INTRODUCTION

Coxiella burnetii is a Gram-negative intracellular bacterium and the aetiological agent of the zoonotic disease Q fever. Q fever is a zoonotic disease with almost worldwide distribution and has a broad host range, including humans, farm animals (e.g. cattle, sheep and goats), wildlife and arthropods (McQuiston & Childs, 2002). Between 2007 and 2010, the largest described outbreak of Q fever occurred in the Netherlands, resulting in >4000 human cases, and >50 000 goats were culled in order to control the spread of the disease (Whelan et al., 2011; Dijkstra et al., 2012). In addition, there are several recent reports of outbreaks amongst military personnel serving in Iraq and Afghanistan, and C. burnetii is listed as a category B agent by the US Centers for Disease Control and Prevention (Moodie et al., 2008; Faix et al., 2008; Bailey et al., 2011). Infection of humans usually occurs by inhalation of bacterial aerosols, but transmission by ingestion of contaminated dairy products has been reported (Madariaga et al., 2003). Clinical presentation of Q fever in humans ranges from an acute self-limiting febrile disease to a chronic, sometimes life-threatening, disease often involving endocarditis or hepatitis (Maurin & Raoult, 1999).

Although Q fever was first described in 1937, there is still limited understanding of the virulence factors produced by C. burnetii (Derrick, 1983). C. burnetii exists in two phases: phase I where the bacteria express a complete LPS and phase II where a truncated LPS is produced (Hackstadt et al., 1985). Phase I bacteria are virulent, in contrast to phase II bacteria which are avirulent in immunocompetent animal models of disease (Moos & Hackstadt, 1987; Andoh et al., 2007; Marmion, 2007). The difference in virulence of these strains has been defined as a consequence of differential O-antigen expression (Hoover et al., 2002). One potential reason for our poor understanding of C. burnetii pathobiology is a historical lack of molecular tools for genetic manipulation of the organism. Recently, the development of axenic growth media, manipulation plasmids, protocols to enable transposon mutagenesis and the production of defined genetic mutants (Beare et al., 2009, 2011a, 2012; Omsland et al., 2009) has provided tools that can be utilized to further characterize the virulence determinants of C. burnetii. However, these developments have been carried out using phase II C. burnetii [Nine Mile (NM)II] and there are limited animal models available to characterize C. burnetii NMII mutant phenotypes in vivo. In contrast, there are several animal models used to characterize phase I C. burnetii (NMI) virulence, including guinea pigs, mice and primates (Scott et al., 1987; Wang et al., 1999; Russell-Lodrigue et al., 2006).

Mammalian models of infection are associated with high cost, ethical constraints and specialized training requirements. Therefore, alternative infection models using insects
are being increasingly employed to characterize virulence of bacterial pathogens and to evaluate novel therapeutics, prior to characterization in mammalian models (Glavis-Bloom et al., 2012). Larvae of the greater wax moth, Galleria mellonella, have been reported as a model for several intracellular bacteria, such as Burkholderia pseudomallei and Campylobacter jejuni, and also for Francisella tularensis and Legionella pneumophila, which are closely related to C. burnetti (Aperis et al., 2007; Schell et al., 2008; Champion et al., 2010; Wand et al., 2011; Harding et al., 2012). G. mellonella can be maintained at mammalian body temperature (37 °C), are cheap, do not require feeding and are simple to manipulate at high containment. In addition, insects such as G. mellonella possess several functional homologues of components of the innate immune response of mammals, such as specialized phagocytic cells (termed haemocytes) that ingest pathogens and produce bactericidal compounds (Bergin et al., 2005). In addition, the G. mellonella immune response extends to the production of antimicrobial peptides, peptidoglycan recognition proteins, haemolymph coagulation and phenol oxidase-based melanization (Seitz et al., 2003; Vogel et al., 2011). In this study, we report that G. mellonella are susceptible to infection with C. burnetti NMI and additionally to C. burnetti NMII. This model has been used to provide the first characterization of C. burnetti NMII genetic mutants in vivo and has also been investigated as an alternative approach to determine antibiotic efficacy against C. burnetti.

**METHODS**

**Bacterial strains.** Bacterial strains used in this work are listed in Table 1. All phase II C. burnetti (NMII) strains were cultured axenically in ACCM-2 in 75 cm² tissue culture flasks containing 20 ml medium (Omsland et al., 2011). Cultures were incubated statically for 6 days in a Galaxy 170 R incubator (New Brunswick Scientific) at 37 °C adjusted to 5 % CO₂ and 2.5 % O₂. C. burnetti NMI cultures were incubated at 37 °C, shaking at 75 r.p.m. for 6 days, with a GENbox microer generator (bioMérieux) to displace oxygen. To kill C. burnetti, bacteria were heated at 80 °C for 4 h. For strains expressing mCherry or GFP, bacteria were grown in ACCM-2 supplemented with 3 µg chloramphenicol ml⁻¹. For infections, bacteria were adjusted to OD₅₉₀ 0.1 in PBS, equivalent to 1 × 10⁸ genome equivalents (GE) ml⁻¹. Challenge dose was confirmed by real-time PCR. All manipulations of C. burnetti NMI and NMII were carried out in a class III or I microbiological safety cabinet, respectively, complying with British Standard EN12469:2000.

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**Bacterial enumeration.** C. burnetti was enumerated using real-time PCR targeting the com1 gene (forward primer, GGCCGCAAACAT-AAAATGCAATG; reverse primer, ATTTGACATTTGCTGTGCTT-ACAAAG; probe, TTAGCTTCGTTTCAACTACATTCTCA). The probe was covalently labelled at the 5’ end with the reporter dye FAM and at the 3’ end with the quencher dye BHQ-1. Primers and probe were purchased (ATDBio). Chromosomal DNA was extracted by addition of 100 µl sample to 900 µl Instagene Matrix (Bio-Rad). The Instagene/sample suspension was heated (95 °C; 15 min) and centrifuged (10 000 r.p.m.; 3 min; Eppendorf Microcentrifuge model no. 5424) and supernatant was retained for PCR. Real-time PCRs comprised 12 µl template DNA, forward primer (900 nM), reverse primer (300 nM), probe (200 nM) and PCR master mix containing 0.04 U JumpStart Taq DNA polymerase µl⁻¹ (Sigma-Aldrich), 0.2 mM dNTPs, 8 % w/v glycerol, 4 mM MgCl₂, 50 mM Tris/HCl, 1 µg BSA µl⁻¹ and 0.5 µM EGTA. PCR cycling conditions comprised 3 min at 95 °C, 30 s at 60 °C, followed by 50 two-step cycles of 15 s at 95 °C and 30 s at 60 °C.

**Infection of G. mellonella with C. burnetti.** G. mellonella were purchased from Live Foods UK and maintained on wood chips at 14 °C. Groups of 10 larvae weighing 0.25–0.35 g were injected with 10 µl of various concentrations of C. burnetti into the uppermost right proleg. The larvae were incubated at 37 °C and survival was monitored at 24 h intervals. Larvae were scored as dead when they displayed no movement in response to gentle manipulation with a pipette tip. PBS-injected controls were included and each experiment was carried out in triplicate.

**Treatment of infected G. mellonella with doxycycline.** Groups of 10 larvae were injected with 10⁶ GE ml⁻¹ into the uppermost right proleg and 24 h post-infection larvae were injected with 50 mg doxycycline hydroclate kg⁻¹ (Sigma-Aldrich) into the uppermost left proleg. Untreated controls were injected with PBS at 24 h post-infection. Uninfected controls were injected with doxycycline only. Each experiment was carried out in triplicate.

**Association of C. burnetti with haemocytes.** To determine bacterial numbers, larvae were infected with 10⁶ GE ml⁻¹ and, at 48 and 96 h post-infection, three larvae were placed on ice for 10 min to prevent movement. The bottom 2 mm of each larva was aseptically removed and haemolymph was drained into a sterile microcentrifuge tube. Bacterial burden was determined using real-time PCR. For visualization of intracellular bacteria in haemocytes, haemolymph was placed onto a sterile glass slide, stained with DAPI and overlayed with a coverslip. Slides were examined using a LSM710 confocal microscope (Carl Zeiss). Twenty random fields of view per slide were viewed for presence of bacteria.

**Statistical analysis.** All graphs were produced using Graphpad Prism version 5. Analysis of survival curves was carried out using a log-rank (Mantel–Cox) test. Bacterial burden data were first subjected to log₁₀ transformation and at the 3 °C end with the quencher dye BHQ-1. Primers and probe were purchased (ATDBio). Chromosomal DNA was extracted by addition of 100 µl sample to 900 µl Instagene Matrix (Bio-Rad). The Instagene/sample suspension was heated (95 °C; 15 min) and centrifuged (10 000 r.p.m.; 3 min; Eppendorf Microcentrifuge model no. 5424) and supernatant was retained for PCR. Real-time PCRs comprised 12 µl template DNA, forward primer (900 nM), reverse primer (300 nM), probe (200 nM) and PCR master mix containing 0.04 U JumpStart Taq DNA polymerase µl⁻¹ (Sigma-Aldrich), 0.2 mM dNTPs, 8 % w/v glycerol, 4 mM MgCl₂, 50 mM Tris/HCl, 1 µg BSA µl⁻¹ and 0.5 µM EGTA. PCR cycling conditions comprised 3 min at 95 °C, 30 s at 60 °C, followed by 50 two-step cycles of 15 s at 95 °C and 30 s at 60 °C.

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**Table 1.** C. burnetti strains used in this study

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RESULTS

G. mellonella are susceptible to lethal infection with C. burnetii

To characterize G. mellonella as an infection model for C. burnetii, NMI and NMII strains were used to infect larvae (Fig. 1). Larvae were injected with C. burnetii at ~10^6 GE ml⁻¹ and survival was monitored for 216 h post-infection. Both NMI and NMII C. burnetii infection resulted in death of the larvae within 9 days, with no significant difference between strains at any concentration (P=0.2756; 10^6 GE ml⁻¹). In addition, the median lethal dose was calculated for both C. burnetii NMI (4.2 x 10^2 GE ml⁻¹) and NMII (9.1 x 10^2 GE ml⁻¹). Macroscopic changes were observed over the course of the infection, such as change in colour of the larvae cuticle from cream to grey, reduced movement and dehydration (data not shown). No macroscopic changes or deaths were observed in the groups injected with PBS or heat-killed C. burnetii NMII. These results demonstrated that G. mellonella were susceptible to C. burnetii and there was no significant difference observed in virulence between C. burnetii NMI and NMII strains in this model.

Dose-dependent killing of G. mellonella by C. burnetii

To determine if the lethality observed with C. burnetii was dependent on the numbers of bacteria in the challenge inoculum, larvae were injected with C. burnetii NMI or NMII at 10^2, 10^3, 10^4, 10^5 or 10^6 GE ml⁻¹ and survival was monitored for 216 h (Fig. 2). No significant difference between NMI and NMII was observed at any dose (P>0.1). All larvae challenged with 10^6 GE ml⁻¹ had succumbed to infection by 216 h post-challenge, whereas mortality was reduced to <50% in larvae infected with 10^4 GE ml⁻¹. Less than 10% of deaths were observed in larvae injected with ≤10^3 GE ml⁻¹, demonstrating that C. burnetii induced dose-dependent killing of G. mellonella.

Treatment of C. burnetii-infected G. mellonella with doxycycline

Doxycycline is the recommended antibiotic for treatment of Q fever (Anderson et al., 2013). Therefore, the efficacy of doxycycline was determined in our G. mellonella model of C. burnetii infection. Larvae were injected with C. burnetii NMII at 10^6 GE ml⁻¹ and monitored for 24 h. At this time point, larvae were administered with 50 mg doxycycline kg⁻¹ and survival was monitored for 216 h. Whilst the median time to death for untreated larvae was 96 h, larvae treated with doxycycline had a significantly extended median time to death of 180 h (Fig. 3; P<0.0001). These results indicated that a G. mellonella model may be suitable for testing novel antibiotics to treat Q fever.

Dot/Icm type 4 secretion system (T4SS) is required for C. burnetii NMII virulence in G. mellonella

The Dot/Icm T4SS has been shown previously to be important for intracellular replication in C. burnetii NMII

![Fig. 1.](http://mic.sgmjournals.org) (a) Survival of G. mellonella following challenge with C. burnetii NMI or NMII at 10^6 GE ml⁻¹. The results shown are the means of three replicates, each with 10 larvae. (b) Macroscopic changes in G. mellonella at various time points following challenge with C. burnetii NMII at 10^6 GE ml⁻¹.

![Fig. 2.](http://mic.sgmjournals.org) Survival of G. mellonella 216 h after challenge with C. burnetii NMI or NMII at 10^2–10^6 GE ml⁻¹. The results shown are the means ± SE of three replicates, each with 10 larvae.
(Beare et al., 2011b). The C. burnetii NMII T4SS genetic mutants used in this study had a transposon inserted into the dotA or dotB gene, and had been shown to be defective in replication within epithelial cells (Martinez et al., 2014). In order to determine if the T4SS was required for in vivo infection, larvae were injected with C. burnetii NMII dotA::Tn or dotB::Tn at 10^6 GE ml^-1, and survival was monitored for 216 h. All larvae infected with WT bacteria died by 216 h post-challenge, whereas 100% of larvae infected with C. burnetii dotA::Tn and 95% of larvae infected with C. burnetii dotB::Tn survived the course of the experiment (Fig. 4; P<0.0001). C. burnetii intergenic::Tn killed larvae at a similar rate to WT bacteria, indicating that constitutive expression of GFP alone did not affect virulence. These results confirmed, using an in vivo model of C. burnetii infection, that T4SS was required for full virulence. In addition, the G. mellonella model was suitable for evaluating the role of virulence factors in infection.

To determine if G. mellonella mortality depended on C. burnetii replication and persistence, larvae were infected with C. burnetii NMII expressing mCherry or T4SS dotA or dotB genetic mutants expressing GFP at 10^6 GE ml^-1. Haemolymph was extracted aseptically at 48 and 96 h post-challenge, and bacterial load determined using real-time PCR and confocal microscopy. At 96 h post-challenge, an increase in the number of C. burnetii NMII was detected by real-time PCR (1.2 × 10^7 GE per larvae; 95% confidence interval 1.1 × 10^6–1.3 × 10^7). In contrast, there were significantly fewer mutant bacteria (dotA::Tn: 1.2 × 10^7 GE per larvae; 95% confidence interval 1.1 × 10^6–1.3 × 10^7; dotB::Tn: 2.5 × 10^7 GE per larvae; 95% confidence interval 2.3 × 10^7–2.7 × 10^7, P<0.05). To analyse if C. burnetii NMII was replicating intracellularly within the haemocytes, confocal microscopy was used to visualize fluorescent C. burnetii in haemocytes (Fig. 5). In larvae infected with WT C. burnetii, large parasitophorous vacuoles filled with bacteria were formed by 48 h post-challenge. In contrast, low numbers of bacteria were observed in larvae infected with C. burnetii dotA::Tn or dotB::Tn at 48 h post-challenge, and by 96 h post-challenge no bacteria could be found. No differences in virulence were observed with strains expressing mCherry or with an intergenic GFP-expressing control (Fig. 4). These results suggested that C. burnetii induced killing was associated with bacterial persistence within haemocytes, which was at least partially dependent on the T4SS.

**DISCUSSION**

There are several animal models of virulent phase I C. burnetii infection available, including guinea pig, mouse and primate models; however, SCID mice are the only lethal animal model of C. burnetii NMII (Islam et al., 2013). C. burnetii NMII strains are used commonly by research laboratories at containment level 2 to characterize host–pathogen interactions in vitro and genetic manipulation has been achieved recently which allows specific virulence determinants to be evaluated (Voth and Heinzen, 2007; Beare et al., 2012). Whilst there are several in vitro cell infection models available to characterize the phenotype of C. burnetii NMII mutants, extrapolating this information into a whole host model has not been undertaken previously. We report the development of an insect model of C. burnetii infection that is susceptible to C. burnetii NMII and the characterization of T4SS mutants in an in vivo system.
Insect models have been employed to investigate pathogenesis of several bacterial species. The nematode Caenorhabditis elegans is used to model bacterial infections, such as Yersinia pestis, B. pseudomallei and L. pneumophila (Gan et al., 2002; Joshua et al., 2003; Komura et al., 2010). Whilst there is currently no reported Caenorhabditis elegans model of C. burnetii infection, insect larvae, such as G. mellonella, have several advantages over Caenorhabditis elegans. Unlike nematodes, G. mellonella can survive at 37 °C, which allows optimal expression of bacterial virulence factors that are active in a mammalian host (Laws et al., 2005). Caenorhabditis elegans is infected typically by allowing the nematodes to graze on a lawn of bacteria, resulting in an uncontrolled infectious dose. In contrast, G. mellonella are infected by subcutaneous injection, allowing determination of precise infectious dose per larva. In addition, G. mellonella possess haemocytes that function in the same way as mammalian macrophages, by phagocytosing bacteria and killing via an oxidative burst (Bergin et al., 2005). Similar to mammalian infection, we have shown that C. burnetii resides and replicates within these phagocytes, suggesting that this model may be used to investigate the role of genes associated with intracellular infection of C. burnetii.

Using C. burnetii NMII, we have demonstrated that G. mellonella could survive a low infectious dose, but the larvae succumbed to infection at higher doses. Taken together with the T4SS mutant data, these results indicate that larvae mortality is associated with specific protein virulence factors but that a threshold of bacterial burden is required to cause death. These results are similar to mammalian models of C. burnetii infection, where the severity of infection has been shown to be dose-dependent, with high levels of bacteria residing in mononuclear phagocytes (Russell-Lodrigue et al., 2006). In addition, the mean time to death following infection of larvae with C. burnetii is extended compared with other G. mellonella bacterial infection models. For example, G. mellonella infected with B. pseudomallei at 10^5 c.f.u. ml^-1 exhibit 100% mortality by 24 h post-challenge (Wand et al., 2011), whereas no clinical signs or deaths were observed until 72 h post-challenge with C. burnetii at 10^6 GE ml^-1. Similarly, Burkholderia mallei, which is a host-restricted obligate pathogen, exhibits slower killing of G. mellonella compared with B. pseudomallei (Schell et al., 2008). This observation may be due to the slow intracellular replication of C. burnetii. Antibiotic activity against C. burnetii can be determined in embryonated eggs, tissue culture and more recently axenic media (Maurin & Raoult, 1999; Omsland et al., 2011). There is only one report, to date, evaluating antibiotic treatment in an animal model of Q fever (Huebner et al., 1948). The recommended antibiotic for treatment of human Q fever is doxycycline and we have demonstrated that a single doxycycline treatment of C. burnetii-infected larvae significantly extends time to death. G. mellonella have been shown to be a useful model for testing antibiotic efficacy against other bacteria (Desbois & Coote, 2011; Thomas et al., 2013). Therefore, this model could be used as a rapid screen to determine the in vivo efficacy of novel antimicrobials to treat Q fever.

The Dot/Icm T4SS of C. burnetii has been shown to deliver bacterial effector proteins into the host cytosol during infection, required for establishing a mature vacuole to allow intracellular replication (Newton et al., 2013; Weber et al., 2013). In addition, a T4SS transposon mutant has been shown to be incapable of replication within cells (Beare et al., 2011b). However, the T4SS has previously not been implicated in infection of a whole organism. Using the G. mellonella infection model, we have demonstrated that the T4SS is required for virulence in larvae and intracellular replication within haemocytes. These results are similar to those observed with an L. pneumophila T4SS mutant, which was avirulent at 10^7 c.f.u. ml^-1 (Harding et al., 2012).

Whilst the G. mellonella model demonstrated the attenuation of C. burnetii mutants lacking virulence proteins, it is interesting to note that there is no significant difference observed in virulence between NMI and NMII strains, indicating that the LPS is not important in infection of G. mellonella. This may be due to the reduced cellular and humoral immune responses in G. mellonella compared with the mammalian system (Glavis-Bloom et al., 2012). The difference in virulence of C. burnetii NMI and NMII has been attributed to the different susceptibilities of these strains to the mammalian immune response. For example, C. burnetii NMII is sensitive to complement-mediated serum killing, induces cytokine production and induces dendritic cell maturation in mammals (Vishwanath & Hackstadt, 1988; Shannon et al., 2005). In addition, C. burnetii NMII can replicate in vitro cultures lacking host immunity and can colonize mice lacking IFN-γ or T- and B-cells (Moos & Hackstadt, 1987; Andoh et al., 2003;
Ochoa-Repáraz et al., 2007). In addition, G. mellonella have been shown to be susceptible to several bacteria that are usually attenuated or avirulent in immunocompetent mammalian models, such as Burkholderia thailandensis (Wand et al., 2011).

In conclusion, this study demonstrates that G. mellonella are susceptible to C. burnetii infection in a dose-dependent manner. Virulence depends on intracellular replication with haemocytes and the Dot/Icm T4SS. In addition, we report the first non-mammalian in vivo model of C. burnetii infection, suitable for rapidly characterizing mutant phenotypes and screening of novel antimicrobials.

ACKNOWLEDGEMENTS

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