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

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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.
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 pneumoniae* 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; kanamycin (Km), 100 µg/ml; and genamycin (Gm), 500 µg/ml. Bacterial strains and plasmids used in this study are listed in Table 1.

### TABLE 1 Bacterial strains and plasmids used in this study

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<tr>
<th>Bacterial strain or plasmid</th>
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<td><strong>Klebsiella pneumoniae strains</strong></td>
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<td><strong>Plasmids</strong></td>
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<td>pFPV 25.1</td>
<td>rpsM::gfpmut3; Amp'</td>
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*Δ*, deletion; RP*, rifampin resistance; Km*, kanamycin resistance; Amp*, ampicillin resistance; Gm*, gentamicin resistance. The ϕca62 gene is manC.
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 growth 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 of 600 nm (OD600) of 1, which corresponds to approximately 1 × 10⁶ CFU/ml. After surface disinfection using ethanol (70% [vol/vol]), larvae were injected with 10 μl of bacterial suspension, containing approximately 1 × 10⁶ 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 infection 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 (LD50) experiments, a series of 10-fold serial dilutions containing 10⁹ to 10⁴ 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 LD50s were calculated according to the formula of Reed and Muench (39). Results are expressed as log LD50.

**Determination of in vivo bacterial loads.** Insects were infected with approximately 1 × 10⁶ CFU per larva of *K. pneumoniae* 52145 or 52145 ΔwcaK₂, per larva. At different time points postinfection (5, 10, and 24 h), insects were surface sterilized 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 CFUs were recovered from noninfected insects in LB agar supplemented with rifampin.

**PO activity assay.** Insects were infected with approximately 1 × 10⁶ CFU of *K. pneumoniae* 52145 or 52145 ΔwcaK₂ 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 1:1 (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 OD490/min.

**Hemocyte quantification and viability staining.** Larvae were infected with 1 × 10⁶ CFU of *K. pneumoniae* 52145 or 52145 ΔwcaK₂, 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 × 10⁶ CFU per larva of *K. pneumoniae* 52145 or 52145 ΔwcaK₂ carrying pFPV251. 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 trypsin 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 × 10⁵ CFU/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 × 10⁶ CFU of *Klebsiella as a 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 polymixin 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 × 10⁶ 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 10⁶ 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 × 10⁶ 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-isopropl alcohol protocol, and the obtained RNA was fur-
ther purified using a Nucleospin RNAII kit (Macherey-Nagel) that in-
cluded one step of on-column DNase treatment, following the manufac-
turer’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 mate-
rial. 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 (ΔΔCT) method, with normalization to the 18S rRNA and actin genes.

Construction of pmrC:lucFF reporter fusion. A 445-bp DNA frag-
ment containing the promoter region of the pmrC operon was ampli-
fied by PCR using Vent polymerase, digested with EcoRI, gel purified, and
cloned into EcoRI-Smal-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 pmrC promoter region was identified by restriction digestion analysis
and named pGPLKpnpmrC. This plasmid was introduced into E. coli SY327-Δpir, from which it was mobilized into K. pneumoniae 52145 by triparental conjugation using the helper strain E. coli HB101/pRK2313. 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
CheckLucFRR (data not shown).

In vivo monitoring of gene expression during G. mellonella infec-
tion. Insects were infected with approximately 1 × 10^8 CFU K. pneu-
moniae 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 microli-
ters 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 mea-
sured 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-express-
ings K. pneumoniae were used to normalize the control expression levels. Notably, three
K. pneumoniae CPS mutant strains are avirulent, as
K. pneumoniae K52145 and MGH78578 were not significantly differ-
cent K. pneumoniae 52145 (O1:K2 serotype) (Table 1), which is used in molec-
ular 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. pneu-
moniae strains were tested (Table 1). Strains ATCC 43816 (sero-
type 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 (sero-
type K52) (Table 1). MGH78578 also killed 100% of infected lar-
vae at 72 h postinfection (Fig. 1B).

To determine whether K. pneumoniae-induced lethality was depen-
dent on the number of injected bacteria, larvae were in-
jected 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 10^6 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. pneu-
moniae 52145 and MGH78578 were not significantly differ-
ent. 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 labora-
tory-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 viru-
ulence 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 Δwca_{Gm}, the isogenic cps mutant of K. pneumoniae 52145 (Table 1), did not cause any mortality of the
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/H9004/manC, killed 50% of *G. mellonella* larvae within this time (Fig. 2A). The LD$_{50}$ of the *cps* mutants, i.e., strains 52145/ΔwcaK2 and 43816/ΔmanC ($6.97 \pm 0.24$ and $5.67 \pm 0.03$, respectively), were significantly higher than those of the isogenic wild-type strains, *K. pneumoniae* 52145 and 43816 ($4.94 \pm 0.11$ and $4.49 \pm 0.03$)

### TABLE 2 LD$_{50}$ of *K. pneumoniae* strains in *G. mellonella* at 72 postinfection

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<thead>
<tr>
<th>Strain</th>
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<td><em>K. pneumoniae</em></td>
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<td>MGH78578</td>
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<td>4.49 ± 0.03</td>
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<td>4.94 ± 0.11</td>
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<td>USA1850</td>
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<tr>
<td>2330</td>
<td>6.56 ± 0.06</td>
</tr>
<tr>
<td>2073</td>
<td>5.55 ± 0.17</td>
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<tr>
<td><em>K. aerogenes</em></td>
<td>6.00 ± 0.01</td>
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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.

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 ΔwcaK2 or 43816 ΔmanC, 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.
We sought to determine whether G. mellonella mortality is associated with the growth of Klebsiella in infected larvae. Larvae were infected with 10^8 CFU of K. pneumoniae 52145 or the cps mutant strain 52145 ΔwcaK2. 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 hemolymph of 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). 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 postinfection. In contrast, infection with the cps mutant resulted in an increase in bacterial numbers, 99% clearance of the inoculum from the hemolymph, and a decrease in 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).
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
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 5***

Wild-type *K. pneumoniae* does not upregulate the expression of *G. mellonella* antimicrobial peptides. (A) Larvae were infected with 10⁶ 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 3-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 (**wca**). 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 from 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 (**wca**), 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); Δ, 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.
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 pmrA/BH operon is required for the synthesis and addition of aminoarabinose to lipid A (54). Strains 52145 ΔpmrF, 52145 ΔpagP, and 52145 ΔpmrF ΔpagP 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Log LD_{50}^a</th>
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<tbody>
<tr>
<td>52145</td>
<td>4.94 ± 0.11</td>
</tr>
<tr>
<td>52145 ΔwcaK2</td>
<td>6.97 ± 0.24*</td>
</tr>
<tr>
<td>52145 ΔpmrF</td>
<td>5.74 ± 0.12*</td>
</tr>
<tr>
<td>52145 ΔpagP</td>
<td>5.53 ± 0.06*</td>
</tr>
<tr>
<td>52145 ΔpagP ΔpmrF</td>
<td>5.50 ± 0.06*</td>
</tr>
<tr>
<td>52145 ΔpxsO</td>
<td>5.61 ± 0.06*</td>
</tr>
<tr>
<td>52OmpA2</td>
<td>5.84 ± 0.08*</td>
</tr>
<tr>
<td>52OmpK36</td>
<td>6.06 ± 0.17*</td>
</tr>
<tr>
<td>52145 ΔphaQGB</td>
<td>5.76 ± 0.41*</td>
</tr>
<tr>
<td>52145 ΔpmrAB</td>
<td>5.80 ± 0.36*</td>
</tr>
<tr>
<td>52145 ΔpmrAB ΔphaQGB</td>
<td>5.75 ± 0.04*</td>
</tr>
<tr>
<td>52145 ΔrcB</td>
<td>5.57 ± 0.01*</td>
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^a^, results are significantly different (P < 0.05; one-tailed t test) from the results for strain 52145.

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. Twenty larvae were infected in each experimental group.
The virulence gene expression in 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 pmrF::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 ΔphoQGB and 52145 ΔpmrAB 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 ΔrcsB 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 material).
Larvae were infected with *K. pneumoniae* 52145 (WT), 52145, *phoQ* (H), *phoQ*::*pmrAB*, and *rcsB* mutants. The LD_{50} 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, 52). 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 transcripational 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.
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.

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