Video Article

The Insect *Galleria mellonella* as a Powerful Infection Model to Investigate Bacterial Pathogenesis

Nalini Ramarao[1], Christina Nielsen-Leroux[1], Didier Lereclus[1]

[1]INRA, Micalis UMR1319, France

Correspondence to: Nalini Ramarao at nalini.ramarao@jouy.inra.fr

URL: https://www.jove.com/video/4392
DOI: doi:10.3791/4392

Keywords: Infection, Issue 70, Microbiology, Immunology, Molecular Biology, Bacteriology, Entomology, Bacteria, *Galleria mellonella*, greater wax moth, insect larvae, intra haemocoelic injection, ingestion, animal model, host pathogen interactions

Date Published: 12/11/2012

Citation: Ramarao, N., Nielsen-Leroux, C., Lereclus, D. The Insect *Galleria mellonella* as a Powerful Infection Model to Investigate Bacterial Pathogenesis. *J. Vis. Exp.* (70), e4392, doi:10.3791/4392 (2012).

Abstract

The study of bacterial virulence often requires a suitable animal model. Mammalian models of infection are costly and may raise ethical issues. The use of insects as infection models provides a valuable alternative. Compared to other non-vertebrate model hosts such as nematodes, insects have a relatively advanced system of antimicrobial defenses and are thus more likely to produce information relevant to the mammalian infection process. Like mammals, insects possess a complex innate immune system[1]. Cells in the hemolymph are capable of phagocytosing or encapsulating microbial invaders, and humoral responses include the inducible production of lysozyme and small antibacterial peptides[2,3]. In addition, analogies are found between the epithelial cells of insect larval midguts and intestinal cells of mammalian digestive systems. Finally, several basic components essential for the bacterial infection process such as cell adhesion, resistance to antimicrobial peptides, tissue degradation and adaptation to oxidative stress are likely to be important in both insects and mammals[1]. Thus, insects are polyvalent tools for the identification and characterization of microbial virulence factors involved in mammalian infections.

Larvae of the greater wax moth *Galleria mellonella* have been shown to provide a useful insight into the pathogenesis of a wide range of microbial infections including mammalian fungal (*Fusarium oxysporum*, *Aspergillus fumigatus*, *Candida albicans*) and bacterial pathogens, such as *Staphylococcus aureus*, *Proteus vulgaris*, *Serratia marcescens Pseudomonas aeruginosa*, *Listeria monocytogenes* or *Enterococcus faecalis*[4-7]. Regardless of the bacterial species, results obtained with *Galleria* larvae infected by direct injection through the cuticle consistently correlate with those of similar mammalian studies: bacterial strains that are attenuated in mammalian models demonstrate lower virulence in *Galleria*, and strains causing severe human infections are also highly virulent in the *Galleria* model[8-11]. Oral infection of *Galleria* is much less used and additional compounds, like specific toxins, are needed to reach mortality.

G. mellonella larvae present several technical advantages: they are relatively large (last instar larvae before pupation are about 2 cm long and weight 250 mg), thus enabling the injection of defined doses of bacteria; they can be reared at various temperatures (20 °C to 30 °C) and infection studies can be conducted between 15 °C to above 37 °C[12,13], allowing experiments that mimic a mammalian environment. In addition, insect rearing is easy and relatively cheap. Infection of the larvae allows monitoring bacterial virulence by several means, including calculation of LD_{50}[14], measurement of bacterial survival[15,16] and examination of the infection process[17]. Here, we describe the rearing of the insects, covering all life stages of *G. mellonella*. We provide a detailed protocol of infection by two routes of inoculation: oral and intra haemocoelic. The bacterial model used in this protocol is *Bacillus cereus*, a Gram positive pathogen implicated in gastrointestinal as well as in other severe local or systemic opportunistic infections[18,19].

Video Link

The video component of this article can be found at https://www.jove.com/video/4392/

Protocol

1. Insect Rearing

The whole cycle from egg to last instar larvae lasts about 5 weeks at 25 °C. One or 2 additional weeks are needed to obtain adult butterflies.

1. Place at least 100 pupae or newly merged adult *G. mellonella* butterflies in a 5-liter wire-mesh cage. Male butterflies measure 10 to 15 mm. The adult male moth is beige with faint light and dark markings. Female butterflies measure around 20 mm. Females are darker than males with a brown/grey color.
2. Suspend two packs of four-layered paper in the cage for egg-laying. After 2 days, the adult female will lay eggs on the edge between the papers.
3. Twice a week, place the egg-paper packs in new plastic rearing boxes with grids to let air circulate, containing pollen and bee wax. Eggs hatch in about 3 days and small larvae start to develop by feeding on wax and pollen. Alternatively, larvae can be placed on an artificial

diet consisting of a mix of 500 g liquid honey, 400 g glycerin, 100 g dried beer yeast, 250 g wheat flour, 200 g skim milk powder and 400 g polenta. To provide appropriate ratio of food per larva, regularly separate the larvae into new boxes and replenish the food every two days.
4. Larvae are reared during the six stages of larva stadium. Each stadium is characterized by the slippage of the head capsule of larvae. When larvae reach the last stage before pupation, they stop feeding and start building a light silk cocoon.
5. In their protective cocoons, the larvae will rest and transform into pupae. Wax worms remain in the pupa stage for one to two weeks to emerge into adult moths. The adult moths do not drink nor eat. Mating occurs and the wax worms' life cycle begins again.
6. To standardize the pathology assay, take the larvae during the last larval stage. During this stage they stop feeding, move to the cover of the rearing box and start to produce silk. This stage lasts around 5 days.

2. Insect Selection for Infection

1. Select last instar larvae, which are 2-3 cm long and 180-250 mg in weight, 24 hr before infections and put them into an empty box to starve them.
2. Remove the nascent silk cocoon around the larvae.

3. Bacterial Preparation

1. Prepare *Bacillus* bacterial suspensions to obtain final concentrations ranging from 10^4 colony forming units (cfu)/ml to 10^8 cfu/ml for intra haemocoelic injection and from $3x10^6$ cfu/ml to $1x10^8$ cfu/ml for ingestion (the cfu to obtain a LD_{50} depends on the bacterial species). In our case, *B. cereus* is grown in Luria-Bertani broth medium (LB, 10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl) at 37 °C under agitation until entry into stationary phase, corresponding to the incurvation of the growth curve. Measure the OD 600 nm (between 1 and 2 for *B. cereus*) and use it to evaluate bacterial concentration, using a previously made titration curve. Harvest bacteria by centrifugation at 5,000 x g for 10 min at room temperature and resuspend in Phosphate Buffered Saline (PBS: 1M KH_2PO_4, 1M K_2HPO_4, 5M NaCl, pH 7.2). Each larva will receive 10 µl of bacterial suspension at the desired concentration.
2. Alternatively, spore suspension can be prepared for infection. Obtain spores by culturing the bacteria in the sporulation medium HCT (5 g/L tryptone, 2 g/L casein hydrolysate, 12.5 mM K_2HPO_4, 12.5 mM $MgSO_4$, 0.05 mM $MnSO_4$, 1.2 mM $ZnSO_4$, 1.2 mM $Fe^2(SO_4)^3$, 0.5% H_2SO_4, 25 mM $CaCl_2$)[20] at 30 °C for 3 days. Harvest spores by centrifugation (10,000 x g, 15 min), and wash twice in sterile distilled water. Resuspend the spore pellet in sterile distilled water and heat for 15 min at 78 °C to remove remaining vegetative bacteria. Numerate the suspension by plating serial dilutions on LB agar plates. Each larva will receive 10 µl of spore suspension at the desired concentration.

4. Cry Toxin Preparation

1. Prepare the Cry1C toxin from the *B. thuringiensis* strain 407 transformed with the plasmid pHTF31C[21] carrying the gene encoding Cry1C. Culture the strain in 100 ml HCT medium at 30 °C for 72 hr with antibiotic (erythromycin 10mg/ml) to allow full sporulation, toxin crystal production and liberation in the culture supernatant.
2. Purify crystals from the supernatant on a 72%-79% sucrose gradient. First add 17 ml of 79% sucrose in a 40 ml tube. Carefully layer 17 ml of 72 % sucrose on top of the 79% sucrose. Finally, layer 5-6 ml of the 10X concentrated sporulated culture and close the tube. Centrifuge the tube for 14 hr at 20,000 x g, 4 °C, to separate the crystals from the spores. Collect the crystals at the gradient interface. Resuspend the crystal pellet in 25 ml cold sterile water to wash it. Centrifuge at 8,000 x g for 20 min. This step is repeated three times to remove all sucrose sticking to the crystals. Resuspend the crystals in 5 ml sterile water and observe under the microscope to evaluate purity.
3. Evaluate toxin protein concentration by classic Bradford staining on crystal solution presolubilized in 50 mM NaOH. Toxin crystals can be stored at -20 °C until use.

5. Injector Preparation

1. To control the precise injection volume, use an automated syringe pump (*e.g.* KD Scientific KDS 100) with setup speed to 1 µl/sec (**Figure 1A**).
2. Fill a 1 ml hypodermic syringe with 300 µl water or the bacterial solution (1 syringe per condition) for infection of 20-25 larvae. Remove the bubbles by carefully tapping the syringe, then attach a 0.45 x 12 mm needle to the syringe.
3. Place the syringe in the injector.
4. Perform a blank injection of 10 µl in an empty tube to control the injected volume.

6. Insect Intrahaemocoelic Injection

1. Place the insect manually between the thumb and forefinger. Then insert the needle fixed in the injector in the larva skin (cuticle) (**Figure 1C**).
2. Keep the insect in place while injecting the 10 µl bacterial solution (spore or vegetative bacteria).
3. Carefully remove the insect from the needle.
4. Place the infected insect in a small Petri dish (5 cm in diameter), 5 larvae per dish.
5. Infect at least 20 larvae for each experimental condition.
6. Place the dishes at 37 °C in an incubator for 48 hr.

7. Insect Force Feeding (Ingestion)

1. In a 2 ml Eppendorf tube, mix 0.2 µg/µl of Cry1C toxin with either spore or vegetative cell suspension to obtain final concentrations ranging from $3x10^4$ to $1x10^7$ per 10 µl.

2. Fill a 1 ml syringe with a 30G, 25 mm hypodermic needle with 300 µl of the bacterial-Cry1C toxin solution for the infection of 20-25 larvae. Remove the bubbles from the syringe.
3. Place the insect manually so that its mouth enters the needle fixed in the injector (**Figure 1D**), and force-feed it with 10 µl of the bacterial solution using the automated syringe pump.
4. Infect at least 20 larvae for each experimental condition.
5. Place the infected insect in a small 5 cm Petri dish (5 larvae per dish). Place the dishes at 37 °C in an incubator for 48 hr.

8. Recording Insect Mortality

1. After force-feeding or injection experiments, keep the larvae in the Petri dish without food at 25 °C or 37 °C. Check larval mortality regularly over a 48 hr period. Dead larvae are inert and usually turn black (**Figure 1B**).
2. Mortality data can be analyzed using the log-Probit program[14]. This program tests the linearity of dose mortality curves and provides lethal doses (LD_{50}). Alternatively, other programs, such as Prism (Graph Pad), analyzing survival or mortality data can be used.

Representative Results

Intra haemocoelic injection of bacteria into *G. mellonella* has been proven very useful for the identification of many virulence factors dealing with tissue damage and resistance to innate immune factors of several human pathogens. As an example, **figure 2A** represents insect mortality after injection of various doses of *B. cereus* bacteria (wild type and mutant strains)[22]. **Figure 2B** represents bacterial survival after infection of *G. mellonella* by *Pseudomonas aeruginosa*[4].

The use of *Galleria* as an oral infection model for *B. cereus* and *B. thuringiensis* relies on the evaluation of the synergistic effect of the vegetative bacteria or spores on the insecticidal activity of the Cry1C toxin[10]. The larvae are susceptible to the ingestion of spore/toxin mixture but are only weakly susceptible to the ingestion of Cry1C toxin alone. The larval death results from septicemia after bacteria have reached the haemocoel following the breakdown of the intestinal barriers. **Figure 3** represents larval mortality after ingestion of crystals and either wild type or mutant strains of *Bacillus* bacteria[10].

Few bacteria have been tested for oral infection in *Galleria*. Amongst the human pathogens tested, *B. cereus* strains with Cry1C toxin were the most virulent for *G. mellonella* after oral infection[24]. This method can be used to monitor infection/colonization of the gastro intestinal tract. Indeed, as shown in **Figure 4**, the larva digestive tract can be extracted after infection, and host cell modifications as well as bacterial colonization can be visualized on histological paraffin sections. In addition, ingestion of fluorescent bacteria can be used to follow the trajectory and the colonization process along the different compartments of the larvae. Finally, the specific expression of a gene in the different insect compartments can be monitored by fluorescent microscopy[25].

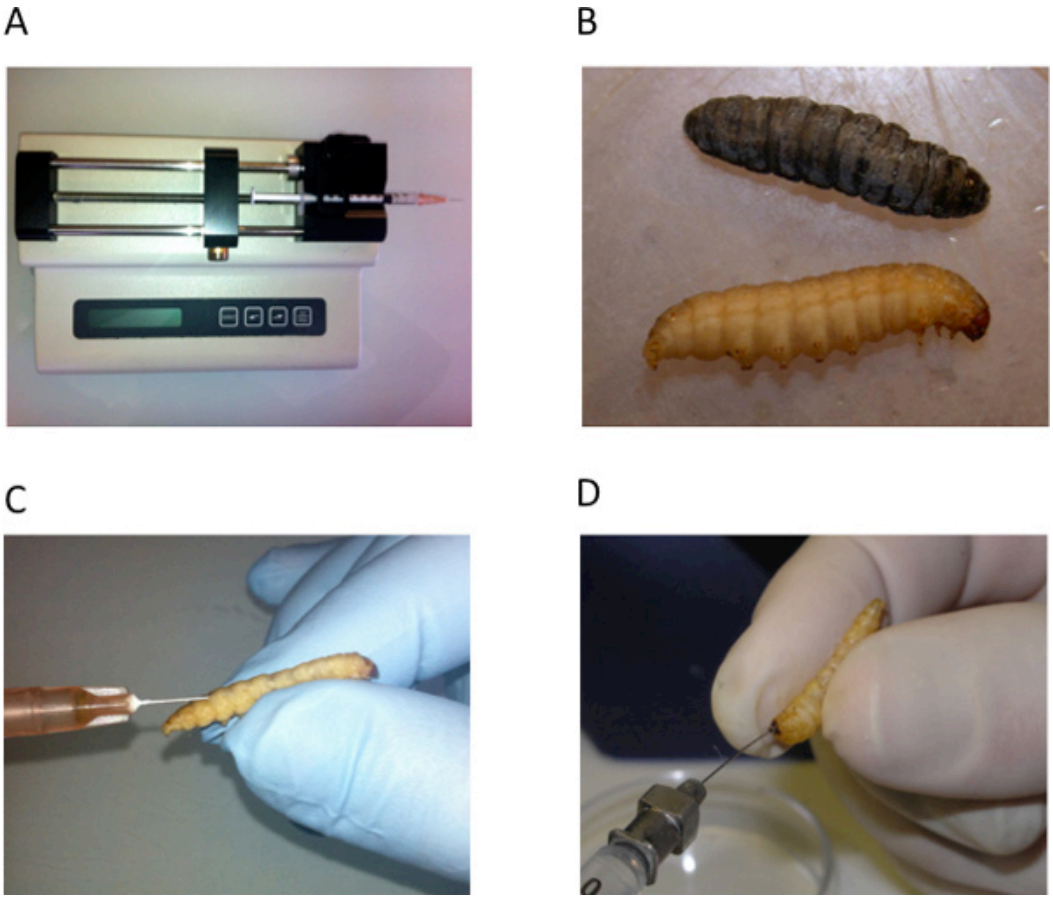

Figure 1. (A) Automated syringe pump injector. (B) Dead (top, black) and alive (bottom, white) *G. mellonella* larvae. (C) Bacteria are injected in the haemocoel of *G. mellonella* larvae. (D) *G. mellonella* larvae are force-fed with bacterial preparation: the needle enters the mouth of the insect larvae.

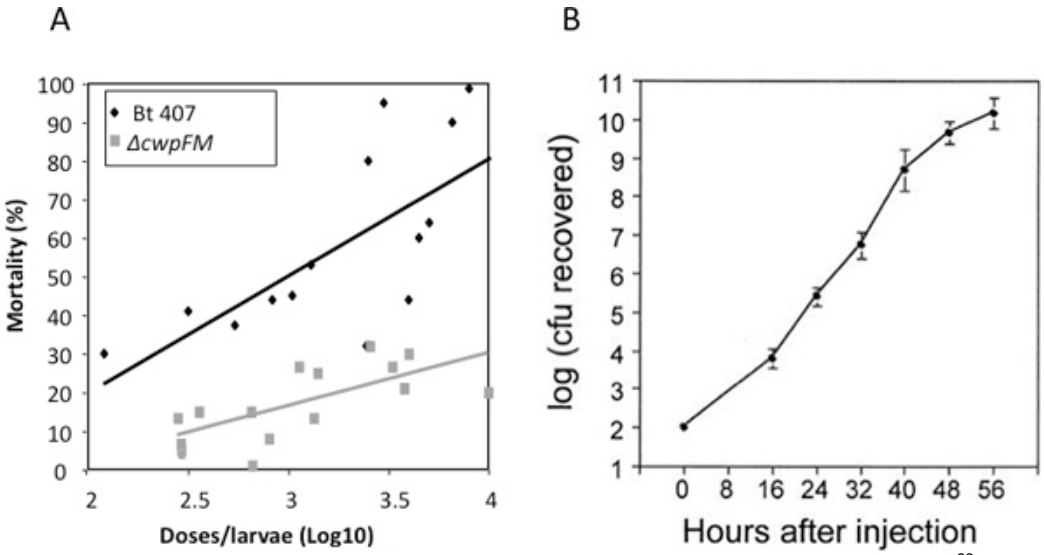

Figure 2. Results after intra haemocoelic injection. (A) CwpFM virulence against *G. mellonella* larvae[22]. Various concentrations of *B. cereus* wild-type (Bt 407) and ΔcwpFM mutant strains were inoculated into the haemocoel of 20 *G. mellonella* larvae. Mortality was recorded after 24 hr at 37 °C. (B) Time course of *Pseudomonas aeruginosa* PA14 infection in *G. mellonella*[4]. Twenty-five bacteria per larva were injected at time zero. Larvae were crushed at the indicated time points post-infection and bacterial counts were determined by plating. Each data point represents the mean and standard deviation of bacterial counts from 10 groups of 10 larvae. Larvae died approximately 48 hr after injection, when bacterial concentrations reached 10^9/g.

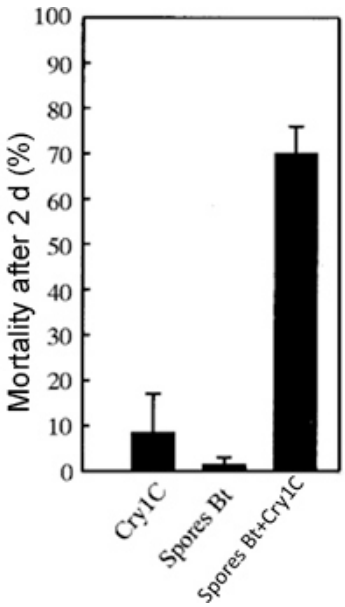

Figure 3. Synergism of spores and Cry1C toxin crystals as an evaluation of pathogenicity in *G. mellonella* larvae after oral ingestion[10]. *G. mellonella* larvae were orally infected with either Cry1C toxin crystals (1 µg per larva), or with 10^6 *B. thuringiensis* spores, or with a mix of Cry1C toxin and *B. thuringiensis* spores. Larvae mortality was evaluated after 48 hr at 25 °C.

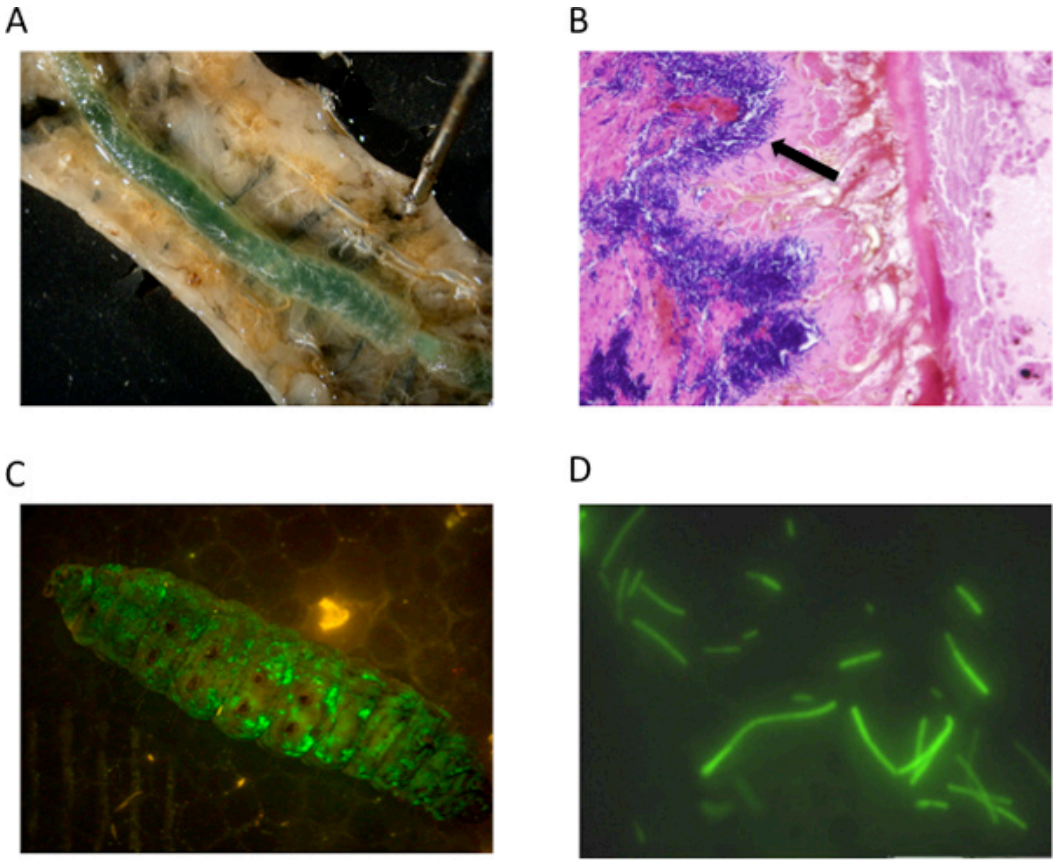

Figure 4. Oral infection of larvae: colonization and tissue localization of bacteria. (A) 10 µl of blue dye solution was introduced by force-feeding into the mouth of a *G. mellonella* larva. The larva was dissected to reveal the intestine filled with the blue dye. (B) A mixture of Bt 407 strain and Cry1C toxin was introduced into *G. mellonella* larvae by force-feeding. After 24 hr, whole larvae were paraffin embedded, cut and fixed. Histological longitudinal sections were stained (hematoxylin & Eosine and Gram straining). The light microscopic photograph (400X) shows the gut (dark purple) and the Gram stained *Bacillus* (dark violet) localized at the intestinal surface. (C) A mixture of Bt 407-Gfp fluorescent strain and Cry1C toxin was introduced into *G. mellonella* larvae by force-feeding. After 24 hr, the bacteria have reached the haemocoel and the insect cadaver is filled with *B. cereus* expressing the green fluorescent protein. (D) Analysis of *ilsA* expression *in vivo*[25]. Microscopic observations by epifluorescence were performed on *B. cereus* carrying pHT315-*pilsA'gfp*. Bacteria were isolated from the hemocoel of dead larvae 24 hr after oral infection. Green bacteria indicate expression of gfp due to the activation of the *ilsA* promoter. This shows the specific expression of this gene (*ilsA*) when the bacteria have reached the haemocoel.

Movie 1. Spreading of solution in the insect gut following oral ingestion. 10 µl of blue dye solution was introduced by force-feeding into the mouth of the European corn borer *Ostrinia nubilalis* larva. The dye quickly fills in the intestinal lumen of the insect larvae. Click here to view movie.

Discussion

The use of insects and especially the larval stage, as infection models for several pathogens, is becoming frequent. A model of choice for some aspects is *Drosophila* (the fly model) used as both adults and larval stage[1,2]. The lepidopteran insect *G. mellonella* has also been mainly used for assaying bacterial virulence by injection. The advantage of tolerating higher temperatures (above 37 °C) than *Drosophila* (maximum 25 °C) is important when mammal pathogens are to be studied. Also the size of *Galleria* is more convenient for studying infection kinetics and tissue damage. Finally, our protocol describes the possibility to use *Galleria* for oral infection, which is an advantage for studies related to the digestive tract. Similarly, the larval stage of another large caterpillar, Manduca sexta, is used to study infections by injection into the haemocoel and more recently for oral infection[26]. *B. mori* and *Spodoptera* species, which genomes are sequenced[27] also offer interesting alternatives, and host genes can be shut down by RNAi methods or other gene manipulations[28]. Here, we describe in a simple way, how to rear *Galleria* larvae, how to infect them and some examples on how to follow the infection process and score mortality in order to show the potential of such models.

Disclosures

No conflicts of interest declared.

Acknowledgements

We would like to thank Elisabeth Guillemet, Christophe Buisson and Ludovic Bridoux for excellent technical assistance. We are greatly indebted to Sylvie Salamitou and Sinda Fedhila for the initial system setup.

References

1. Lemaitre, B. & Hoffmann, J. The host defense of *Drosophila melanogaster. Annu. Rev. Immunol*. **25**, 697-743 (2007).
2. Vodovar, N., Acosta, C., Lemaitre, B., & Boccard, F. *Drosophila*: a polyvalent model to decipher host-pathogen interactions. *Trends Microbiol*. **12**, 235-242 (2004).
3. Dalhammar, G. & Steiner, H. Characterization of inhibitor A, a protease from *Bacillus thuringiensis* which degrades attacins and cecropins, two classes of antibacterial proteins in insects. *Eur. J. Biochem.* **139**, 247-252 (1984).
4. Jander, G., Rahme, L.G. & Ausubel, F.M. Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. *J. Bacteriol.* **182**, 3843-3845 (2000).
5. Purves, J., Cockayne, A., Moody, P.C., & Morrissey, J.A. Comparison of the regulation, metabolic functions, and roles in virulence of the glyceraldehyde-3-phosphate dehydrogenase homologues gapA and gapB in Staphylococcus aureus. *Infect. Immun.* **78**, 5223-5232 (2010).
6. Chadwick, J.S. Serological responses of insects. *Fed. Proc.* **26**, 1675-1679 (1967).
7. Chadwick, J.S., Caldwell, S.S. & Chadwick, P. Adherence patterns and virulence for *Galleria mellonella* larvae of isolates of Serratia marcescens. *J. Invertebr. Pathol.* **55**, 133-134 (1990).
8. Gao, W., *et al.* Two novel point mutations in clinical *Staphylococcus aureus* reduce linezolid susceptibility and switch on the stringent response to promote persistent infection. *PLoS Pathog.* **6**, e1000944 (2010).
9. Peleg, A.Y., *et al.* Reduced susceptibility to vancomycin influences pathogenicity in *Staphylococcus aureus* infection. *J. Infect. Dis*. **199**, 532-536 (2009).
10. Salamitou, S., *et al.* The *plcR* regulon is involved in the opportunistic properties of *Bacillus thuringiensis* and *Bacillus cereus* in mice and insects. *Microbiology.* **146**, 2825-2832 (2000).
11. Cadot, C., *et al.* InhA1, NprA and HlyII as candidates to differentiate pathogenic from non-pathogenic *Bacillus cereus* strains. *J. Clin. Microbiol.* **48**, 1358-1365 (2010).
12. Rejasse, A., *et al.* Temperature-dependent production of various PlcR-controlled virulence factors in *Bacillus* weihenstephanensis strain KBAB4. *Appl. Environ. Microbiol.* **78**, 2553-57 (2012).
13. Jones, R.T., *et al.* Photorhabdus adhesion modification protein (Pam) binds extracellular polysaccharide and alters bacterial attachment. *BMC Microbiol*. **10**, 141 (2010).
14. Finney, D.J. *Probit analysis.* Cambridge University Press, (1971).
15. Fedhila, S., Nel, P., & Lereclus, D. The InhA2 metalloprotease of *Bacillus thuringiensis* strain 407 is required for pathogenicity in insects infected via the oral route. *J. Bacteriol.* **184**, 3296-3304 (2002).
16. Guillemet, E., *et al.* The InhA metalloproteases of *Bacillus cereus* contribute concomitantly to virulence. *J. Bacteriol.* **192**, 286-294 (2010).
17. Nielsen-LeRoux, C., Gaudriault, S., Ramarao, N., Lereclus, D., & Givaudan, A. How the insect pathogen bacteria *Bacillus thuringiensis* and Xenorhabdus/Photorhabdus occupy their hosts. *Curr. Opin. Microbiol.* **15**, 1-12 (2012).
18. Bottone, E.J. *Bacillus cereus*, a volatile human pathogen. *Clin. Microbiol. Rev.* **23**, 382-398 (2010).
19. Stenfors Arnesen, L., Fagerlund, A., & Granum, P. From soil to gut: *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiol. Rev*. **32**, 579-606 (2008).
20. Lecadet, M., Blondel, M.O., & Ribier, J. Generalized transduction in *Bacillus thuringiensis* var. berliner 1715, using bacteriophage CP54 Ber. *J. Gen. Microbiol.* **121**, 203-212 (1980).
21. Sanchis, V., Agaisse, H., Chaufaux, J., & Lereclus, D. Construction of new insecticidal *Bacillus thuringiensis* recombinant strains by using the sporulation non-dependent expression system of cryIIIA and a site specific recombination vector. *J. Biotechnol.* **48**, 81-96 (1996).
22. Tran, S.L., Guillemet, E., Gohar, M., Lereclus, D., & Ramarao, N. CwpFM (EntFM) is a *Bacillus cereus* potential cell wall peptidase implicated in adhesion, biofilm formation and virulence. *J. Bacteriol.* **192**, 2638-2642 (2010).
23. Tran, S.L., *et al.* Hemolysin II is a *Bacillus cereus* virulence factor that induces apoptosis of macrophages. *Cell Microbiol.* **13**, 92-108 (2011).
24. Fedhila, S., *et al.* Comparative analysis of the virulence of invertebrate and mammalian pathogenic bacteria in the oral insect infection model *Galleria mellonella*. *J. Invertebr. Pathol.* **103**, 24-29 (2010).
25. Daou, N., *et al.* IlsA, a unique surface protein of *Bacillus cereus* required for iron acquisition from heme, hemoglobin and ferritin. *PLoS Pathog*. **5**, e1000675 (2009).
26. Mason, K.L., *et al.* From commensal to pathogen: translocation of Enterococcus faecalis from the midgut to the hemocoel of Manduca sexta. *MBio*. **2**, e00065-00011 (2011).
27. Goldsmith, M.R., Shimada, T., & Abe, H. The genetics and genomics of the silkworm, *Bombyx mori*. *Annu. Rev. Entomol.* **50**, 71-100 (2005).
28. Fraser, M.J., Jr. Insect transgenesis: current applications and future prospects. *Annu. Rev. Entomol.* **57**, 267-289 (2012).

