfection in the infected larvae (Fig. 8B), whereas the *pagP*::*lucFF* fusion levels decreased over time in the infected larvae (Fig. 8C). The levels of the *lpxO* transcriptional fusion were 6-fold higher in the hemolymph than in culture medium at 1 h postinfection (Fig. 8D), and the expression of the fusion was nearly undetectable at 12 h postinfection. The reduced expression of all transcriptional fusions over time was not due to changes in bacterial growth in the infected larvae, since the strains harboring the tested fusions replicated to the same levels as *K. pneumoniae* 52145 in *G. mellonella* (data not shown).

We recently showed that PhoPQ, PmrAB, and Rcs systems govern the expression of *cps* and of the loci implicated in lipid A remodeling (54; our unpublished data). To define the contributions of these systems to the expression of the *cps* operon, the *pmrF* operon, *lpxO*, and *pagP* in *G. mellonella*, we investigated the transcription of these loci in isogenic mutants upon *G. mellonella* infection. The levels of the *cps* transcriptional fusion were lower in the 52145 Δ*phoQ*GB and 52145 Δ*pmrAB* backgrounds than in *K. pneumoniae* 52145 (Fig. 8E) and were nearly abolished in the *phoQ-pmrAB* double mutant. In contrast, the levels obtained in the 52145 Δ*rcsB* background were similar to those found in *K. pneumoniae* 52145 (Fig. 8E). The expression of the *pmrH* transcriptional fusion was not affected in the *phoQ*, *pmrAB*, and *rcsB* mutant backgrounds, but it was shut off in the *phoQ-pmrAB* double mutant (Fig. 8F). PhoPQ governs the expression of *pagP* and *lpxO in vivo*, since the levels of the transcriptional fusions were affected only in the *phoQ* mutant backgrounds (Fig. 8G and H). The facts that the expression levels found in the *pmrAB* background were not different from those found in the wild type and the expression levels of both loci were not significantly different in the *phoQ* and *phoQ-pmrAB* double mutant backgrounds are consistent with the notion that PmrAB does not control the expression of *pagP* and *lpxO* in the hemolymph of infected larvae.

On the whole, these data indicate that PhoPQ and PmrAB are necessary for *cps* and *pmrH* expression in *Galleria*, whereas the expression of *pagP* and *lpxO* is controlled by PhoPQ. Interestingly, the expression of the regulators is also affected by the environment found within the hemolymph (see Fig. S2 in the supplemental

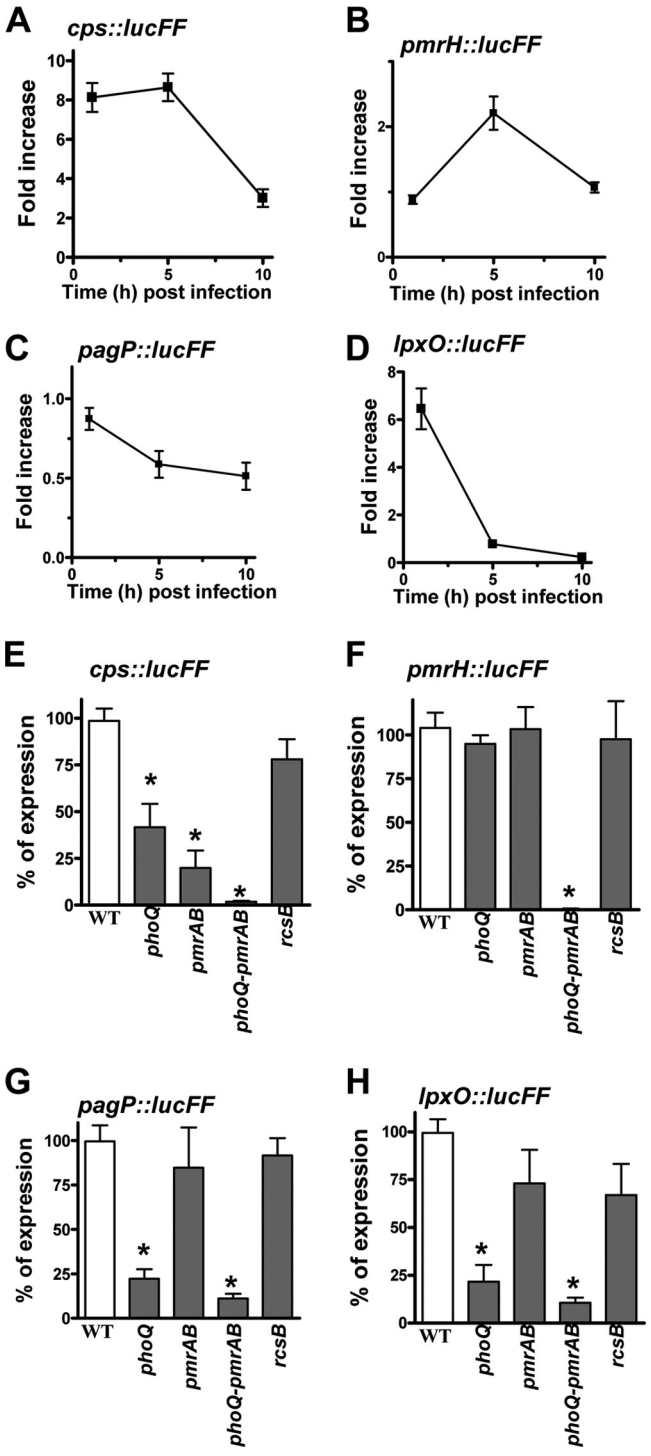

FIG 8 *In vivo* monitoring of *K. pneumoniae* gene expression. Luminescence in the hemolymph of infected larvae was determined at different time points postinfection (five larvae per time point) and corrected by the number of CFU. Larvae were infected with *K. pneumoniae* 52145 carrying the transcriptional fusion *cps*::*lucFF* (A), *pmrH*::*lucFF* (B), *pagP*::*lucFF* (C), or *lpxO*::*lucFF* (D). Results are expressed as fold increases from the luminescence per CFU of the inoculum. Luminescence in the hemolymph of infected larvae was determined at 5 h postinfection (E, F, and G) or 1 h postinfection (H) (five larvae per time point) and corrected by the number of CFU. Larvae were infected with *K. pneumoniae* 52145 (WT), 52145 Δ*pho*QGB (*phoQ*), 52145 Δ*pmrAB* (*pmrAB*), 52145 Δ*pmrAB* Δ*pho*QGB (*phoQ-pmrAB*), or 52145 Δ*rcsB* (*rcsB*) carrying the transcriptional fusion *cps*::*lucFF* (E), *pmrH*::*lucFF* (F), *pagP*::*lucFF* (G), or *lpxO*::*lucFF* (H). Results are expressed as percentages of the luminescence per CFU of *K. pneumoniae* 52145. *, results are significantly different ($P < 0.05$; one-tailed *t* test) from the results for *K. pneumoniae* 52145.

material). The expression of *phoP*::*lucFF* peaked at 1 h postinfection in the infected larvae, whereas the *pmrC*::*lucFF* fusion levels decreased over time in the infected larvae (see Fig. S2). To monitor transcription of the Rcs system, we analyzed the expression of *rcsD*::*lucFF* and *rcsC*::*lucFF* transcriptional fusions. The expression of both fusions was similar in the hemolymph of larvae and in culture medium, and moreover, it did not change over time in the infected insects (see Fig. S2).

Finally, we determined the LD_{50}s of *phoQ*, *pmrAB*, *phoQ-pmrAB*, and *rcsB* mutants. The LD_{50}s of the four mutants were higher than that of the wild type (Table 3).

DISCUSSION

The implementation of infection models that approximate human disease is essential not only for understanding pathogenesis at the molecular level but also for testing new therapies before they are entered into clinical stages. While the infection of higher animals, including knockout animals, has provided invaluable information, alternative infection models providing comparable information, and at the same time being easier to use and ethically acceptable, would be highly useful. The fact that the immune system of insects approximates early stages of mammalian defenses upon infection has led to the use of insects, chiefly *D. melanogaster*, as surrogate hosts. Within the field of infection biology, the larva of the wax moth *G. mellonella* is emerging as an attractive infection model for human pathogens.

In this study, we provide evidence demonstrating that *K. pneumoniae* infection of *G. mellonella* models some of the known features of *K. pneumoniae*-triggered pneumonia. This infection process is characterized by cellular necrosis as a result of a fierce inflammatory response triggered by high bacterial burdens (57). Likewise, infection of *G. mellonella* by *K. pneumoniae* resulted in larval death due to bacterial replication in the hemolymph, increased PO activity (at 12 h postinfection), which is a typical *G. mellonella* response upon septic injury (45), and hemocyte depletion. Moreover, differential immunostaining experiments revealed that *G. mellonella* hemocytes did not engulf *K. pneumoniae* 52145. Similar observations were made by infecting human cell cultures and mouse macrophages with this pathogen (35, 36, 51, 58). Strikingly, the *G. mellonella* model also recapitulates additional aspects of the interplay between *K. pneumoniae* and the lung innate immune system. Mounting evidence indicates that activation of early inflammatory responses is essential for clearing *K. pneumoniae* infections (21–28), and augmentation of this protective immune response decreases the mortality associated with *Klebsiella* infection (29–32). Similarly, we showed in this work that an induced response composed of antimicrobial factors provided protection against subsequent infection by a lethal dose of *Klebsiella*. Of particular interest, we found that the antimicrobial factors present in the hemolymph of preimmunized larvae killed wild-type bacteria. Therefore, our findings demonstrating that wild-type *K. pneumoniae* did not elicit the production of antimicrobial factors at early time points postinfection could be considered a strategy to survive in *G. mellonella*. Notably, we obtained

similar results in human cell models and mice and in tests of *K. pneumoniae* susceptibility to antimicrobial factors upregulated during pneumonia (34, 54). On the whole, our data support the notion that *K. pneumoniae* employs conceptually similar subversion strategies in both the lung and *G. mellonella* innate immune systems to create a niche favorable for replication. Further studies are warranted to decipher whether *K. pneumoniae* targets the same host determinants in *G. mellonella* and mammalian models (34, 59, 60). Nonetheless, to address this question rigorously, further advances in our knowledge of *G. mellonella* cell biology are needed.

Another important finding of our study is that the *G. mellonella* model is useful for assessing the virulence potential of *K. pneumoniae*. It is becoming evident that there are differences in the virulence potential of *K. pneumoniae* strains (61–63). Our data revealed that the *G. mellonella* model discriminates strains regarded as highly virulent from others considered less virulent, and even pinpoints differences among highly virulent strains. To explain these differences, a systematic comparison of the genome features of strains together with an analysis of virulence features of these isolates in mammalian models should be carried out. Note that the genome sequences of *K. pneumoniae* 43816 and 52145 are not yet available. Nevertheless, our findings support the notion that the *G. mellonella* model is an easy-to-handle system for comparing the virulence of different *K. pneumoniae* isolates or *Klebsiella* species. Furthermore, in this work we found that *K. pneumoniae* mutants previously known to be attenuated in the mouse pneumonia model were also attenuated in the *G. mellonella* model. In further support of the hypothesis that prevention of host responses is an important feature of *K. pneumoniae* pathogenesis, we found that all mutants activated *G. mellonella* defensive responses. Moreover, this was also true for the *lpxO* mutant, for which there was no previous information on its possible contribution to *K. pneumoniae* virulence. Studies are ongoing to characterize in depth the contribution of LpxO-dependent lipid A modification to *K. pneumoniae* virulence.

The *G. mellonella* model also allowed us to monitor *K. pneumoniae* gene expression. We found that the expression levels of *cps* and the loci implicated in lipid A remodeling peaked during the first hours postinfection, further emphasizing the importance of these determinants for *K. pneumoniae* survival in *G. mellonella*. At present, we can only speculate on which signal(s) within the hemolymph of infected larvae is sensed by *Klebsiella* to upregulate gene expression. However, since antimicrobial peptides upregulate the expression of these loci *in vitro* (54), it is tempting to speculate that *G. mellonella* antimicrobial factors may represent this *in vivo* signal. In support of this hypothesis, we found that the PhoPQ and PmrAB two-component systems, which control antimicrobial peptide-induced transcriptional changes *in vitro* (54), also governed the expression of *cps* and the loci implicated in lipid A remodeling in *G. mellonella*. Although our data indicate that the Rcs system does not contribute to the expression of these loci in *G. mellonella*, it is evident that Rcs-controlled systems are needed for *K. pneumoniae* survival in *G. mellonella*, since the *rcsB* mutant is attenuated.

Despite the clear utility of *G. mellonella* as a surrogate host for assessing infections with *K. pneumoniae*, it is worth commenting on the limitations of the *G. mellonella* model. The processes that are reproduced in mice and *G. mellonella* may represent ancient mechanisms of pathogen interaction with the innate immune system. However, the evolutionary distance between these models also makes it clear that many host-specific phenomena are likely to exist. Further impediments are the reduced knowledge about the *G. mellonella* immune gene repertoire, cell death pathways, and hemocyte biology and the nonexistence of a well-established method to generate *G. mellonella* mutants. Advances in these areas will facilitate three-dimensional analysis of host-pathogen interactions, i.e., testing a panel of *G. mellonella* mutants versus a panel of bacterial mutants over time.

ACKNOWLEDGMENTS

We are grateful to members of the Bengoechea lab for helpful discussions and to Brent Weber for careful editing of the manuscript. We are indebted to Nicholas Tucker and Paul Hoskisson (University of Strathclyde) for their help in setting up the *G. mellonella* model.

Fellowship support to J.L.I. from the Conselleria d'Educació, Cultura i Universitats Govern Illes Balears (cofunded by the European Regional Development Fund) is gratefully acknowledged. Part of this work was supported by grants from the Biomedicine Program of the Ministerio de Economía y Competitividad (grants SAF2009-07885 and SAF2012-39841) to J.A.B. The Laboratory of Microbial Pathogenesis is supported by a grant to competitive groups (project reference 46/2011) from the Conselleria d'Educació, Cultura i Universitats Govern Illes Balears (cofunded by the European Regional Development Fund). CIBERES is an initiative from the Instituto de Salud Carlos III.

REFERENCES

1. **Reis e Sousa C.** 2004. Activation of dendritic cells: translating innate into adaptive immunity. Curr. Opin. Immunol. **16:**21–25.
2. **D'Argenio DA, Gallagher LA, Berg CA, Manoil C.** 2001. *Drosophila* as a model host for *Pseudomonas aeruginosa* infection. J. Bacteriol. **183:**1466–1471.
3. **Feinbaum RL, Urbach JM, Liberati NT, Djonovic S, Adonizio A, Carvunis AR, Ausubel FM.** 2012. Genome-wide identification of *Pseudomonas aeruginosa* virulence-related genes using a *Caenorhabditis elegans* infection model. PLoS Pathog. **8:**e1002813. doi:10.1371/journal.ppat.1002813.
4. **Mahajan-Miklos S, Tan MW, Rahme LG, Ausubel FM.** 1999. Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa-Caenorhabditis elegans* pathogenesis model. Cell **96:**47–56.
5. **Aballay A, Yorgey P, Ausubel FM.** 2000. *Salmonella typhimurium* proliferates and establishes a persistent infection in the intestine of *Caenorhabditis elegans*. Curr. Biol. **10:**1539–1542.
6. **Brandt SM, Dionne MS, Khush RS, Pham LN, Vigdal TJ, Schneider DS.** 2004. Secreted bacterial effectors and host-produced Eiger/TNF drive death in a *Salmonella*-infected fruit fly. PLoS Biol. **2:**e418. doi:10.1371/journal.pbio.0020418.
7. **Labrousse A, Chauvet S, Couillault C, Kurz CL, Ewbank JJ.** 2000. *Caenorhabditis elegans* is a model host for *Salmonella typhimurium*. Curr. Biol. **10:**1543–1545.
8. **Uehlinger S, Schwager S, Bernier SP, Riedel K, Nguyen DT, Sokol PA, Eberl L.** 2009. Identification of specific and universal virulence factors in *Burkholderia cenocepacia* strains by using multiple infection hosts. Infect. Immun. **77:**4102–4110.
9. **Meijer AH, Spaink HP.** 2011. Host-pathogen interactions made transparent with the zebrafish model. Curr. Drug Targets **12:**1000–1017.
10. **Pradel E, Ewbank JJ.** 2004. Genetic models in pathogenesis. Annu. Rev. Genet. **38:**347–363.
11. **Garcia-Rodas R, Casadevall A, Rodriguez-Tudela JL, Cuenca-Estrella M, Zaragoza O.** 2011. *Cryptococcus neoformans* capsular enlargement and cellular gigantism during *Galleria mellonella* infection. PLoS One **6:**e24485. doi:10.1371/journal.pone.0024485.
12. **Harding CR, Schroeder GN, Reynolds S, Kosta A, Collins JW, Mousnier A, Frankel G.** 2012. *Legionella pneumophila* pathogenesis in the *Galleria mellonella* infection model. Infect. Immun. **80:**2780–2790.
13. **Jander G, Rahme LG, Ausubel FM.** 2000. Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. J. Bacteriol. **182:**3843–3845.

14. **Mukherjee K, Altincicek B, Hain T, Domann E, Vilcinskas A, Chakraborty T.** 2010. *Galleria mellonella* as a model system for studying *Listeria* pathogenesis. Appl. Environ. Microbiol. **76:**310–317.
15. **Peleg AY, Jara S, Monga D, Eliopoulos GM, Moellering RC, Jr, Mylonakis E.** 2009. *Galleria mellonella* as a model system to study *Acinetobacter baumannii* pathogenesis and therapeutics. Antimicrob. Agents Chemother. **53:**2605–2609.
16. **Seed KD, Dennis JJ.** 2008. Development of *Galleria mellonella* as an alternative infection model for the *Burkholderia cepacia* complex. Infect. Immun. **76:**1267–1275.
17. **Thomaz L, Garcia-Rodas R, Guimaraes AJ, Taborda CP, Zaragoza O, Nosanchuk JD.** 2013. *Galleria mellonella* as a model host to study *Paracoccidioides lutzii* and *Histoplasma capsulatum*. Virulence **4:**139–146.
18. **Junqueira JC.** 2012. *Galleria mellonella* as a model host for human pathogens: recent studies and new perspectives. Virulence **3:**474–476.
19. **Sahly H, Podschun R.** 1997. Clinical, bacteriological, and serological aspects of *Klebsiella* infections and their spondylarthropathic sequelae. Clin. Diagn. Lab. Immunol. **4:**393–399.
20. **Nordmann P, Cuzon G, Naas T.** 2009. The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. Lancet Infect. Dis. **9:**228–236.
21. **Aujla SJ, Chan YR, Zheng M, Fei M, Askew DJ, Pociask DA, Reinhart TA, McAllister F, Edeal J, Gaus K, Husain S, Kreindler JL, Dubin PJ, Pilewski JM, Myerburg MM, Mason CA, Iwakura Y, Kolls JK.** 2008. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. Nat. Med. **14:**275–281.
22. **Greenberger MJ, Strieter RM, Kunkel SL, Danforth JM, Laichalk LL, McGillicuddy DC, Standiford TJ.** 1996. Neutralization of macrophage inflammatory protein-2 attenuates neutrophil recruitment and bacterial clearance in murine *Klebsiella* pneumonia. J. Infect. Dis. **173:**159–165.
23. **Greenberger MJ, Kunkel SL, Strieter RM, Lukacs NW, Bramson J, Gauldie J, Graham FL, Hitt M, Danforth JM, Standiford TJ.** 1996. IL-12 gene therapy protects mice in lethal *Klebsiella* pneumonia. J. Immunol. **157:**3006–3012.
24. **Happel KI, Zheng M, Young E, Quinton LJ, Lockhart E, Ramsay AJ, Shellito JE, Schurr JR, Bagby GJ, Nelson S, Kolls JK.** 2003. Cutting edge: roles of Toll-like receptor 4 and IL-23 in IL-17 expression in response to *Klebsiella pneumoniae* infection. J. Immunol. **170:**4432–4436.
25. **Happel KI, Dubin PJ, Zheng M, Ghilardi N, Lockhart C, Quinton LJ, Odden AR, Shellito JE, Bagby GJ, Nelson S, Kolls JK.** 2005. Divergent roles of IL-23 and IL-12 in host defense against *Klebsiella pneumoniae*. J. Exp. Med. **202:**761–769.
26. **Moore TA, Perry ML, Getsoian AG, Monteleon CL, Cogen AL, Standiford TJ.** 2003. Increased mortality and dysregulated cytokine production in tumor necrosis factor receptor 1-deficient mice following systemic *Klebsiella pneumoniae* infection. Infect. Immun. **71:**4891–4900.
27. **Moore TA, Lau HY, Cogen AL, Monteleon CL, Standiford TJ.** 2003. Anti-tumor necrosis factor-alpha therapy during murine *Klebsiella pneumoniae* bacteremia: increased mortality in the absence of liver injury. Shock **20:**309–315.
28. **Zeng X, Moore TA, Newstead MW, Deng JC, Kunkel SL, Luster AD, Standiford TJ.** 2005. Interferon-inducible protein 10, but not monokine induced by gamma interferon, promotes protective type 1 immunity in murine *Klebsiella pneumoniae* pneumonia. Infect. Immun. **73:**8226–8236.
29. **Standiford TJ, Wilkowski JM, Sisson TH, Hattori N, Mehrad B, Bucknell KA, Moore TA.** 1999. Intrapulmonary tumor necrosis factor gene therapy increases bacterial clearance and survival in murine gram-negative pneumonia. Hum. Gene Ther. **10:**899–909.
30. **Tsai WC, Strieter RM, Wilkowski JM, Bucknell KA, Burdick MD, Lira SA, Standiford TJ.** 1998. Lung-specific transgenic expression of KC enhances resistance to *Klebsiella pneumoniae* in mice. J. Immunol. **161:**2435–2440.
31. **Deng JC, Moore TA, Newstead MW, Zeng X, Krieg AM, Standiford TJ.** 2004. CpG oligodeoxynucleotides stimulate protective innate immunity against pulmonary *Klebsiella* infection. J. Immunol. **173:**5148–5155.
32. **Karaolis DK, Newstead MW, Zeng X, Hyodo M, Hayakawa Y, Bhan U, Liang H, Standiford TJ.** 2007. Cyclic di-GMP stimulates protective innate immunity in bacterial pneumonia. Infect. Immun. **75:**4942–4950.
33. **March C, Moranta D, Regueiro V, Llobet E, Tomas A, Garmendia J, Bengoechea JA.** 2011. *Klebsiella pneumoniae* outer membrane protein A is required to prevent the activation of airway epithelial cells. J. Biol. Chem. **286:**9956–9967.
34. **Moranta D, Regueiro V, March C, Llobet E, Margareto J, Larrate E, Garmendia J, Bengoechea JA.** 2010. *Klebsiella pneumoniae* capsule polysaccharide impedes the expression of β-defensins by airway epithelial cells. Infect. Immun. **78:**1135–1146.
35. **Regueiro V, Campos MA, Pons J, Alberti S, Bengoechea JA.** 2006. The uptake of a *Klebsiella pneumoniae* capsule polysaccharide mutant triggers an inflammatory response by human airway epithelial cells. Microbiology **152:**555–566.
36. **March C, Cano V, Moranta D, Llobet E, Perez-Gutierrez C, Tomas JM, Suarez T, Garmendia J, Bengoechea JA.** 2013. Role of bacterial surface structures on the interaction of *Klebsiella pneumoniae* with phagocytes. PLoS One **8:**e56847. doi:10.1371/journal.pone.0056847.
37. **Lawlor MS, Handley SA, Miller VL.** 2006. Comparison of the host responses to wild-type and *cpsB* mutant *Klebsiella pneumoniae* infections. Infect. Immun. **74:**5402–5407.
38. **Banville N, Browne N, Kavanagh K.** 2012. Effect of nutrient deprivation on the susceptibility of *Galleria mellonella* larvae to infection. Virulence **3:**497–503.
39. **Thakur AK, Fezio WL.** 1981. A computer program for estimating LD50 and its confidence limits using modified Behrens-Reed-Muench cumulant method. Drug Chem. Toxicol. **4:**297–305.
40. **Eleftherianos I, Millichap PJ, Ffrench-Constant RH, Reynolds SE.** 2006. RNAi suppression of recognition protein mediated immune responses in the tobacco hornworm *Manduca sexta* causes increased susceptibility to the insect pathogen *Photorhabdus*. Dev. Comp. Immunol. **30:**1099–1107.
41. **Valdivia RH, Falkow S.** 1996. Bacterial genetics by flow cytometry: rapid isolation of *Salmonella typhimurium* acid-inducible promoters by differential fluorescence induction. Mol. Microbiol. **22:**367–378.
42. **Valdivia RH, Falkow S.** 1997. Fluorescence-based isolation of bacterial genes expressed within host cells. Science **277:**2007–2011.
43. **Lehrer RI, Rosenman M, Harwig SS, Jackson R, Eisenhauer P.** 1991. Ultrasensitive assays for endogenous antimicrobial polypeptides. J. Immunol. Methods **137:**167–173.
44. **Llobet E, Tomas JM, Bengoechea JA.** 2008. Capsule polysaccharide is a bacterial decoy for antimicrobial peptides. Microbiology **154:**3877–3886.
45. **Kavanagh K, Reeves EP.** 2004. Exploiting the potential of insects for in vivo pathogenicity testing of microbial pathogens. FEMS Microbiol. Rev. **28:**101–112.
46. **Gunn JS, Miller SI.** 1996. Pho-PhoQ activates transcription of *pmrAB*, encoding a two-component regulatory system involved in *Salmonella typhimurium* antimicrobial peptide resistance. J. Bacteriol. **178:**6857–6864.
47. **Cano V, Moranta D, Llobet-Brossa E, Bengoechea JA, Garmendia J.** 2009. *Klebsiella pneumoniae* triggers a cytotoxic effect on airway epithelial cells. BMC Microbiol. **9:**156. doi:10.1186/1471-2180-9-156.
48. **Cortes G, Alvarez D, Saus C, Alberti S.** 2002. Role of lung epithelial cells in defense against *Klebsiella pneumoniae* pneumonia. Infect. Immun. **70:**1075–1080.
49. **Froquet R, Lelong E, Marchetti A, Cosson P.** 2009. *Dictyostelium discoideum*: a model host to measure bacterial virulence. Nat. Protoc. **4:**25–30.
50. **Camprubi S, Merino S, Benedi VJ, Tomas JM.** 1993. The role of the O-antigen lipopolysaccharide and capsule on an experimental *Klebsiella pneumoniae* infection of the rat urinary tract. FEMS Microbiol. Lett. **111:**9–13.
51. **Cortes G, Borrell N, de Astorza B, Gomez C, Sauleda J, Alberti S.** 2002. Molecular analysis of the contribution of the capsular polysaccharide and the lipopolysaccharide O side chain to the virulence of *Klebsiella pneumoniae* in a murine model of pneumonia. Infect. Immun. **70:**2583–2590.
52. **Lawlor MS, Hsu J, Rick PD, Miller VL.** 2005. Identification of *Klebsiella pneumoniae* virulence determinants using an intranasal infection model. Mol. Microbiol. **58:**1054–1073.
53. **Gillespie JP, Kanost MR, Trenczek T.** 1997. Biological mediators of insect immunity. Annu. Rev. Entomol. **42:**611–643.
54. **Llobet E, Campos MA, Gimenez P, Moranta D, Bengoechea JA.** 2011. Analysis of the networks controlling the antimicrobial peptide-dependent induction of *Klebsiella pneumoniae* virulence factors. Infect. Immun. **79:**3718–3732.
55. **Hernandez-Alles S, Alberti S, Alvarez D, Domenech-Sanchez A, Martinez-Martinez L, Gil J, Tomas JM, Benedi VJ.** 1999. Porin expression in clinical isolates of *Klebsiella pneumoniae*. Microbiology **145:**673–679.
56. **Llobet E, March C, Gimenez P, Bengoechea JA.** 2009. *Klebsiella pneu-*

moniae OmpA confers resistance to antimicrobial peptides. Antimicrob. Agents Chemother. **53:**298–302.
57. **Podschun R, Ullmann U.** 1998. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin. Microbiol. Rev. **11:**589–603.
58. **Alvarez D, Merino S, Tomas JM, Benedi VJ, Alberti S.** 2000. Capsular polysaccharide is a major complement resistance factor in lipopolysaccharide O side chain-deficient *Klebsiella pneumoniae* clinical isolates. Infect. Immun. **68:**953–955.
59. **Frank CG, Reguerio V, Rother M, Moranta D, Maeurer AP, Garmendia J, Meyer TF, Bengoechea JA.** 2013. *Klebsiella pneumoniae* targets an EGF receptor-dependent pathway to subvert inflammation. Cell. Microbiol. **15:**1212–1233.
60. **Regueiro V, Moranta D, Frank CG, Larrarte E, Margareto J, March C, Garmendia J, Bengoechea JA.** 2011. *Klebsiella pneumoniae* subverts the activation of inflammatory responses in a NOD1-dependent manner. Cell. Microbiol. **13:**135–153.
61. **Bachman MA, Oyler JE, Burns SH, Caza M, Lepine F, Dozois CM, Weiser JN.** 2011. *Klebsiella pneumoniae* yersiniabactin promotes respiratory tract infection through evasion of lipocalin 2. Infect. Immun. **79:**3309–3316.
62. **Brisse S, Fevre C, Passet V, Issenhuth-Jeanjean S, Tournebize R, Diancourt L, Grimont P.** 2009. Virulent clones of *Klebsiella pneumoniae*: identification and evolutionary scenario based on genomic and phenotypic characterization. PLoS One **4:**e4982. doi:10.1371/journal.pone.0004982.
63. **Putze J, Hennequin C, Nougayrede JP, Zhang W, Homburg S, Karch H, Bringer MA, Fayolle C, Carniel E, Rabsch W, Oelschlaeger TA, Oswald E, Forestier C, Hacker J, Dobrindt U.** 2009. Genetic structure and distribution of the colibactin genomic island among members of the family *Enterobacteriaceae*. Infect. Immun. **77:**4696–4703.
64. **Fang CT, Chuang YP, Shun CT, Chang SC, Wang JT.** 2004. A novel virulence gene in *Klebsiella pneumoniae* strains causing primary liver abscess and septic metastatic complications. J. Exp. Med. **199:**697–705.
65. **Hsieh PF, Lin TL, Yang FL, Wu MC, Pan YJ, Wu SH, Wang JT.** 2012. Lipopolysaccharide O1 antigen contributes to the virulence in *Klebsiella pneumoniae* causing pyogenic liver abscess. PLoS One **7:**e33155. doi:10.1371/journal.pone.0033155.
66. **Nassif X, Fournier JM, Arondel J, Sansonetti PJ.** 1989. Mucoid phenotype of *Klebsiella pneumoniae* is a plasmid-encoded virulence factor. Infect. Immun. **57:**546–552.