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 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...
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 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; kanamycin (Km), 100 μg/ml; and kanamycin (Km), 100 μg/ml.

**G. mellonella larvae.** *G. mellonella* larvae were acquired at Alcôtà 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, 24ºC), 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 (OD600) of 1, which corresponds to approximately 1 × 10⁸ CFU/ml. After surface disinfection using ethanol (70% [vol/vol]), larvae were injected with 10 µl of bacterial suspension, containing approximately 1 × 10⁸ 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 (LD50) experiments, a series of 10-fold serial dilutions containing 10⁴ to 10⁷ 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 LD5₀, 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 × 10⁸ CFU per larva of K. pneumoniae 52145 or 52145 Δωtrk₂. 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 × 10⁶ CFU of K. pneumoniae 52145 or 52145 Δωtrk₂ 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 overlying 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 and used for all the experiments, data from three independent experiments were combined, and LD50 were calculated according to the formula of Reed and Muench (39). Results are expressed as log LD50.

RNA extraction and RT-PCR. Larvae 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). 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 19 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 analysis. 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 antimicrobial agents. All measurements were done in triplicate on at least three separate occasions.

Immunoﬂuorescence assay with extracted hemocytes. Insects were infected with approximately 1 × 10⁶ CFU per larva of K. pneumoniae 52145 or 52145 Δωtrk₂ 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 electrodendosmosis (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 × 10⁵ CFU per ml of molten gel. This 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 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 19 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.
chloroform-isopropyl alcohol protocol, and the obtained RNA was fur-
ther purified using a Nucleospin RNAII kit (Macherey-Nagel) that
included 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 thermostatic 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::lacFF 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-SmaI-digested pGPL01 suicide vector (46). This vector
furnished by PCR using CheckProkpnPmrCF and CheckProkpnPmrCR
primers. This plasmid was introduced into E. coli
and named pGPLKpnPmrC. This plasmid was introduced into
Klebsiella
pir
and E. coli
into the genome by homologous recombination was selected. This was con-
firmation by Southern blotting and a PCR using primers CheckProkpnPmrCF
and CheckLucFFR (data not shown).

In vivo monitoring of gene expression during G. mellonella infec-
tion. Insects were infected with approximately 1 × 10^6 CFU K. pneu-
moniae carrying a lacFF-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
100-μl of a saturated solution of xylazine (5 mg/kg). Overnight bacterial cultures were centrifuged (2,500
g

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K. pneumoniae Pathogenesis in G. mellonella

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 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-
infected 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 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 10^6 CFU of K. pneumoniae strain 52145,
43816, or MGH78578. The LD50 of strain MGH78578 was not significantly differ-
tent from that of strain 52145 (O1:K2 serotype) (Table 1), which is used in molec-
ular pathogenesis studies. Larvae were injected with 10^6 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. The LD50 was not significantly different
from that of strain 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 LD50s. 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 capsular 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 Δcps
, the isogenic cps mutant of K. pneu-
moniae 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 ± 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 ±

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Log LD_{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. pneumoniae</em> strains</td>
<td></td>
</tr>
<tr>
<td>MGH78578</td>
<td>4.82 ± 0.16</td>
</tr>
<tr>
<td>43816</td>
<td>4.49 ± 0.03</td>
</tr>
<tr>
<td>NTUH-K2044</td>
<td>4.14 ± 0.26</td>
</tr>
<tr>
<td>52145</td>
<td>4.94 ± 0.11</td>
</tr>
<tr>
<td>USA1850</td>
<td>5.55 ± 0.05</td>
</tr>
<tr>
<td>2330</td>
<td>6.56 ± 0.06</td>
</tr>
<tr>
<td>2073</td>
<td>5.55 ± 0.17</td>
</tr>
<tr>
<td><em>K. aerogenes</em></td>
<td></td>
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<tr>
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<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.
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 \(\Delta\)manC was significantly lower than that of 52145 \(\Delta\)wcaK2 \((P < 0.05\) 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 \(\Delta\)wcaK2. 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

![FIG 3](http://iai.asm.org/)

**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 \(\Delta\)wcaK2 \((\Delta\)psi\) 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).
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 *E. coli*-infected larvae 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
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 (\( P \leq 0.05 \) 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 _K. pneumoniae_ 52145 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 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, or the _cps_ mutant strain 52145 ΔwcaK2 (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 ΔwcaK2 (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); Δ, results are significantly different from the results for 52145 ΔwcaK2-injected larvae by one-way ANOVA; n.s., \( P > 0.05 \) for the indicated comparison.
Larvae were inoculated with 10^6 heat-killed E. coli MG1655 cells and, after 24 h, infected with 10 times the LD50 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 metallopeptase 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 LD50 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 LD50% 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 ΔpmrF, 52145 ΔpagPGB, and 52145 ΔpmrF ΔpagPGB 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 LD50% of the three lipid A mutants (Table 3). The LD50% of the K. pneumoniae infections were 4.94 ± 0.11 (52145) and 4.09 ± 0.03 (ΔpmrF), 4.01 ± 0.02 (ΔpagPGB), and 4.02 ± 0.01 (ΔpmrF ΔpagPGB) (54).

**TABLE 3** LD50% of K. pneumoniae mutants in G. mellonella at 72 postinfection

<table>
<thead>
<tr>
<th>Strain</th>
<th>Log LD50 ± SD</th>
</tr>
</thead>
<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 ΔpagPGB</td>
<td>5.53 ± 0.06*</td>
</tr>
<tr>
<td>52145 ΔpmrF ΔpagPGB</td>
<td>5.50 ± 0.04*</td>
</tr>
<tr>
<td>52145 ΔpxsO</td>
<td>5.61 ± 0.06*</td>
</tr>
<tr>
<td>52145 ΔpmrF ΔpxsO</td>
<td>5.84 ± 0.08*</td>
</tr>
<tr>
<td>52145 ΔpmrF ΔpmrK36</td>
<td>6.06 ± 0.17*</td>
</tr>
<tr>
<td>52145 ΔpmQGB</td>
<td>5.76 ± 0.41*</td>
</tr>
<tr>
<td>52145 ΔpmrAB ΔpmQGB</td>
<td>5.80 ± 0.36*</td>
</tr>
<tr>
<td>52145 ΔpmrAB ΔpmrK36</td>
<td>5.75 ± 0.04*</td>
</tr>
<tr>
<td>52145 ΔpxsO ΔpmrK36</td>
<td>5.57 ± 0.01*</td>
</tr>
</tbody>
</table>

*, results are significantly different (P < 0.05; one-tailed t-test) from the results for strain 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 LD50s of the OMP mutants were significantly higher than those found in K. pneumoniae 52145-infected larvae (Fig. S1 in the supplemental material). The facts that the expression levels found in the pmrAB mutant backgrounds were similar to 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 these systems to the expression of the lpxO 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 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 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).

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 in 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 lpxO 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 postinfection (five larvae per time point) and corrected by the number of CFU. The hemolymph of infected larvae was determined at different time points (5 h postinfection (E, F, and G) or 1 h postinfection (H)) (five larvae per time point). Results are expressed as fold increases from the luminescence per CFU of the inoculum. Luminescence in the hemolymph of infected larvae was determined in vivo monitoring of K. pneumoniae 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} 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 interaction 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 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.

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 ΔphoQGB (ΔphoQGB), 52145 ΔpmrAB (ΔpmrAB), 52145 ΔpmrAB ΔphoQGB (ΔphoQGB-ΔpmrAB), or 52145 ΔrcsB (ΔrcsB) 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.
similar results in human cell models and mice and in tests of *P. aeruginosa* susceptibility to antimicrobial factors upregulated during pneumonia (34, 54). On the whole, our data support the notion that *P. aeruginosa* 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 *P. aeruginosa* 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 regarding 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 sequence 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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