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Molecular pathogenesis of *Listeria monocytogenes* in the alternative model host *Galleria mellonella*

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Larvae of *Galleria mellonella*, the greater wax moth, provide an alternative infection model for many human pathogens as they are amenable to use at elevated incubation temperatures (37 °C). This study and a parallel study by Mukherjee *et al.* [Mukherjee, K., Altincicek, B., Hain, T., Domann, E., Vilcinskas, A. & Chakraborty, T. (2010). *Appl Environ Microbiol* **76**, 310–317] establish this insect host as an appropriate model to investigate the pathogenesis of *Listeria* species. In this study we show that inoculation with *Listeria monocytogenes* initiates a dynamic infection in *G. mellonella* and that production of the cytolysin listeriolysin O (LLO) is necessary for toxicity and bacterial growth. Production of LLO by the non-pathogenic species *Lactococcus lactis* is sufficient to induce mortality in the insect model. We employed real-time bioluminescence imaging to examine the dynamics of listerial growth and virulence gene expression in the *G. mellonella* model. Analysis of *lux* promoter fusions demonstrated significant induction of virulence gene expression upon introduction of the pathogen into insects at both 30 and 37 °C. The host response to listerial infection was examined which demonstrated that haemocyte destruction accompanies *L. monocytogenes* pathogenesis and is preceded by activation of the phenoloxidase system. Furthermore, we demonstrate that *Listeria innocua* is pathogenic to *G. mellonella* through a persistence mechanism that implicates an alternative mechanism for pathogenicity in this model.

INTRODUCTION

Listeria monocytogenes is an invasive foodborne Gram-positive pathogen that causes sporadic and epidemic disease (listeriosis) that is often fatal (20–30 % case fatality rates). The pathogen has a facultative intracellular life cycle with the capacity for cellular invasion, intracellular replication and movement from cell to cell without an extracellular phase. The intracellular pathogenesis of *L. monocytogenes* is mainly attributed to the presence of a multi-gene virulence island that is regulated by the CRP/FNR-type protein PrfA (Freitag *et al.*, 2009; Greene & Freitag, 2003; Sheehan *et al.*, 1995). PrfA also regulates expression of factors required for cellular invasion (InlA and InlB) and intracellular growth (Hpt) that are located elsewhere on the chromosome (Hamon *et al.*, 2006). Virulence factors encoded on the virulence gene island include listeriolysin O (LLO, encoded by *hly*), a protein that is absolutely required for phagosomal escape and pathogenesis of *L. monocytogenes* in mammalian models of disease (Freitag *et al.*, 2009; Hamon *et al.*, 2006). Also present at this locus is a gene encoding ActA, a protein required for actin polymerization and intracytoplasmic movement (Hamon *et al.*, 2006). Clearly PrfA is essential in order to direct virulence gene expression in *L. monocytogenes* during the invasive and systemic phases of infection and PrfA deletion mutants are significantly attenuated in the murine model of disease (Chakraborty *et al.*, 1992). In addition, the stress response factor SigB plays a significant role within the gastrointestinal tract and also positively regulates PrfA and other factors during adaptation to stress (Garner *et al.*, 2006; Nadon *et al.*, 2002). The non-pathogenic species *Listeria innocua* appears to have evolved from *L. monocytogenes* and lacks the virulence locus as well as specific genes (*inlA*, *inlB*, *bsh* and *hpt*) that encode virulence factors (Buchrieser *et al.*, 2003).

Many studies have established the murine model as a useful model for analysis of the systemic phase of listerial pathogenesis (Lecuit, 2007). However, the murine model has a number of limitations including poor interaction between bacterial InlA and the cognate host receptor (E-cadherin), relatively high cost and ethical considerations associated with the analysis of mammalian hosts (Disson *et al.*, 2008, 2009; Lecuit, 2007). Alternative model systems

Abbreviations: AMP, antimicrobial peptide; BHI, brain heart infusion; CFSE, carboxylfluorescein succinimydyl ester; IVIS, *in vitro* imaging system; LLO, listeriolysin O; PGRP-LE, peptidoglycan recognition protein; PO, phenoloxidase; PPO, pro-phenoloxidase.

Two supplementary tables and two supplementary figures are available with the online version of this paper.

have contributed to our understanding of the infectious process of *L. monocytogenes* species. These include *Drosophila melanogaster* (Cheng & Portnoy, 2003; Mansfield *et al.*, 2003), *Caenorhabditis elegans* (Thomsen *et al.*, 2006), *Acanthamoeba polyphaga* (Akya *et al.*, 2009a) and *Danio rerio* embryos (Levraud *et al.*, 2009), all of which have well-developed innate immunity but which lack adaptive immunity. Recently, *D. melanogaster* has contributed significantly as a model for *Listeria* infection revealing a Toll- and IMD-independent system, autophagy, which is induced by a long form secreted and intracellular peptidoglycan recognition protein (PGRP-LE) as a first line defence mechanism against *L. monocytogenes*. PGRP-LE also induces melanization and the cellular response in insects. The activity of this enzyme appears crucial for host survival in the presence of *L. monocytogenes* (Yano *et al.*, 2008).

The *G. mellonella* insect model system has a significant advantage over other alternative models in that the larvae can be incubated at human core temperature (37 °C). Very recently, Mukherjee and coworkers examined larvae of *G. mellonella* and validated this species as an alternative host model for the analysis of *Listeria* pathogenesis (Mukherjee *et al.*, 2010). We have concurrently performed independent experiments to determine the nature of the interaction between *Listeria* species and this insect model and our work extends the analysis of *Listeria* infection in this system. We confirm that *Listeria* species are pathogenic to *G. mellonella*. Furthermore, *lux* promoter fusion experiments show that virulence genes required for *L. monocytogenes* infection of mice are highly induced during infection of *G. mellonella* larvae. We also show that LLO production is a key mechanism for induction of insect mortality by *L. monocytogenes* and that infection of insects with a non-insect-pathogenic *Lactococcus* (*Lact.*) *lactis* expressing LLO is directly toxic to insects. On examining the host response we find that the phenoloxidase system mounts a response to the presence of *Listeria* species within 4 h of insect infection. Taken together, we demonstrate that pathogenesis of *L. monocytogenes* infection in the *G. mellonella* model results from high level expression of virulence genes and a dependence upon LLO for haemocyte toxicity and insect mortality.

METHODS

Maintenance of strains and cultures. Strains and plasmids utilized in this study are listed in Supplementary Table S1, available with the online version of this paper. *Listeria* strains were routinely cultured in brain heart infusion (BHI; Oxoid) unless otherwise stated. *Escherichia coli* was routinely cultured using Luria–Bertani broth and *Lactococcus* strains were cultured in M17 (Oxoid) media supplemented with 0.5 % D-Glucose. *Listeria* and *E. coli* strains were grown with agitation at 37 °C while *Lactococcus* strains were cultivated at 28 °C under static conditions unless otherwise stated. Media were supplemented with the appropriate antibiotic where required; chloramphenicol 7.5 μg ml^{-1}; erythromycin 5 μg ml^{-1}. Nisin-inducible promoters required 20 μl (for 10 ml of media) of filter-sterilized nisin for induction.

Infection of *G. mellonella*. Overnight cultures of *Listeria* wild-type and mutant strains were inoculated as single colonies into 3 ml BHI and agitated at 37 °C overnight. These cultures were used to re-inoculate fresh medium and allowed to proceed to exponential growth (OD_{600} 0.5–0.7, unless otherwise indicated) before infection of *G. mellonella* larvae. *E. coli* and *Lactococcus* strains were treated in the same manner in their respective media and temperatures. Bacteria were collected by centrifugation at room temperature, rinsed with PBS then resuspended in sterile PBS to 1 OD_{600} unit. Where required, serial dilutions were performed in sterile PBS or PBS containing the appropriate antibiotic. Insects were obtained from Livefood, UK, and stored at room temperature in the dark until use. Ten individual insects were injected with 10 μl of the relevant bacterial suspension into the first right pro-leg of the second set of pro-legs using a sterile Hamilton syringe and a 30 gauge disposable needle. For gavage, the 30 gauge needle was inserted between the open insect mandibles for delivery of the dosage. All ten insects were placed in a 9.0 cm Petri-dish lined with 8.5 cm Whatman paper, then incubated at the appropriate temperature, usually 37 °C, in the dark. The same number of insects was injected with PBS alone and with PBS containing antibiotic where relevant. Insects were individually examined for the production of pigmentation and the time of death was recorded. Death assays performed at 37 °C were allowed to proceed for 5 days only as edycis (pupa formation) was evident at this temperature by day 6. Bacterial suspensions were serially diluted and plated on one of BHI, LB or SW17 agar to allow the number of c.f.u. for each inoculation to be determined. Three independent replicates of each infection experiment were performed per infection strain.

Determination of bacterial numbers in *G. mellonella*. One hundred insects per variant (10 Petri-dishes per variant) were injected with the relevant dose of exponentially growing bacteria as described above. The initial inoculation was termed $t=0$ and was serially diluted and plated to determine the c.f.u. injected per variant. Infection was allowed to progress and at various time points (1, 2, 4, 5, 6, 20, 24 h) post-infection, five insects were removed and individually surface sterilized using 70 % ethanol. Each insect was added to a 30 ml sterile tube containing 10 ml sterile PBS and 10 3 mm glass beads. The individual insects were crushed by vortexing for 2 min. The mixture was serially diluted and plated onto the relevant agar medium to determine the exact number of c.f.u. present in each insect at each time point.

Real-time visualization of *L. monocytogenes* infection. *L. monocytogenes* EGDe has been chromosomally tagged with the *Photorhabdus luminescens* bioluminescence operon (LuxABCDE) in the absence of an upstream promoter (negative control) and in the presence of nine different promoters (Bron *et al.*, 2006; Riedel *et al.*, 2007, 2009). Promoters analysed represent well-characterized virulence genes of *L. monocytogenes* as well as the constitutive promoter P_{help}; EGDe::luxP_{help} was used as a positive control (see Supplementary Table S1). This promoter fusion was also applied to *L. innocua* strain Clip 11262 to allow detection of infection by either *Listeria* species under different infection and temperature conditions. Each of the ten bioluminescent-tagged strains was grown for injection as described above. Ten individual insects were injected with each variant (OD_{600} 0.1) and placed in duplicate on 4.0 cm Petri-dishes. The insects were incubated at 37 °C in the chamber of an *in vitro* imaging system (IVIS-100). Bioluminescence levels (490 nm) were recorded at 30 min intervals for 1 min over a 24 h time period. Light production was quantified using the Living Image programme (Xenogen) from each Petri-dish and therefore from each of the different promoters over the time-course of an infection in *G. mellonella*. Experiments were repeated three times independently.

Phenoloxidase (PO) activity. Insects were infected as described above. At different time points during the infectious process, insects

were chilled on ice for 15 min, surface sterilized with 70 % ethanol and then dissected to collect the insect haemolymph to a pre-chilled Eppendorf containing the same volume of Graces insect media (GIM). The plasma was isolated following centrifugation of the mixture at 14 000 r.p.m. for 10 min at 4 °C and retained on ice. The PO activity in the haemolymph plasma of final instar (last moult) *G. mellonella* larvae was quantified by using a microplate enzyme assay as described by Eleftherianos *et al.* (2006). Briefly, a reaction mixture containing 115 µl 50 mM PBS buffer (pH 6.5), 10 µl diluted haemolymph plasma, 2 µl *E. coli* LPS (5 mg ml^{-1}, in control reactions only) (Sigma) was left for 1 h on a shaker at slow speed (1 r.p.m.), at room temperature, to allow the activation of the enzyme; the reaction was started by adding 25 µl 20 mM 4-methyl catechol (Sigma). The change in absorbance was read at 490 nm for 1 h at room temperature with a reading taken every 1 min. Three insects were used per treatment and the experiment was repeated three times.

Haemocyte viability. At 24 h post-infection, larvae were chilled, surface sterilized and bled into pre-chilled GIM and the cell density was adjusted accordingly to 5×10^6 ml^{-1}. Cells were washed three times with GIM and trypan blue (0.02 % in PBS) stained for 10 min at room temperature, washed three times with PBS and then fixed with 4.0 % paraformaldehyde in PBS. Trypan-blue-stained and total cell counts were visualized and recorded with a light microscope at × 200 magnification using a haemocytometer and a Countess Cell Counter (Invitrogen).

Confocal imaging of *Listeria* infection of insect haemocytes. Differential labelling of *Listeria* species allows the distinctive detection of intracellular and surface-bound bacteria. Carboxylfluorescein succinimydyl ester (CFSE) attaches to bacterial cells by forming stable conjugates with aliphatic amines (Karrer *et al.*, 1992) to monitor the Gram-positive bacterial population. In order to distinguish between internalized and extracellular bacterial cells the bacterial population was labelled with CFSE, then linked with biotin before injection into *G. mellonella*. Briefly, overnight culture (OD_{600} 2) was pelleted then washed twice in PBS, then incubated with CFSE for 15 min in the dark and washed three times in PBS. The cells were biotinylated by addition of No-Weigh Sulpho-NHS-Biotin (Pierce Scientific), incubated at room temperature for 40 min and washed three times with PBS. Approximately 10 µl of each treated culture (OD_{600} 1) was injected into each of 10 insects as described above. Infection was continued to the following time points: 1, 2, 4, 7, 24 h post-infection. At the different time points, an average of five insects were bled (as described above) and 200 µl of the total haemolymph was added to an L-lysine-coated slide and incubated at 4 °C for 30 min. Unattached cells were washed from the slides with PBS and cells were fixed in formalin/formaldehyde (10.0 %/4.0 %, Sigma) for 20 min at room temperature and washed again. Bound cells were treated with streptavidin–allophycocyanin (Pierce) for 30 min at 4 °C. Cells were permeabilized by addition of 0.2 % Triton X-100 (Sigma) for 5 min then washed with PBS. The slides were mounted using fluorescent mounting media (DakoCytomation) and allowed to dry. Cells were visualized by confocal microscopy at the following wavelengths: 488 nm for CFSE (Fig. 6), 543–550 nm for rhodamine–phalloidin (actin), which stained any region producing actin, and 543–550 nm for streptavidin–allophycocyanin to detect non-internalized cells.

RNA extraction and RT-PCR. Approximately 7 h after infection with *Listeria*, insects were chilled on ice for 15 min, surface sterilized with 70 % ethanol and then dissected to collect the fat body tissue. Fat bodies from three individual insects were pooled (100 mg). To isolate total RNA, extracted tissues were homogenized in TRI reagent (Sigma). Single-step RT-PCR was performed using the 'OneStep' RT-PCR kit (Qiagen). Each reaction was carried out in a 50 µl volume containing 0.6 pM of forward and reverse gene primers and 2 µg of RNA template. All primers were gene-specific (Supplementary Table S2). Reverse transcription was allowed to proceed for 30 min at 50 °C, reverse transcriptases were inactivated and DNA polymerase was activated by incubation for 15 min at 95 °C. Amplifications were performed on a PTC-100 thermal controller (MJ Research) under the

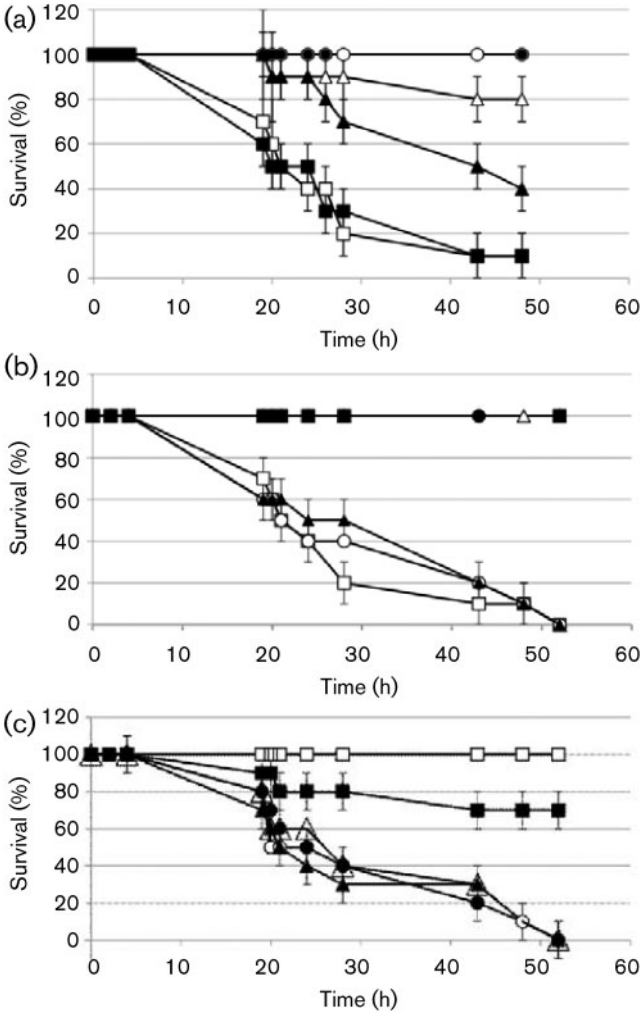

Fig. 1. *Listeria* species are pathogenic to *G. mellonella.* (a) The lethal dose of *L. monocytogenes* (filled symbols) and *L. innocua* (open symbols) required to kill 50 % of *G. mellonella* insects (LD_{50}) corresponds to 300 000 c.f.u. delivered in 10 µl for *L. monocytogenes* EGDe and 420 000 c.f.u. for *L. innocua* species. Squares, 1×10^{-2} ; triangles, 1×10^{-3} ; circles, 1×10^{-4}. (b) *L. monocytogenes* EGDe : : $luxP_{help}$ (▲) is equally as pathogenic as EGDe wild-type strain to *G. mellonella*; *L. innocua* F4 (○) is pathogenic to *G. mellonella* at the same dose, while *Bifidobacterium breve* (△) and *Lact. lactis* (■) are avirulent to the insect. □, *L. innocua* CLIP 11262; ●, PBS control. (c) *L. monocytogenes* EGDe mutated in *prfA* (■) or *hly* (□) is attenuated in *G. mellonella*, while mutants in *actA* (▲) and *sigB* (●) have no effect on virulence in this model. ○ and △, *L. monocytogenes* EGDe wild-type. For (a–c), $n=10$ per data point and error bars represent SD. Experiments were replicated three times independently and a representative experiment is shown.

following cycling conditions: 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C for 35 cycles. RT-PCR control reactions for actin protein were performed as described by Bergin *et al.* (2006). Reactions were assessed by 1.0 % agarose gel electrophoresis (90 V cm^{-1}) in TAE and visualized by ethidium bromide staining.

RESULTS

Establishing *G. mellonella* as a model system for analysis of *Listeria* infection

In order to determine if *G. mellonella* could be used as an infection model for *Listeria* infection, the human pathogenic species *L. monocytogenes* EGDe (serotype 1/2a) and the non-human pathogenic *L. innocua* Clip 11262 were used to infect insects. In initial dose–response experiments, *Listeria* species were serially diluted then injected into each of 10 individual insects and monitored for the time taken to kill 50 % of insects (LT_{50}) by each dose over a 48 h period at 37 °C (Fig. 1a–c). Both *L. monocytogenes* and *L. innocua* species reproducibly killed 100 % of insects within 48 h at 37 °C (Fig. 1a) with the highest doses at 10^7 c.f.u. per OD_{600} unit per ml and 10^6 c.f.u. per OD_{600} unit per ml, respectively. The exact LT_{50} for both of these strains was recorded as 22 ± 1 h at these dosages. Below this threshold, with 10^5 c.f.u., *L. innocua* was lethal to only 20 % of the insect larvae over 50 h while an LT_{50} of 43 h was recorded for *L. monocytogenes*. More specifically, 3×10^5 c.f.u. and 4.2×10^5 c.f.u. *L. monocytogenes* strain EGDe and *L. innocua*, respectively, were required to kill 50–100 % of insects within 24 h at 37 °C (Fig. 1b).

L. monocytogenes mutated in specific virulence genes has previously been analysed using other model systems (Levraud *et al.*, 2009; Mansfield *et al.*, 2003; Thomsen *et al.*, 2006). In order to examine the efficacy of *G. mellonella* larvae as a model system for *Listeria* infection, we examined the infectivity of LO28 and EGDe strains that are mutated for known virulence genes (Fig. 1c and Table 1). In all cases, the LT_{50} at 37 °C was recorded where *G. mellonella* insects were injected with the lowest possible wild-type killing dose (3×10^5 c.f.u.) for each variant. Here, the wild-type strain and mutants in *actA*, *inlAB* and *sigB* elicited an LT_{50} of 21–25 h and were therefore equally as virulent as the wild-type parent in the insect model. A delay of 101 h (greater than 4 days) was evident for mutants in PrfA indicating significant attenuation in this model. Strains mutated in haemolysin (LLO) production were completely attenuated for virulence; these strains did not lead to mortality in any insect in any of three replicates. Comparative LT_{50} was also calculated for other *L. monocytogenes* strains with mutations in *hly* and *prfA* (Table 1). *L. monocytogenes* LO28 (serotype 1/2c), F2365 (serotype 4b) and EGDe (serotype 1/2a) wild-type strains showed similar LT_{50} values of 21–25 h on injection of equivalent c.f.u. values. However, the LT_{50} value calculated for the F2365 PrfA mutant was 46–48 h, which was twofold slower than the wild-type parent strain but 2.5-fold faster than a PrfA mutant in strain EGDe. An LLO^- strain of *L. monocytogenes* F2365 (Δ*hly*) was completely attenuated in *G. mellonella*. *L. monocytogenes* strain F2365 mutated in *sigB* demonstrated no attenuation in this model system. Finally, non-pathogenic commensal bacteria, *Bifidobacterium breve* UCC2003, *Lact. lactis* NZ9000 and *E. coli* EC101 were non-pathogenic to *G. mellonella*; no larvae were killed 5 days post-injection (Table 1).

Temperature affects *Listeria* infection of *G. mellonella*

The *G. mellonella* insect model system has the advantage of being amenable to a broad range of incubation temperatures following infection. It has been well documented that many of the known virulence genes in *L. monocytogenes* exhibit temperature-dependent expression (McGann *et al.*, 2007; Peel *et al.*, 1988). In order to determine whether such effects could be determined in the *G. mellonella* insect model system, 10 individual insects were infected with one of *L. innocua*, *L. monocytogenes* EGDe or with *L. monocytogenes* mutant strains as described above, and then monitored hourly during incubation at 30 and 37 °C. The mutants chosen were of two types (i) temperature-dependent genes that require elevated temperatures for optimal expression *in vitro*: *hly*, *prfA*, *sigB*, *inlAB*; and (ii) temperature-dependent genes that require lower temperatures for expression: *cheA* and *fliA*. *L. monocytogenes* was capable of causing insect death at both temperatures,

Table 1. LT_{50} of *G. mellonella* at 30 and 37 °C when injected with 10 µl of exponentially growing cells (OD_{600} 0.1) for different strains

Experiments were performed in triplicate; values shown are mean ± SD. ND, Not done.

Bacterial strain	LT_{50} (h) at 37 °C	LT_{50} (h) at 30 °C
L. monocytogenes EGDe	23 ± 3	29 ± 3
EGDeΔ*inlA*	23 ± 2	32 ± 3
EGDeΔ*sigB*	23 ± 2	28 ± 2
EGDeΔ*actA*	23 ± 2	32 ± 2
EGDeΔ*fur*	23 ± 3	ND
EGDeΔ*prfA*	124 ± 7	No death
EGDeΔ*hly*	No death	No death
EGDeΔ*cheA*	36 ± 5	32 ± 2
EGDeΔ*fliA*	36 ± 5	48 ± 3
L. monocytogenes F2365	22 ± 3	ND
F2365Δ*sigB*	22 ± 2	ND
F2365Δ*hly*	No death	ND
F2365Δ*prfA*	48 ± 3	ND
L. monocytogenes LO28	21 ± 3	ND
L. innocua Clip 11262	23 ± 2	22 ± 1
L. innocua F4	23 ± 3	32 ± 2
B. breve UCC2003	No death	ND
Lact. lactis NZ9000	No death	ND
E. coli DH10B	No death	ND

although a 5–7 h delay in mortality was evident at 30 °C (Table 1). Infection of larvae with an *L. monocytogenes* mutant in *prfA* incubated at 30 °C did not result in insect mortality even after 5 days, while at 37 °C this mutant showed an LT_{50} of 124 h. Strains with mutations in *inlA* and *inlB* showed no difference in LT_{50} at 30 and 37 °C and mirrored wild-type infections at these temperatures. *L. monocytogenes* EGDe strains mutated in either *cheA* or *fliA* were equally as pathogenic as *G. mellonella* at 37 °C, although a delay of 10–13 h was evident when compared with the wild-type strain. A differential effect was clear when the LT_{50} of these mutants was examined at 30 °C. Here, the *cheA* mutant was equally as pathogenic as the wild-type strain (LT_{50} of 32 h) to the insect larvae while the LT_{50} of the *fliA* mutant increased to 48 h, indicating that the presence of flagella plays a role in pathogenicity at 30 °C. *L. monocytogenes* is motile at temperatures below 30 °C. Taken together, these data implicate a role for temperature in the resolution of virulence of *Listeria* species during infection of *G. mellonella* larvae and suggest an optimal temperature for *L. monocytogenes* pathogenesis of 37 °C in this model system.

L. monocytogenes infection of *G. mellonella* is accompanied by bacterial growth

We utilized bioluminescent *L. monocytogenes* (Riedel *et al.*, 2007, 2009) to monitor growth in the insect. Our data represent a summary of three independent experiments. We have shown that *L. monocytogenes* EGDe::luxP$_{help}$ (Lux-tagged strain) is equally as pathogenic as the wild-type is to *G. mellonella* insects (Fig. 1b). Similarly survival of insects in the model is identical following infection with the Lux-tagged *L. innocua*::luxP*help* strain as with the wild-type *L. innocua* strain (data not shown). Following infection with Lux-tagged *Listeria*, luminescence was clearly detectable using the Xenogen IVIS system (Fig. 2a). *L. monocytogenes* EGDe::luxP$_{help}$ and *L. innocua* 11262::luxP$_{help}$ were used to infect insects by either injection or oral gavage (Fig. 2b). During the first 2 h after infection, the level of bioluminescence decreased rapidly for both strains irrespective of the means of infection. For the injected *L. monocytogenes*, an increase in light emission was seen at 4 h post-infection that persisted for 10 h before reaching a basal level until death of the insects. Death of the insects coincided with an increase in the relative luminescence. *L. innocua* also demonstrated a twofold increase in light levels between 15 and 35 h post-infection. In contrast, gavaged *L. monocytogenes* EGDe::luxP$_{help}$ and *L. innocua* 11262::luxP$_{help}$ both showed decreasing light production throughout the course of infection.

In order to confirm the growth kinetics of *L. monocytogenes* and *L. innocua* in *G. mellonella*, insects were crushed and the bacteria were harvested and quantified. Analysis of bacterial numbers confirmed that wild-type *L. monocytogenes* numbers decreased for the first 2 h post-injection, with a rapid increase in bacterial numbers thereafter (Fig. 2c). For *L. innocua*, c.f.u. counts also fell significantly within the first 2 h post-infection but rapid growth was never achieved, rather the bacteria persisted in the insect until death. We also examined the growth of the *L. monocytogenes* mutant variants deleted in *sigB*, *actA*, *prfA* and *hly* (Fig. 2d). Mutants were reduced following initial inoculation and failed to recover to the same levels as the wild-type over the course of the infection with only the *hly* (LLO) mutant appearing completely defective for growth. The data indicate that specific mutations affect the capacity for *L. monocytogenes* to grow in the insect host but that mortality is not directly related to only bacterial numbers *in vivo*.

Haemolysin production is critical to *L. monocytogenes* pathogenesis in *G. mellonella*

The fact that mutants in haemolysin production were completely attenuated in this model system (Table 1) suggests that haemolysin (LLO) is a dominant factor for *L. monocytogenes* pathogenicity in this insect model. In order to determine if the presence of this cytolysin alone is sufficient to cause insect death, the non-insect-pathogenic *Lact. lactis* strain NZ9000 (Fig. 1c and Table 1) was used to deliver LLO to *G. mellonella* insects. *Lact. lactis* containing a plasmid either expressing LLO constitutively (pQE30/*hly*) or expressing LLO under the control of a nisin-inducible promoter (pNZ8048) was injected into *G. mellonella* larvae and insect death was monitored as described above. At the highest dose (OD_{600} 0.1), strains either that constitutively expressed LLO or where expression was pre-induced by addition of nisin caused insect death 3 days post-infection (Fig. 3a) with up to 70 % of insects killed by 4 days post-infection. An LT_{50} of 73 h was recorded following infection at 37 °C. Control inoculants (*Lact. lactis* with empty vector, *Lact. lactis* carrying LLO in pNZ8048 without nisin induction, PBS containing nisin and/or antibiotic) had no effect on *G. mellonella* mortality. The growth of *Lact. lactis* expressing LLO was examined over time in the insect host, as described above (Fig. 3b). Within 2 h post-infection, all of the *Lact. lactis* strains had decreased in number from 1×10^8 c.f.u. to 2×10^3 c.f.u. and they persisted at this level irrespective of the presence of haemolysin. Haemocyte viability was then examined (Fig. 3c). Here, haemocyte viability decreased to 60 % compared with control PBS injection in the presence of wild-type *Lact. lactis*, which could indicate haemocyte migration to the insect fat body (primitive liver). However, the presence of LLO further reduced this level to between 30 and 40 %. Taken together, these data indicate that haemolysin production alone is sufficient to cause insect death within 72 h when expressed in high-density infections and that its presence does not confer any growth advantage on *Lact. lactis* in this model.

In vivo monitoring of *L. monocytogenes* virulence gene expression during *G. mellonella* infection

Virulence factors in *L. monocytogenes* are expressed at low levels during growth in laboratory medium but are

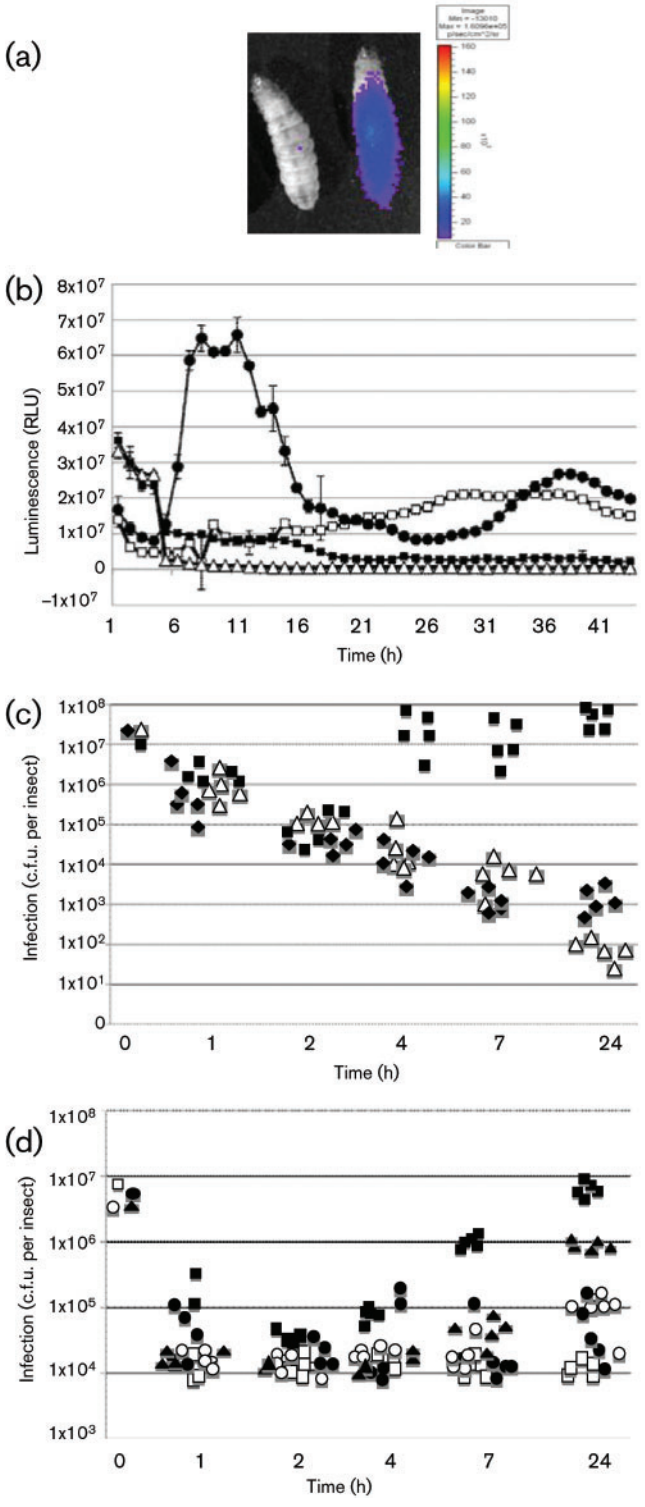

Fig. 2. Growth of injected and gavaged *L. monocytogenes* EGDe::luxP$_{help}$ and injected and gavaged *L. innocua* 11262 EGDe::luxP$_{help}$ in *G. mellonella*. (a) *In vivo* luminescence imaging was used to analyse host–pathogen interactions in the insect host. *G. mellonella* were injected with PBS and *L. monocytogenes* EGDe::luxP$_{help}$ and visualized using IVIS at 1 h post-infection. (b) Emission of light using Xenogen IVIS at 37 °C over 42 h representing the growth of injected and gavaged *L. monocytogenes* EGDe::luxP$_{help}$ (● and ■, respectively) and injected and gavaged *L. innocua* 11262 EGDe::luxP$_{help}$ (□ and △, respectively) in *G. mellonella*. Ten individual insects were monitored per infection. Experiments were performed three times independently and a single experiment is presented. Error bars, SEM. (c, d) Insects were infected via injection and bacterial growth was monitored over time by homogenization of five individual insects per treatment at each time point and subsequent plate counting. (c) ■, *L. monocytogenes* EGDe::luxP$_{help}$; ◆, *L. innocua* 11262 EGDe::luxP$_{help}$; △, *E. coli*. (d) ■, *L. monocytogenes* EGDe; mutant strains: ●, *prfA*; ▲, *sigB*; □, *hly*; ○, *actA*.

transcriptionally induced within host cells or during murine infection (Bubert *et al.*, 1999; Camejo *et al.*, 2009; Ermolaeva *et al.*, 2004; Riedel *et al.*, 2009; Wilson *et al.*, 2001). We investigated whether *L. monocytogenes* infection in the insect model induces similar levels of virulence gene expression upon entry into the insect host. We utilized chromosomally integrated promoter fusions to a *lux* reporter system to monitor expression from virulence gene promoters in *L. monocytogenes* strain EGDe (see Supplementary Table S1) during infection of *G. mellonella* larvae at 37 °C (Fig. 4a and b). Control strains contained either no promoter (negative control) or the constitutive P$_{help}$ promoter (positive control) (Riedel *et al.*, 2007, 2009) and insects were monitored every 30 min over 22 h for the production of light in a temperature-controlled enclosed IVIS. We detected significant activity from a number of promoters resulting in luminescence in the insect model. Prior to infection of insects, activity from the P$_{hly}$ promoter was undetectable. However, within 30 min of infection, activity from the P$_{hly}$ promoter was clearly detectable, reaching a peak of 8.5×10^7 RLU at 7 h post-infection. This activity was twofold higher than the maximum recorded for the constitutive P$_{help}$ promoter. P$_{prfA}$, P$_{mpl}$, P$_{actA}$ and P$_{plcA}$ promoters were also activated during infection of the insect. However, P$_{hly}$ was the most highly expressed promoter during insect infections. No light detection was evident from the P$_{hpt}$, P$_{inlA}$ or P$_{p60}$ promoter constructs or the negative control (no promoter). These data indicate that many of the key *L. monocytogenes* virulence genes are significantly induced in the *G. mellonella* insect model at 37 °C.

When these experiments were replicated at 30 °C (Fig. 4c and d), no detectable activity was evident for the P$_{prfA}$, P$_{hpt}$, P$_{inlA}$ and P$_{p60}$ promoter fusion strains. Whilst significant expression of P$_{hly}$ was detected during insect infection at 30 °C, levels were lower than those seen during infection at 37 °C. Taken together, these data demonstrate strong induction of expression of specific virulence genes in *L. monocytogenes* during infection of the insect host at both 37 and 30 °C.

G. mellonella response to infection by *Listeria* species

We have shown that *L. monocytogenes* infects and kills *G. mellonella* insect larvae using many of the genetic factors

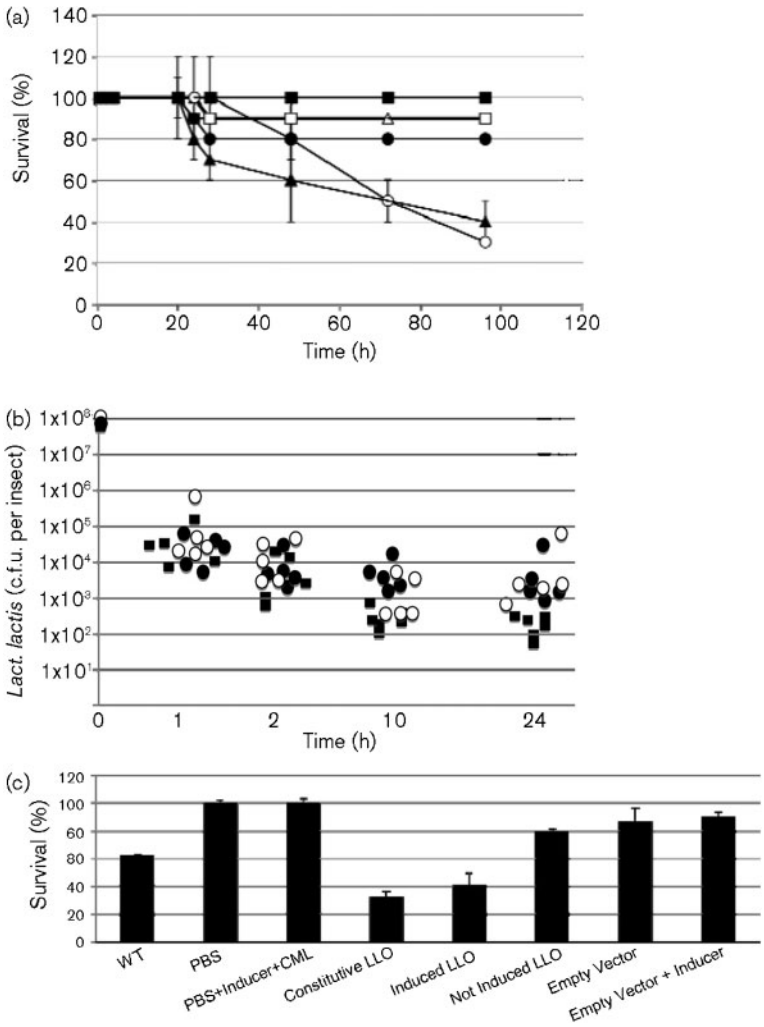

Fig. 3. The production of haemolysin is sufficient to induce *G. mellonella* insect mortality when produced using an engineered *Lact. lactis* strain. (a) Fifty per cent of insects were killed 72 h after infection with *Lact. lactis* producing haemolysin. An equivalent amount (OD_{600} 0.1) of each variant was injected into each of ten insects in the presence or absence of antibiotic and/or nisin to induce LLO production. ▲, *Lact. lactis* LLO-induced; ●, *Lact. lactis* LLO-non-induced. Controls were the empty vector (□), *Lact. lactis* with no vector (△), *Lact. lactis* constitutively expressing LLO (○) and PBS (■) containing antibiotic alone or in combination with nisin. (b) C.f.u. of *Lact. lactis* from homogenized *G. mellonella* insects following infection in the presence and absence of LLO production (five replicates for each treatment) at different time points during infection. Data shown are representative of three independent experiments. ■, LLO-induced; ●, empty vector; ○, constitutive LLO. (c) Haemocyte viability assays performed 24 h after infection with *Lact. lactis* wild-type and *Lact. lactis* containing plasmids harbouring inducible LLO or constitutively produced LLO, as well as the empty plasmid. Three individual insects were examined per treatment and the experiment was replicated three times independently. Bars in (a) and (c) indicate SEM.

attributed to listeriosis in humans and mice. The progress of infection of *G. mellonella* by *Listeria* is accompanied by an increase in pigmentation (Fig. 5a), usually indicative of pro-phenoloxidase (PPO) induction causing melanization (Kanost *et al.*, 2004). In order to determine if the PPO cascade is induced in insects in response to *L. monocytogenes* infection we examined the level of PO produced by *G. mellonella* plasma throughout the course of an infection. PO production was detected as described above from plasma isolated from insects at 1, 2, 4, 7 and 24 h post-inoculation with either PBS or *L. monocytogenes* strain EGDe (Fig. 5b). Throughout the course of the assay, the level of PO in PBS-infected insects remained constant at OD_{490} 0.22. The presence of *L. monocytogenes* had no effect on PO levels for the first 2 h of infection (levels of OD_{490} 0.2). By 4 h, the level of PO detected had doubled (to OD_{490} 0.4) in infected insects and continued to increase to OD_{490} 1.2 at 24 h, indicating that the presence of *L. moncytogenes* induces the PPO cascade in insects.

The insect innate immune system functions at both the cellular and the humoral level. To determine the effects of *L. monocytogenes* at the cellular level, insects were infected with *L. monocytogenes* wild-type or mutant strains and the effect on insect haemocytes was examined at 24 h post-infection as described above. Infection of insects with wild-type *L. monocytogenes* EGDe resulted in a 90 % decrease in numbers of viable haemocytes compared with those injected with PBS alone (Fig. 5c and d). This phenomenon was also apparent when insects were infected at 30 °C (Supplementary Fig. S1). In insects infected with *L. monocytogenes* mutants, the effect upon haemocyte numbers varied according to the nature of the mutation. Insects infected with highly attenuated *hly* mutants demonstrated only a moderate decrease in haemocyte numbers (a drop of 10–20 % at 24 h post-infection). Insects infected with the PrfA mutant strain elicited a drop of 57 % in viable haemocytes. These data implicate *L. monocytogenes* in the destruction of the insect haemocytes, which is followed by insect death. Insects infected with strains that are attenuated for virulence in the insect model (mutated in PrfA or Hly) maintained a high level of haemocyte viability. Therefore, we postulate that insect death is directly linked to haemocyte destruction in *G. mellonella*.

To further analyse the pathogen–host interaction in the insect model, we followed the interaction between insect

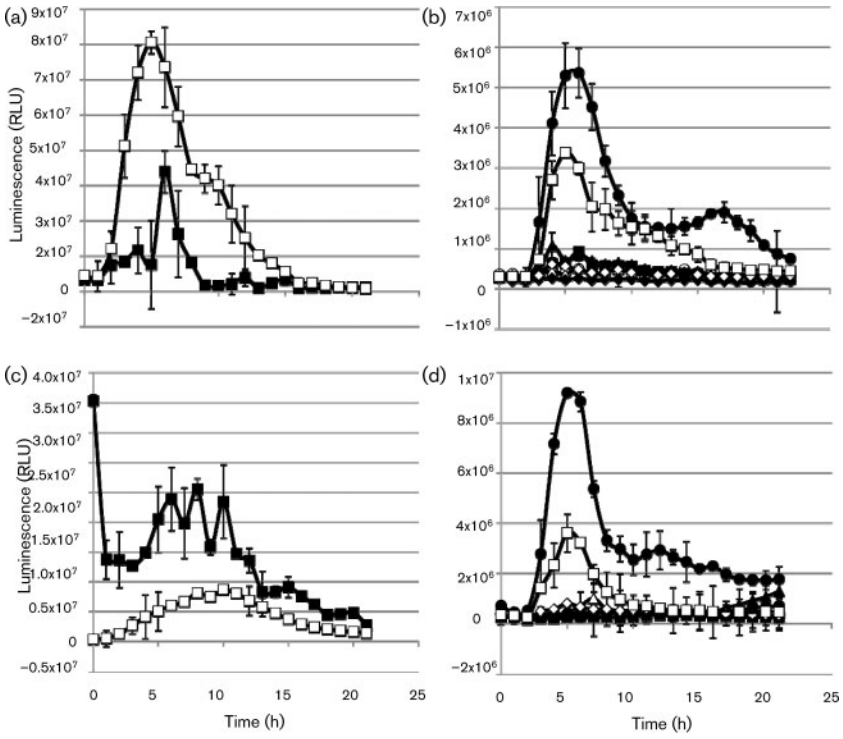

Fig. 4. Real-time monitoring of gene expression using virulence gene promoter fusions to the *lux* operon in *L. monocytogenes* during infection in *G. mellonella*. Promoter expression was monitored by the production of light at 37 °C (a and b) and at 30 °C (c and d). (a and c) Promoter activity for P_{help} (■) and P_{hly} (□) at the relevant temperature. (b and d) Promoter activity for P_{prfA} (○), P_{mpl} (●), P_{p60} (△), no promoter (◆), P_{inlA} (■), P_{hpt} (▲), P_{plcA} (◇) and P_{actA} (□) at the relevant temperature. Experiments were performed independently in triplicate. Error bars indicate SEM.

haemocytes and *Listeria* species and mutant strains throughout the course of infection using confocal microscopy. Bacteria were CFSE-stained and used to infect insects as described in Methods. At 1, 2, 4, 7 and 24 h post-infection, haemolymph was isolated from insects, mounted and stained and then examined by confocal microscopy (Fig. 6). Differential staining and imaging showed that within 1 h of infection, the majority of *L.*

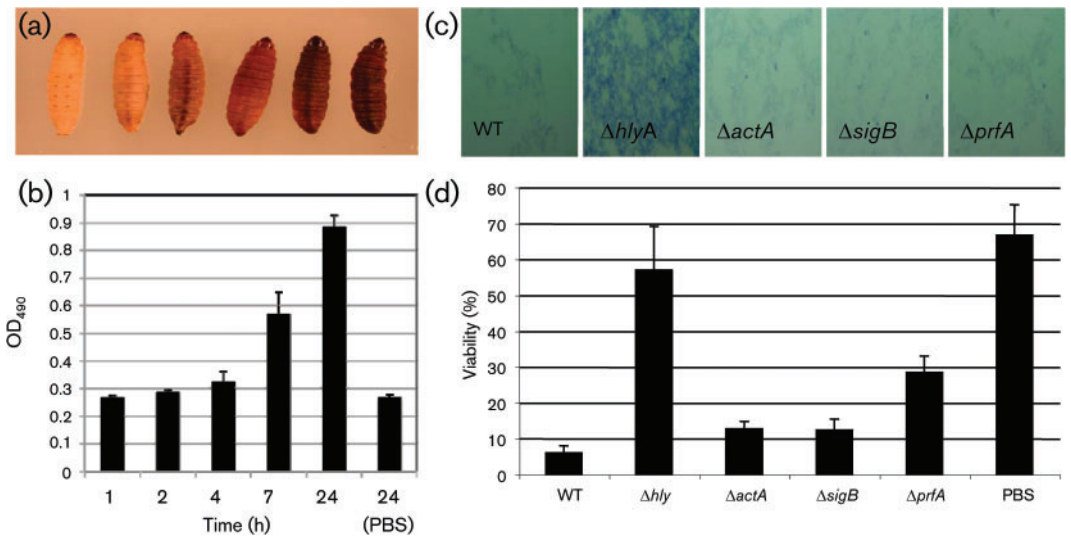

Fig. 5. *G. mellonella* response to the presence of *Listeria*. (a) The progress of infection by *L. monocytogenes* EGDe over the 24 h after infection in *G. mellonella*. Images are from 0 h to 24 h at 5 h intervals, from left to right. (b) PPO production in response to the presence of *L. monocytogenes* EGDe by *G. mellonella* insects over 24 h until insect mortality occurs. The level of PPO remained constant throughout the assay when injected with PBS (data shown for PBS is at 24 h). Assays were conducted three times independently. Bars indicate SD. (c) *G. mellonella* haemocyte viability recorded 24 h after infection with *L. monocytogenes* EGDe and mutant strains. Visual representation of live haemocytes stained with 2.0 % trypan blue. (d) The number of live haemocytes assessed using a Countess viability counter. Three representatives were taken for each variant; error bars show SD.

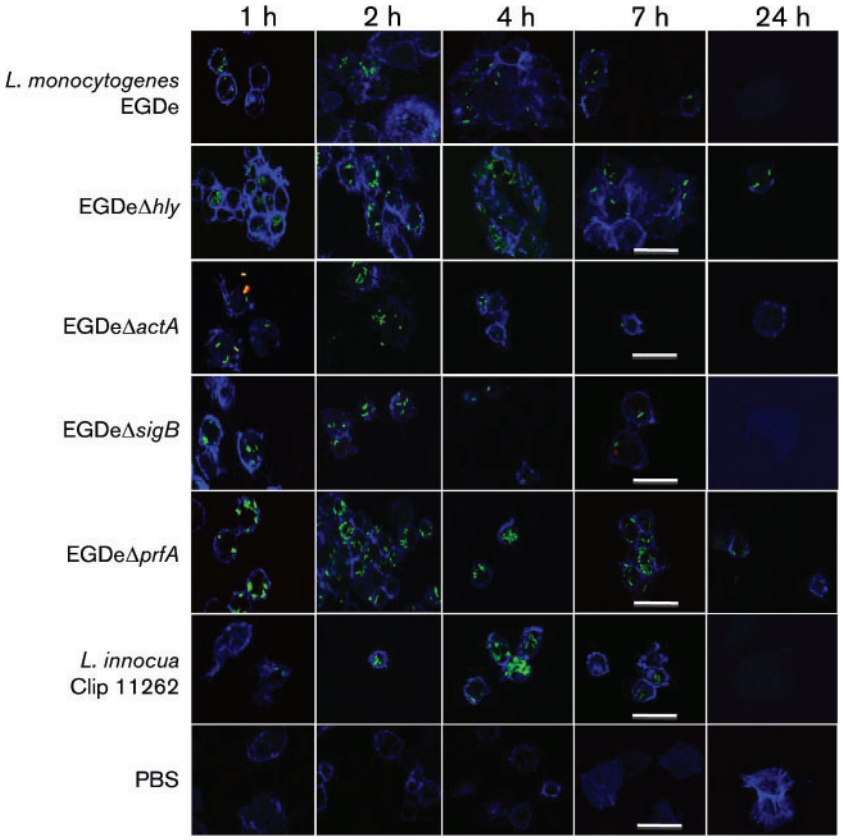

Fig. 6. *G. mellonella* response to the presence of *Listeria*. Visualization of *L. monocytogenes* and *L. innocua* in *G. mellonella* insects over 24 h using differential staining and confocal microscopy at the following wavelengths: 488 nm for CFSE-stained bacteria (green), 543–550 nm for rhodamine–phalloidin (blue), which stains any region producing actin, and 543–550 nm for streptavidin–allophycocyanin to detect non-internalized cells (red). Five individual insects for each strain were examined at each time point during the infectious process. Bars, 30 μm.

innocua and wild-type and mutant *L. monocytogenes* strains are intracellularly localized. In the case of the ActA mutant, some bacteria were present on the surface of the cell (stained red) although the majority are intracellular. We found no evidence for actin-based motility irrespective of the time at which samples were taken and of the strain examined. All strains appeared intracellular at 2 h post-infection with nodule formation evident in each case. By 4 h post-infection both nodule formation and cell size were reduced, where infection occurred with the ActA, SigB and PrfA mutant strains and with *L. innocua* when compared with PBS-injected control cells. Seven hours post-infection, bacterial numbers in haemocytes appeared severely reduced for all infections. Twenty-four hours post-infection, nodules were completely absent for all bacterial infections. Furthermore, in infections with wild-type and *actA* and *sigB L. monocytogenes* mutants and for *L. innocua*, haemocyte numbers were significantly reduced due to either migration of the nodules to the fat body or haemocyte destruction. However, viable haemocytes containing low bacterial numbers were evident in insects infected with the *prfA* and *hly* mutants; no haemocytes containing bacteria were observed for wild-type *L. monocytogenes* (Fig. 5c and d).

The innate immune cellular response in insects is accompanied by secretion of antimicrobial peptides (AMPs) in response to either injury or invasion by a pathogen. Since haemocyte destruction accompanies listerial infection we investigated whether *G. mellonella* mounts an immune response to the presence of *L. monocytogenes* EGDe via the production of AMPs and other factors. We isolated insect fat bodies to examine their production at 7 h post-infection for the following reasons: bacterial growth in insects is optimal (Fig. 2b), virulence gene expression is maximal (Fig. 4a and b), the PPO cascade had been activated (Fig. 5b) and nodulation of insect cells was evident (Fig. 6). RT-PCR showed that the following genes of the immune response are activated in the presence of *L. monocytogenes* EGDe: gallerimycin, PPO subunit 2, galliomycin, inducible metalloproteinase inhibitor, gloverin, transferrin and peptidoglycan recognition protein B. The expression of *β*-actin was used as an internal control (Supplementary Fig. S2). The control inoculation of PBS alone elicited no AMP response (Supplementary Fig. S2).

DISCUSSION

The greater wax moth (*G. mellonella*) has been developed as an infection model for a number of human pathogens (Aperis *et al.*, 2007; Jander *et al.*, 2000; Kavanagh & Reeves, 2004). This insect species has an active phagocytic cell system within the haemolymph that is capable of defence against infecting bacteria. We, and others, have shown that the larvae are capable of resisting inoculation with non-pathogenic bacterial species (non-pathogenic *E. coli*, *Bifidobacterium* spp. and *Lact. lactis*) (this work and Mylonakis *et al.*, 2005).

However, infection with specific pathogens results in a fulminant overwhelming infection that causes death of the insect despite the induction of an innate immune response (Kurz & Ewbank, 2007). The system therefore provides a model with which to study host–pathogen interactions and cellular infection in the presence of innate immunity. Unlike other alternative infection models (*D. melanogaster* and *C. elegans*) *G. mellonella* can be incubated at 37 °C and therefore permits the analysis of human pathogens that preferentially express specific virulence factors at this temperature. Other significant benefits of this model include the relatively low cost of the insect larvae and the ease of infection, which allows for their use in relatively high-throughput molecular screening studies.

Our study and a parallel study by Mukherjee *et al.* (2010) examined infection of *G. mellonella* by *Listeria* species and collectively we show that *L. monocytogenes* causes an active pathogenic infection in *G. mellonella* larvae which is dependent upon intact virulence factor expression. We extend the work of Mukherjee *et al.* (2010) here by demonstrating high levels of virulence gene expression by *L. monocytogenes in situ* in infected insects and by showing that the haemolytic factor LLO is sufficient to cause insect death. Insect death is concomitant with a significant reduction in haemocyte numbers in the insect and is preceded by induction of an immune response to invading *L. monocytogenes*.

We demonstrate similar infectivity for wild-type *L. monocytogenes* in insects compared with the work of Mukherjee *et al.* (2010). However, whilst Mukherjee and coworkers found that the non-pathogenic *L. innocua* is lethal for insects only at high infectious doses, we show lethality at lower infectious doses. This difference in susceptibility may be attributed to the fact that, in contrast with laboratory-reared *G. mellonella*, our out-sourced insects are maintained on an antibiotic-supplemented diet and therefore remain virtually unchallenged by microbes until exposure in our experiments. Indeed, Mukherjee *et al.* (2010) demonstrate that pre-exposure of *G. mellonella* to LPS enhances resistance to *Listeria* infection. In our study, other non-pathogenic bacteria (non-pathogenic *E. coli*, *Bifidobacterium* spp. and *Lact. lactis*) were incapable of causing insect death, suggesting that *L. innocua* possesses a specific mechanism of disease causation in the insect. Significantly, a variety of independent studies have shown that *L. innocua* is lethal in other non-mammalian model host systems [including Zebrafish embryos (*Danio rerio*), the nematode *C. elegans* and the fruit fly *D. melanogaster*] (Levraud *et al.*, 2009; Mansfield *et al.*, 2003; Thomsen *et al.*, 2006). *L. monocytogenes* and *L. innocua* are highly related and through evolutionary adaptation *L. innocua* has lost the genes encoding major virulence factors (including LLO), rendering this species non-pathogenic in mammalian hosts (Buchrieser *et al.*, 2003). However, LLO^{-} mutants in *L. monocytogenes* are completely attenuated in the *G. mellonella* infection model (this study; Mukherjee *et al.*, 2010). This implies that *L. innocua* possesses a specific mechanism for inducing insect mortality that is not active in *L. monocytogenes*. This capacity could be a result of minor genetic variations that exist between these species or it could reside within the 149 genes that are present in *L. innocua* but are absent from *L. monocytogenes* (Buchrieser *et al.*, 2003; Glaser *et al.*, 2001). We suggest that this separate capacity has been retained in the genome of *L. innocua* (or has been acquired) and allows some advantage in soil or saprophytic environments. The fact that *L. innocua* infects insects using a mechanism distinct from that of *L. monocytogenes* does not detract from the usefulness of *G. mellonella* as a model system for the study of *L. monocytogenes* pathogenesis.

In order to further investigate the role of LLO in this model host, we infected insects with *Lact. lactis* strains expressing plasmid-encoded LLO. Whilst wild-type *Lact. lactis* was non-pathogenic in insects, the engineered strains expressing LLO were capable of causing insect death. LLO did not facilitate growth of the *Lact. lactis* strain *in vivo* reflecting previous reports that other factors are required to support intracellular growth of non-pathogenic bacteria (Bahey-El-Din *et al.*, 2008; Goetz *et al.*, 2001). Instead, it is likely that LLO produced *in situ* is directly cytotoxic for insects as shown by the ability of *Lact. lactis* secreting LLO to reduce haemocyte numbers *in vivo*. LLO is not toxic for mammalian cells due to the presence of a PEST sequence that targets the protein for ubiquitination and proteolytic degradation in the cytoplasm of infected cells (Decatur & Portnoy, 2000). It is possible that the LLO PEST sequence does not function in the same manner in insect cells and that the LLO protein is poorly degraded during insect infection. When we followed the growth of *L. monocytogenes* and the isogenic *hly* mutant in insects it was clear that LLO contributes to growth of *L. monocytogenes* in the host. Most likely this reflects a defect in access to the cell cytoplasm as clearly demonstrated in the murine model (Hamon *et al.*, 2006; Portnoy *et al.*, 1988). The data therefore suggest that this virulence factor may have a dual effect during the pathogenesis of *L. monocytogenes* in insects, both in permitting listerial growth and as a toxic factor that is directly lethal for insects when bacterial numbers are elevated.

We have recently developed bioluminescence imaging tools for the analysis of *Listeria* species in complex environments (Bron *et al.*, 2006; Riedel *et al.*, 2007, 2009; Sleator *et al.*, 2009). Here we utilized constitutive *lux* expression (mediated by the P_{help} promoter) to analyse the growth kinetics of *L. monocytogenes* in the insect host. To our knowledge, this is the first use of *in vivo* bioluminescence imaging to monitor host–pathogen (non-commensal) interactions in an insect host in real time and our data suggest that this approach may be amenable to future high-throughput studies or molecular screens. *L. monocytogenes* was capable of replication following injection but not following oral gavage in insects, demonstrating that the pathogen cannot cause infection by this route and supporting a previous study investigating oral gavage of *L. monocytogenes* in insects (Fedhila *et al.*, 2010).

We utilized promoter fusions to *lux* to analyse listerial gene expression profiles *in situ* during infection of the insect host. Whilst expression from the *hly* promoter was undetectable prior to infection, we demonstrated rapid induction of gene expression following infection of insect larvae. Levels of *hly* gene expression were 100-fold greater than those seen *in vitro* in BHI containing activated charcoal (Riedel *et al.*, 2009) and therefore reflect recent transcriptional (array) analyses that showed up to 118-fold induction of *hly* during infection of murine spleens (Camejo *et al.*, 2009). In our study, the greatest levels of expression were seen from the *hly* promoter at 37 °C; however, this gene was also expressed when insects were incubated at 30 °C. In addition, other PrfA-dependent promoters (P_{mpl} and P_{actA}) were also clearly induced upon infection of the insect host, again reflecting high levels of induction seen during murine infections (Camejo *et al.*, 2009). Interestingly, the PrfA- and SigB-dependent gene *inlA* was not upregulated in the insect model in contrast with the mammalian infection model (Camejo *et al.*, 2009). The significance of this finding is currently not clear. However, the data demonstrate that *L. monocytogenes* virulence gene expression profiles are generally similar during infection of both mammalian and non-mammalian hosts. The work also strongly supports previous studies which demonstrate that regulation of specific virulence factors is induced strongly by the *in vivo* (cellular) environment (Bubert *et al.*, 1999; Chatterjee *et al.*, 2006; Joseph *et al.*, 2006), a process which we show is active even at 30 °C.

An obvious phenotype associated with infection of *G. mellonella* by *L. monocytogenes* is a visual colour change indicative of induction of the PPO system, a process triggered by microbial peptides and which itself induces tissue damage. We show that the PPO system becomes activated within 4 h post-infection and remains activated over a 24 h period. Induction of this system is required to induce phagocytosis by granular cells, although mainly plasmatocytes and granulocytes participate in this process, leading to nodule formation and then melanization and ultimately leading to cell destruction (Bidla *et al.*, 2009). We also confirm the production of other AMPs as demonstrated by Mukherjee *et al.* (2010). When we examined haemolymph from infected insects, it was apparent that haemocyte numbers are significantly lower in *Listeria*-infected larvae. The same drop in haemocyte numbers does not occur in insects infected with highly attenuated mutants of *L. monocytogenes*, suggesting that the loss of haemocytes is a direct result of pathogenesis. We postulate that loss of haemocytes may be linked to the death of the insect and is driven by LLO expression. Further work is necessary to demonstrate the mechanisms by which LLO may be toxic to insects *in vivo*.

In conclusion, we have examined the molecular pathogenesis of *L. monocytogenes* infection in the alternative *G. mellonella* model system. The work extends a recent independent study by Mukherjee *et al.* (2010). Both studies demonstrate active infection by *L. monocytogenes* in this model which leads to intracellular growth of the pathogen and is dependent upon production of intact virulence factors. Here, we also show that *L. monocytogenes* responds to infection of the insect host by significant induction of virulence gene expression and that production of LLO alone is sufficient to kill insect larvae. In addition, we have determined that in our model, *L. innocua* is pathogenic for insects, utilizing a mechanism that reduces haemocyte viability. Collectively, the data suggest commonalities in the responses of *L. monocytogenes* to evolutionarily distinct host environments. The work is consistent with a theory that *Listeria* species evolved to take advantage of saprophytic environments where predatory amoeba (and insects) may represent potential hosts (Akya *et al.*, 2009b).

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