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 csp 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 Bioethic Committee of the University of the Balearic Islands. This study was approved by the Bioethic Committee of the University of the Balearic Islands, under authorization number 1748.

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

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

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

<table>
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<th>Bacterial strain or plasmid</th>
<th>Genotype or comments</th>
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<td>DH10B</td>
<td>F′ endA1 recA1 galE15 galK16 napG rpsL ΔlacX74 ∆80dlacZ∆M15 araD139 Δ(ara,leu)/7697 mcrA</td>
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</tr>
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<td><strong>Klebsiella pneumoniae strains</strong></td>
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**Plasmids**

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<tr>
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<td>pFFV25.1</td>
<td>rpsM::gfpmut3; Amp′</td>
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*a* Cm′, chloramphenicol resistance; Rif′, rifampin resistance; Km′, kanamycin resistance; Amp′, ampicillin resistance. The wcaK2 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 phase (2,500 × g, 20 min, 24°C), and washed once with 10 mM phosphate-buffered saline (PBS; pH 6.5). Bacteria were diluted in PBS to an optical density at 600 nm (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 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 10⁰ 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 values 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. 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 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 1:3 (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 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 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 or heat-killed E. coli MG1655 were collected and pooled in an ice-cold microcentrifuge tube containing 10 μl of a saturated solution of N-phenylthiourea (Sigma). Wells were filled with 10-μl aliquots of these samples, and the agar plates were incubated for 3 h at 37°C. After that, a 30-ml overlay gel composed of 1% agarose and 6% TSB powder in water was poured on top of the previous gel, and the plates were incubated at 37°C. After 18 h, the diameters of the inhibition halos were measured to the nearest 1 mm and, after subtraction of the diameter of the well, were expressed in inhibition units (10 units = 1 mm). PBS and 32 μg/ml polymyxin B (Sigma) were used as negative and positive controls, respectively, in the bioassay. All measurements were done in duplicate on at least five separate occasions.

**Antimicrobial peptide resistance assay.** Insects were infected with approximately 1 × 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 10⁵ 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-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-
rual. 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 quanti-
ties 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 pGFP1 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

One strain in which the suicide vector was integrated into the genome
by homologous recombination was selected. This was confirmed by
mating with a mixture containing ketamine (50 mg/kg of body weight) and
noninfected larvae at 72 h postinfection. No mortality was observed in the PBS-
injected G. mellonella larvae (Fig. 1A). Three additional K. pnu-
emoniae 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-
va at 72 h postinfection (Fig. 1B).

To determine whether K. pneumoniae-induced lethality was dependent 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
(Table 1), which is used in molecular pathogenesis studies. Larvae were injected with 10^6 CFU, and their survival was monitored (Fig. 1A). K. pneumoniae strain 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. pne-
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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-
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type K52) (Table 1). MGH78578 also killed 100% of infected lar-
va at 72 h postinfection (Fig. 1B).

We next determined the LD₅₀ of the four strains. As shown in Table 2, NTUH-K2044 had the lowest LD₅₀, followed by strain 43816 and then strains 52145 and MGH78578. The LD₅₀ of K. pneumoniae strains 52145 and MGH78578 were not significantly differ-
ent. Notably, three K. pneumoniae strains with reduced virulence in mammalian models (47, 48) had LD₅₀ significantly higher than those of K. pneumoniae strains 52145 and MGH78578 (P < 0.05 by the one-sample t test) (Table 2). Moreover, a nonpathogenic labora-
tory-adapted strain of Klesbiella 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 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 ∆wcaH, the isogenic cps mutant of K. pneumoniae strain 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}s of the cps mutants, i.e., strains 52145ΔwcaK2 and 43816ΔmanC (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 ± 0.03). The LD_{50}s of the wild-type strains were as follows: *K. pneumoniae* 52145/H9004 4.94 ± 0.11, 43816/H9004 4.49 ± 0.03, NTUH-K2044/H11006 4.14 ± 0.26, and MGH78578/H11006 4.82 ± 0.16.

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

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<th>Strain</th>
<th>Log LD_{50}</th>
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<td><em>K. pneumoniae</em> strains</td>
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<td>43816</td>
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<td>NTUH-K2044</td>
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<td>4.94 ± 0.11</td>
</tr>
<tr>
<td>USA1850</td>
<td>5.55 ± 0.05</td>
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<tr>
<td>2330</td>
<td>6.56 ± 0.06</td>
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<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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</table>

**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 52145ΔwcaK2 or 43816ΔmanC, 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.
numbers over time (Fig. 2B), hence demonstrating that wild-type
K. pneumoniae
larvae at 24 h postinfection. In contrast, infection of
bacterial numbers and 99% clearance of the inoculum from the
5 h postinfection. However, this was followed by a decrease in
activity in the hemolymph of larvae infected with
52145 (Fig. 3B). The levels of PO in PBS-infected insects remained
activity in the plasmas of insects injected with PBS or infected with K.
manC/H9004 was significantly lower than that of 52145 ΔwcaK2 (P <
PBS-injected larvae by the one-tailed t test). Altogether, these findings indicated
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 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 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).

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 (Fig. 4C). The number of hemocytes decreased over time in larvae infected with \( K. \) \textit{pneumoniae} 52145, hence suggesting that wild-type \( K. \) \textit{pneumoniae} does indeed induce cell destruction (Fig. 4C). In contrast, the number of hemocytes was not significantly different between the \( \text{cps} \) 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 \( E. \) \textit{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 \( E. \) \textit{coli}-infected larvae were higher at 24 than at 5 h postinfection (Fig. 5A). We tested the susceptibility of \( K. \) \textit{pneumoniae} to these \( E. \) \textit{coli}-elicited antimicrobial factors. Indeed, survival assays showed that \( K. \) \textit{pneumoniae} 52145 and the \( \text{cps} \) mutant were susceptible to the antimicrobial factors present in the hemolymph of \( G. \) \textit{mellonella} larvae infected with heat-killed \( E. \) \textit{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 \( K. \) \textit{pneumoniae} infection triggers the production of antimicrobial...
As shown in Fig. 5B, the levels of antimicrobials in the hemolymph of larvae infected with \textit{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 \textit{K. pneumoniae} 52145-infected larvae by one-way ANOVA). The lack of induction of antimicrobial factors by \textit{K. pneumoniae} 52145 was dependent on the expression of CPS, since hemolymph from insects infected with \textit{K. pneumoniae} 52145 does not upregulate the expression of \textit{G. mellonella} antimicrobial peptides. (A) Larvae were infected with \(10^6\) heat-killed (65°C, 15 min) \textit{E. coli} MG1655 cells, and at 5 and 24 h postinfection, hemolymph was extracted and the presence of antimicrobial factors assessed by radial diffusion bioassay. The results, expressed as radial diffusion units (10 units = 1 mm), represent means and standard deviations for at least four independent determinations. *, \(P < 0.05\) (results are significantly different from the 5-h postinfection results by the one-tailed \textit{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 \textit{K. pneumoniae} 52415, heat-killed (65°C, 15 min) \textit{K. pneumoniae} 52145, UV-killed (UV irradiated at 1 J for 10 min in a Bio-Link BLX cross-linker [Vilber Lourmat]) \textit{K. pneumoniae} 52145, or the \textit{cps} mutant strain 52145 \(\Delta\text{wca}_K\) \(\Delta\text{cps}\). 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 \textit{t} test). (C) Transcriptional activation of immune-responsive genes following infection. The transcription levels of lysozyme, galiomycin, gallermymycin, 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 \textit{K. pneumoniae} 52415, heat-killed (65°C, 15 min) \textit{K. pneumoniae} 52145, or the \textit{cps} mutant 52145 \(\Delta\text{wca}_K\) \(\Delta\text{cps}\), and after 8 h, total RNA was purified. Results represent means and standard deviations. *, \(P < 0.05\) (results are significantly different from the expression levels in PBS-injected animals by one-way ANOVA); \(\Delta\), results are significantly different from the results for \textit{K. pneumoniae} 52145-injected larvae by one-way ANOVA; n.s., \(P > 0.05\) for the indicated comparison.
Larvae were inoculated with 10^6 heat-killed K. pneumoniae to protect against a subsequent infection by K. pneumoniae whether prior induction of immune responses in E. coli in the hemolymph of G. mellonella 52145 was susceptible to the antimicrobial factors present in lysozyme, no significant differences were found between the levels of antimicrobial effectors found in the hemolymph of insects infected with K. pneumoniae 52145 and 43816 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 (pglF) is required for the addition of palmitate to lipid A (54), whereas the pmrAB operon is required for the synthesis and addition of aminoarabinose to lipid A (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 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 (pglF) is required for the addition of palmitate to lipid A (54), whereas the pmrAB operon is required for the synthesis and addition of aminoarabinose to lipid A (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...
three mutants were higher than that of *K. pneumoniae* 52145 but lower than that of the *cps* mutant (Table 3). No significant differences were found between the three lipid A mutants.

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

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

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

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

We recently showed that PhoPQ, PmrAB, and Rcs systems govern the expression of *cps* and of the loci implicated in lipid A remodeling (54; our unpublished data). To define the contributions of these systems to the expression of the *cps* operon, the *pmrF* operon, *lpxO*, and *pagP* in *G. mellonella*, we investigated the transcription of these loci in isogenic mutants upon *G. mellonella* infection. The levels of the *cps* transcriptional fusion were lower in the 52145 *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).
postinfection (five larvae per time point) and corrected by the number of CFU. Larvae were infected with K. pneumoniae 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. Results are expressed as fold increases from the luminescence per CFU of the inoculum. 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.

**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 (phoQ), 52145 ΔpmrAB (pmrAB), 52145 ΔpmrAR (pmrAR), 52145 ΔphoQGB (phoQ-pmrAB), or 52145 ΔrcsB (rcsB) carrying the transcriptional fusion cps::lucFF (E), pmrH::lucFF (F), pagP::lucFF (G), or lpxO::lucFF (H). Results are expressed as percentages of the luminescence per CFU of *K. pneumoniae* 52145. *, results are significantly different (*P* < 0.05; one-tailed *t*-test) from the results for *K. pneumoniae* 52145.

**DISCUSSION**

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

In this study, we provide evidence demonstrating that *K. pneumoniae* infection of *G. mellonella* models some of the known features of *K. pneumoniae*-triggered pneumonia. This infection process is characterized by cellular necrosis as a result of a fierce inflammatory response triggered by high bacterial burdens (57). Likewise, infection of *G. mellonella* by *K. pneumoniae* resulted in larval death due to bacterial replication in the hemolymph, increased PO activity (at 12 h postinfection), which is a typical *G. mellonella* response upon septic injury (45), and hemocyte depletion. Moreover, differential immunostaining experiments revealed that *G. mellonella* hemocytes did not engulf *K. pneumoniae* 52145. Similar observations were made by infecting human cell cultures and mouse macrophages with this pathogen (35, 36, 51, 58). Strikingly, the *G. mellonella* model also recapitulates additional aspects of the interplay between *K. pneumoniae* and the lung innate immune system. Mounting evidence indicates that activation of early inflammatory responses is essential for clearing *K. pneumoniae* infections (21–28), and augmentation of this protective immune response decreases the mortality associated with *Klebsiella* infection (29–32). Similarly, we showed in this work that an induced response composed of antimicrobial factors provided protection against subsequent infection by a lethal dose of *Klebsiella*. Of particular interest, we found that the antimicrobial factors present in the hemolymph of preimmunized larvae killed wild-type bacteria. Therefore, our findings demonstrating that wild-type *K. pneumoniae* did not elicit the production of antimicrobial factors at early time points postinfection could be considered a strategy to survive in *G. mellonella*. Notably, we obtained...
similar results in human cell models and mice and in tests of K. pneumoniae susceptibility to antimicrobial factors upregulated during pneumonia (34, 54). On the whole, our data support the notion that K. pneumoniae employs conceptually similar subversion strategies in both the lung and G. mellonella innate immune systems to create a niche favorable for replication. Further studies are warranted to decipher whether K. pneumoniae targets the same host determinants in G. mellonella and mammalian models (34, 59, 60). Nonetheless, to address this question rigorously, further advances in our knowledge of G. mellonella cell biology are needed.

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

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

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

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REFERENCES


