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Short communication

Intracellular survival of *Staphylococcus aureus* during persistent infection in the insect *Tenebrio molitor*

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A B S T R A C T

Survival of bacteria within host cells and tissues presents a challenge to the immune systems of higher organisms. Escape from phagocytic immune cells compounds this issue, as immune cells become potential vehicles for pathogen dissemination. However, the duration of persistence within phagocytes and its contribution to pathogen load has yet to be determined. We investigate the immunological significance of intracellular persistence within the insect model *Tenebrio molitor*, assessing the extent, duration and location of bacterial recovery during a persistent infection. Relative abundance of *Staphylococcus aureus* in both intracellular and extracellular fractions was determined over 21 days, and live *S. aureus* were successfully recovered from both the hemolymph and within phagocytic immune cells across the entire time course. The proportion of bacteria recovered from within phagocytes also increased over time. Our results show that to accurately estimate pathogen load it is vital to account for bacteria persisting within immune cells.

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1. Introduction

Bacterial persistence within cells and tissues can establish reservoirs from which reinfection can occur (Clement et al., 2005; Kerrn et al., 2005; Monack et al., 2004), resulting in long term pathogenicity (Freeman and Holland, 2007; Prajsnar et al., 2008). Bacterial persistence is not restricted to susceptible cells and live bacteria have been successfully recovered from multiple phagocytotic cell types including neutrophils and macrophages (Gresham et al., 2000; Kubica et al., 2008), demonstrating a capacity for survival within cells specialized for bacterial clearance. Such persistence within immune cells has been observed across a range of species, including insects (Daborn et al., 2002; Shinzawa et al., 2009; Vlisidou et al., 2010), mice (Gresham et al., 2000) and humans (Kubica et al., 2008), implying that this may be a common mechanism of bacterial evasion from the extracellular immune response (Monack et al., 2004).

Staphylococcus aureus is well known to survive within a range of human cell types, particularly epithelial cells (Clement et al., 2005; Menzies and Kourteva, 1998; Vesga et al., 1996). Furthermore, *S. aureus* is able to survive, escape and proliferate following uptake by human monocyte-derived macrophages *in vitro* within 3–4 days (Kubica et al., 2008). This capacity for immune evasion has been further demonstrated *in vivo* using mouse neutrophils (Gresham et al., 2000). *S. aureus* appeared to convert the neutrophils into pathogen vectors, transporting infective bacteria around the body and resulting in increased host mortality (Gresham et al., 2000). However, this hijacking was only followed over 24 h, leaving uncertainty as to the long term importance of this intracellular persistence. In this study we focus on this capacity of *S. aureus* to survive within cell designed for bacterial destruction.

Insects represent an ideal system in which to model persistent infections, possessing a highly effective innate immune system (Kounatidis and Ligoxygakis, 2012), that exhibits genetic and functional conservation between different taxa (Palmer and Jiggins, 2015). Bacterial pathogens are able to persist within a range of insect host species, with persistence identified in various tissues (Daborn et al., 2002; Zouache et al., 2009), the free hemolymph (blood) (Daborn et al., 2002; Haine et al., 2008a), and phagocytes (Daborn et al., 2002; Shinzawa et al., 2009; Vlisidou et al., 2010).

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Furthermore, recent studies have demonstrated an 'active' method of intracellular persistence in both *Salmonella typhimurium* (Shinzawa et al., 2009) and *Photorhabdus asymbiotica* (Vlisidou et al., 2010) indicating the potential for bacterial dissemination following establishment within these cells, although this has only been demonstrated over short time scales.

Long-term, low-level persistence of the non-natural pathogen *S. aureus* has been observed within *Tenebrio molitor* following sub-lethal inoculation (Haine et al., 2008a, 2008b), with viable bacteria recovered from within the free hemolymph 28 days post-infection (Haine et al., 2008a). Bacterial recovery from within the hemolymph indicates a significant potential for interaction between the processes of immunity and pathogenesis, as it implies continuous direct contact between immune elicitors and the persisting pathogen (Bettencourt et al., 2004). This study aims to investigate the possibility of viable bacteria recovery from within haemocytes over a three week time course, both within phagocytic cells and the extracellular environment in a long term persistent infection to assess the potential importance of intracellular persistence during infection.

2. Materials and methods

2.1. Insect and bacterial stocks

Virgin female *T. molitor* beetles, of ages 7–9 days post-eclosion, were selected from a stock population maintained at 25 °C, under a 12:12hr light/dark photo-cycle. Beetles were fed on a diet rat chow and provided water with apple, ad libitum. For the time series infection we used *S. aureus* JLA 513 (provided by Simon Foster, University of Sheffield), which was produced from the parent strain SH1000 by the addition of a tetracycline cassette into the chromosome that does not affect transcription or growth (Haine et al., 2008a). For the confocal imaging we used *S. aureus* SH1000 (pSB0219), which carries a plasmid-based GFP reporter gene expressed in a growth dependent manner in *S. aureus*, and a chloramphenicol resistance cassette (Qazi, 1999; Qazi et al., 2001).

2.2. T. molitor infection

Female beetles were sexed as pupae and then kept and maintained individually with food provided ad libitum for the duration of the experiment. Beetles were infected with a single inoculum of 5 μl (~2×10^7 CFU) of either *S. aureus* JLA 513 or SH1000(pSB2019) cultures, washed twice in sterile PBS to remove extracellular toxins, 7 days post adult eclosion. Negative controls were injected with 5 μl of sterile PBS. Injections were performed as previously described (Dobson et al., 2014).

2.3. S. aureus persistence time course

Following immune challenge with JLA 513 beetles were assigned a treatment, housed and fed, and their hemolymph collected at the following time points post infection: 2 h, 1, 3, 4, 5, 6, 7, 14, and 21 days post-infection, with at least 20 beetles assayed per treatment. Hemolymph was collected by perfusion bleed (Dobson et al., 2014; Haine et al., 2008a), which allows to collect a very high amount of the hemolymph. This is necessary to increase the detectability of the bacteria, which drop rapidly to very low numbers (Haine et al., 2008a). The resulting extract was divided into one of three separate treatments. Samples were treated with either gentamicin (200 μg/ml) or PBS, according to their treatment, and incubated for 15 h in a shaking incubator at 30 °C. Gentamicin is unable to cross the eukaryotic membrane and has little influence on intracellular bacteria populations (Gresham et al., 2000; Vlisidou et al., 2010). Following incubation, samples were pelleted and their supernatant removed before being washed repeatedly with PBS, to ensure the removal of any remaining gentamicin, before being resuspended in 1000 μl of PBS. One 500 mm sterile stainless steel metal ball was added to 'Intracellular' samples and these samples were placed in a Tissue lyser (QIAGEN, Tissue lyser II) at 25 KHz for 90 s to lyse all haemocytes present. This treatment has previously been shown to have no effect on bacterial number (data not shown). Finally, 250 μl of each sample was spread out onto agar plates (1.5% Agarose, 2% LB) containing 20 μg/ml of both tetracycline and amphotericin B. Plates were incubated for 48 h at 37 °C in a static incubator, following which their CFUs were recorded.

2.4. Confocal imaging

Following immune challenge with SH1000 (pSB2019) beetles were assigned a treatment, housed and fed, and their hemolymph collected at time points post infection: 30 min, 1 day, 3 days and 7 days. Hemolymph was collected via perfusion bleeds (Dobson et al., 2014; Haine et al., 2008a) using 500 μl of PBS + 10% formalin in order to fix the recovered cells. 200 μl of each sample was adhered onto positively charged glass slides using a cytospin centrifuge (400 × g for 4 min) and left to dry. Insect cells were permabilised by adding 100 μl of 1xPBS + 0.1% Triton for 10 min and then rinsed in PBS and stained with both DAPI (300 nM) which stains DNA and a Rhodamine-Phallodin conjugate (10 U/ml) which stains the actin cytoskeleton of eukaryotic cells, for 10–15 min in the dark. Samples were washed 3x in fresh PBS and then set by the addition of fluoromount and mounted under coverslips before viewing by confocal microscopy. Single plane and Z-stacked 3D image series were captured using a Zeiss LSM700 confocal microscope at 63x magnification for each cell analysed and all image and video output was produced using ZEN imaging software (Zeiss).

2.5. Colony counts

CFU counts for all samples were recorded using OpenCFU, an open source programme designed to enumerate bacterial colonies (Geissmann, 2013). Counts were taken using a live video stream with a high resolution webcam (Blackberry WC250, Sweex), and a 'mask' was applied to exclude detection of external noise. Counts were recorded against a black background, with a minimum radius of detection of 2 mm for each object specified.

2.6. Statistical analysis

Colony counts were analysed using the statistical package R (Version 3.13.0) and modelled under a generalised linear model with a quasi-poisson distribution. All comparisons were performed using the Wald t-test. An initial model of Count ~ Treatment*Time was employed and then subsequently simplified using a step algorithm, to remove insignificant interactions, resulting in Count ~ Treatment + Time as a final model estimate for the data.

3. Results & discussion

Viable bacteria were recovered from both extracellular samples of *T. molitor* hemolymph and from within haemocytes following infection by *S. aureus* across the entirety of the time course (Fig. 1, for count data see Table S1). This demonstrates *S. aureus*' capability to survive both inside and outside of *T. molitor* immune cells for up to 21 days post-infection, indicating the potential for long-term bacterial presence in each environment. Bacterial recovery was significantly larger in the extracellular samples (Wald t-test: $t = 24.51$, d. f. $= 1, 464$, $p = <0.001$), implying that a higher bacterial

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