Drosophila melanogaster as a model host for Staphylococcus aureus infection

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INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen, causing a variety of diseases in humans ranging from superficial skin lesions and wound infections to more serious conditions, such as osteomyelitis, endocarditis and septicemia (Waldvogel, 1995). In order to study host–pathogen interaction a number of animal models have been developed (Abdelnour et al., 1993; Cheung et al., 1998; Tarkowski et al., 2001). The use of mouse models of infection has allowed the identification of numerous virulence determinants. However, for functional genomic studies the use of large numbers of mammals is difficult for logistical, ethical and financial reasons. Recently a number of non-mammalian models of infection for human pathogens have been developed. Model hosts include: zebrafish, Danio rerio (Neely et al., 2002; van der Sar et al., 2003); the cress Arabidopsis thaliana (Rahme et al., 1997); the soil-living amoeba Dictyostelium discoideum (Solomon et al., 2000) and the nematode Caenorhabditis elegans (Kurz & Ewbank, 2003; Darby et al., 1999; Tan & Ausubel, 2000; Garsin et al., 2001). C. elegans was used as a model host for S. aureus by Sifri et al. (2003), who found that previously characterized virulence determinants important for mammalian infection are required for full pathogenicity in nematodes. Thus key virulence determinants are necessary for disease in a diverse range of hosts. S. aureus infection has also been modelled in silkworm larvae, Bombyx mori (Kaito et al., 2002), the use of which identified this as a possible screen for antibiotics.

The fruit fly Drosophila melanogaster is genetically well defined, has a short generation time and possesses an innate immune system which is remarkably similar to that of humans (Takeda & Akira, 2003; Leulier et al., 2003). Infection by Gram-positive bacteria induces the Toll signalling cascade, comparable to the Toll-like receptor (TLR) cascade in vertebrates (Takeda & Akira., 2003). In insects this cascade leads to the expression of a number of antimicrobial and antifungal peptides. Previously, the expression of antimicrobial peptides in response to bacterial infection, including S. aureus, has been assessed in D. melanogaster (Lemaitre et al., 1997). A recent study showed the importance of two pattern recognition receptors (PRRs) for the detection of Gram-positive bacteria in D. melanogaster: PGRP-SA, a peptidoglycan recognition protein, and GNBP1, a Gram-negative binding protein now found to be required for Toll activation following infection by the Gram-positive bacteria S. aureus and Enterococcus faecalis (Pili-Floury et al., 2004). The induction of these pathways has been examined in more detail through the injection of purified bacterial cell components of Escherichia coli, Pseudomonas aeruginosa and Bacillus thuringiensis (Leulier et al., 2003).

These factors make D. melanogaster a suitable model for studies of human host–pathogen interaction. D. melanogaster has previously been developed as a model for Pseudomonas aeruginosa (D’Argenio et al., 2001; Fauvarque et al., 2002), Mycobacterium marinum (Dionne et al., 2003) and Listeria monocytogenes (Mansfield et al., 2003). This study investigates the use of D. melanogaster as a model for S. aureus infection. We describe the identification of two
attenuated strains, *pheP* and *perR*, and demonstrate the use of the model to monitor *in vivo* expression and assess the effectiveness of antibiotics.

**METHODS**

**Bacterial strains and growth conditions.** Bacterial strains are listed in Table 1. *Staphylococcus aureus, Staphylococcus epidermidis,* *Listeria monocytogenes, Streptococcus pneumoniae, Streptococcus agalactiae* and *Enterococcus faecalis* were grown in brain heart infusion (BHI) broth (Oxoid). *Bacillus subtilis* and *Bacillus megaterium* were grown in nutrient broth (NB). *E. coli* was grown in Luria broth (LB). All were grown at 37°C. When required, antibiotics were added to media at the following concentrations: chloramphenicol, 5 μg ml⁻¹; erythromycin, 5 μg ml⁻¹; kanamycin, 50 μg ml⁻¹; lincomycin, 25 μg ml⁻¹; neomycin, 50 μg ml⁻¹; and tetracycline, 5 μg ml⁻¹.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or description*</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>S. aureus</strong></td>
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<tr>
<td>SH1000</td>
<td>Functional <em>rsbU</em>⁺ derivative of 8325-4</td>
<td>Horsburgh et al. (2002a)</td>
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<tr>
<td>8325-4</td>
<td>Wild-type strain cured of prophages</td>
<td>Lab. stock</td>
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<tr>
<td>COL</td>
<td>Low enterotoxin B producer, homogeneously methicillin resistant</td>
<td>Compagnone-Post et al. (1991)</td>
</tr>
<tr>
<td>S6</td>
<td>High enterotoxin B producer</td>
<td>Compagnone-Post et al. (1991)</td>
</tr>
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<td>SJF1385</td>
<td><em>pheP::tet</em> (SH1000)</td>
<td>Transduced from MJH600; Horsburgh et al. (2004)</td>
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<td>SJF987</td>
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<td>Transduced from STE029; Horsburgh et al. (2002b)</td>
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<td>SJF1219</td>
<td>SH1000/pSB2035</td>
<td>Transduced from 8325-4/pSB2035; Qazi et al. (2001)</td>
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<td><em>saeR::Tn551</em> (SH1000)</td>
<td>Aish (2003)</td>
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<td><em>sodM::Tn551</em> (SH1000)</td>
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<td><em>sigB::tet</em> (SH1000)</td>
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<td><em>mntA::kan mntH::tet</em> (8325-4)</td>
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**Other bacteria**

* Bacillus megaterium | KM | Lab. stock |
* Bacillus subtilis | HR | Lab. stock |
* Enterococcus faecalis | NCTC 775 | Lab. stock |
* Listeria monocytogenes | EGD | Lab. stock |
* Staphylococcus epidermidis | 138 | Lab. stock |
* Streptococcus agalactiae | 6313 | Valenti-Weigand et al. (1996) |
* Streptococcus pneumoniae | Type 1 | Lab. stock |
* Streptococcus pyogenes | M type 3 | Lab. stock |

*Parental background is shown in parentheses.*
**D. melanogaster strains and maintenance.** Wild-type *D. melanogaster* w¹¹8 (laboratory stock) were cultured on standard fly culture medium (maize molasses medium) at 25°C and 60% humidity.

**D. melanogaster infection.** Cells from overnight bacterial cultures (10 ml) were recovered by centrifugation at 4000 g for 5 min (room temperature); the supernatant was discarded and the pellet resuspended in PBS (10 ml). Cell suspensions were serially diluted in PBS and the concentration of cells determined by plating on appropriate media. For *S. aureus*, typically the inoculum was approximately 2 x 10⁶ c.f.u. ml⁻¹. For injection, adult female flies were used (2–5 days old); for female flies pricking with a sterile needle is consistently harmless (D’Argenio et al., 2001). Flies were anaesthetized with CO₂ and infected via pricking in the dorsal thorax with a needle (25 GA) dipped in the cell suspension. Flies were returned to standard fly culture vials with food and incubated at 30°C.

**Death of flies following infection.** Flies were infected in batches of ten. Initially a single batch was inoculated for each *S. aureus* strain or SH1000 in batches of five. At intervals following infection, all flies had died. A single fly was taken, and the tissue was then washed for 20 min in 0.4% (v/v) paraformaldehyde in PBS for 30 min at room temperature. The tissue was then washed again as described above and rinsed with PBS. Sections, 15 µm thick, were cut with a cryostat, collected on Superfrost Plus slides and allowed to air dry. Sections were mounted in 70% (v/v) glycerol in PBS and observed by fluorescence microscopy (Zeiss: Axiophot 2).

**Growth of S. aureus in vivo.** Flies were infected in batches of ten. At intervals following infection a single batch of infected flies was taken and flies separated into individual microfuge tubes. Dead and living flies were distinguished. Each fly was crushed in 100 µl PBS with a micropestle and the homogenate serially diluted in PBS. C.f.u. per fly was determined through growth on BHI agar plus appropriate antibiotics.

**In vivo expression analysis**

**GFP.** Plasmid pSB2035 (Qazi et al., 2001) was phage-transduced into *S. aureus* SH1000 (Novick, 1967). An inoculum of ~2 x 10¹⁰ c.f.u. ml⁻¹ was typically used to infect flies. Approximately 16 h following infection all flies had died. A single fly was taken, and the head, wings and legs removed. The remaining tissue was fixed in 4% (v/v) paraformaldehyde in PBS for 30 min at room temperature. The tissue was then washed for 20 min in 0.2% (v/v) Triton X in PBS. This washing was repeated three times. The tissue was then incubated at 37°C with 250 µg RNase A ml⁻¹ for 2–3 h followed by 10 µg propidium iodide ml⁻¹ for 30 min. The tissue was then washed again as described above and rinsed with PBS. Sections, 15 µm thick, were cut with a cryostat, collected on Superfrost Plus slides and allowed to air dry. Sections were mounted in 70% (v/v) glycerol in PBS and observed by fluorescence microscopy (Zeiss: Axiophot 2).

**β-Galactosidase activity.** Flies were infected with a lacZ fusion strain or SH1000 in batches of five. At intervals following infection, a batch was taken and flies transferred to a single microfuge tube. The flies were crushed together in 250 µl PBS with a micropestle. Duplicate 100 µl samples of the homogenate were taken and frozen at −70°C. Levels of β-galactosidase activity were measured as described previously (Horsburgh et al., 2001a). Fluorescence was measured using a Victor plate reader (Wallac) with a 0.1 s count time and calibrated with standard concentrations of MU (4-methylumbelliferyl). Background fluorescence, measured from SH1000-infected flies, was deducted from all samples. One unit of β-galactosidase activity was defined as the amount of enzyme that catalysed the production of 1 pmol MU min⁻¹ per c.f.u. The remaining 50 µl was serially diluted in PBS. C.f.u. per fly was determined through growth on BHI agar plus appropriate antibiotics.

**Antibiotic treatment of infected flies.** Flies were infected in batches of ten. Following infection, batches of flies were transferred to standard fly culture vials containing filter paper disks soaked with 10% (w/v) sucrose plus tetracycline or methicillin at a range of concentrations. The number of surviving flies was recorded at intervals.

**RESULTS AND DISCUSSION**

**S. aureus proliferates within and kills D. melanogaster**

*S. aureus* infection has previously been modelled in *C. elegans* (Garsin et al., 2001; Sifri et al., 2003). Infection of *C. elegans* through feeding on a lawn of bacteria caused nematode death due to growth of bacteria in the digestive tract. Heat- or antibiotic-killed *S. aureus* did not result in death, suggesting that lethal infection requires the presence of live bacteria (Sifri et al., 2003). Feeding *D. melanogaster* with *S. aureus* had no effect on the life span of flies (data not shown).

Infection of *D. melanogaster* was obtained through pricking of the dorsal thorax with a needle dipped in a bacterial suspension; flies were not injected with a defined amount of the inoculum. Approximately 25 ml was inoculated into the fly. This value is calculated from the determination of c.f.u. per fly immediately after infection, typically 10⁶ c.f.u. with a 2 x 10⁶ c.f.u. ml⁻¹ inoculum. However, upon inoculation some cells may adhere to the surface of the fly; the infective dose may therefore be less than 10⁴ cells. *S. aureus* SH1000 cells at concentrations between 1·9 x 10⁷ and 1·9 x 10⁹ c.f.u. ml⁻¹ were shown to kill flies, whilst those flies inoculated with sterile PBS survived (Fig. 1). Those flies inoculated with autoclaved *S. aureus* SH1000
(4.6 × 10^9 c.f.u. ml⁻¹) also survived (data not shown). As for C. elegans, this indicates that death of infected flies is due to the presence of live bacteria and not due to shock. The rate of death was proportional to the number of bacteria inoculated, with 100% death achieved after 23 h with an inoculum of 1·9 × 10^8 c.f.u. ml⁻¹ (Fig. 2). Infection of D. melanogaster with Mycobacterium marinum (Dionne et al., 2003) or Listeria monocytogenes (Mansfield et al., 2003), through a similar method of infection, also causes death, due to growth in larval haemolymph and tissues (Kaito et al., 2002). S. aureus grows exponentially in D. melanogaster, with a doubling time of ~80 min and a yield of approximately 2 × 10^7 c.f.u. per fly (Fig. 3). For S. aureus SH1000, stationary phase is reached approximately 15 h after infection. Infection of D. melanogaster infection with P. aeruginosa results in a comparable bacterial titre of 1 × 10^8–40 × 10^6 c.f.u. per fly before fly death (D’Argenio et al., 2000).

**Identification of bacterial components required for pathogenesis**

Batches of ten flies were inoculated with defined mutants of S. aureus SH1000 or 8325-4 in order to identify genes that may have a role in virulence in D. melanogaster. Significant attenuation was not seen in the following mutants: acnA, agr, arlR, fur, katA, mntA, mntAH, mntAR, mntH, mntR, saeR, saeS, sar, sarA agr, sigB, sodA, sodAM, sodM. Two mutants were attenuated in this model, perR and pheP, as measured by increased fly longevity after infection (Fig. 4). Both perR and pheP mutants also show attenuation in the murine skin abscess model of infection (Horsburgh et al., 2001a, 2004). Flies infected with the perR or pheP mutants die up to three times more slowly than those infected with wild-type S. aureus; 50% lethality is reached approximately...
PerR is one of three ferric uptake regulator (Fur) homologues present in the *S. aureus* genome (Horsburgh *et al.*, 2001a). It is involved in the control of expression of a number of oxidative stress resistance and iron storage proteins. Inactivation of PerR results in the deregulation of multiple components with a concomitant attenuation in a mouse subcutaneous abscess model of *S. aureus* infection. PheP is a putative amino acid permease involved in the uptake of phenylalanine (Horsburgh *et al.*, 2004), has a role in starvation survival in carbon-limited conditions, and is important for phenylalanine transport in oxygen-depleted conditions (Horsburgh *et al.*, 2004). A PheP mutant is highly attenuated in the mouse abscess model of infection.

Interestingly, none of the well-characterized regulators of virulence appears to have a major role in the *D. melanogaster* model. Both *agr* and *sara* have been shown to be required for full virulence in several mammalian model systems. This shows that although components like *perR* and *pheP* are important in both the fly and the mouse there is disparity between the systems. Thus the fly model is not directly comparable to mammalian models but does allow the identification of potential novel components involved in pathogenesis.

Bacterial infection is systemic in the fly

The green fluorescent protein (GFP) is encoded on the plasmid pSB2035 with expression under the control of P3 (*agr*) (Qazi *et al.*, 2001). P3, in vitro, is turned on in post-exponential phase. pSB2035 was phage-transduced from *S. aureus* 8325-4 to SH1000 (Novick, 1967). The resulting strain (SJF1219) was used to inoculate flies. An inoculum of $\sim 2 \times 10^8$ c.f.u. ml$^{-1}$ was used to ensure that infection progressed in all flies. Fig. 6 shows a section through the mid-thorax of a fly infected with SJF1219. The fly was fixed and sectioned 16 h after infection. Fig. 6 is typical of sections throughout the fly, where *S. aureus* forms micro-colonies on a range of tissues. Individual cells can be seen embedded in a matrix of material. SH1000-infected flies were also dissected into head, thorax and abdomen and the c.f.u. per section determined (data not shown). *S. aureus* cells were present throughout the fly in numbers proportional to the volume of tissue present, supporting the historical results indicating systemic infection. Such pathology may be analogous to infection observed in other models: in *S. aureus*-infected silkworm larvae, bacterial cells proliferate in blood and tissues, particularly at the epithelial surface of the midgut (Kaito *et al.*, 2002); and in *S. aureus*-fed *C. elegans*, death is correlated with the accumulation of bacteria within the digestive tract (Sifri *et al.*, 2003). A systemic infection in the fly is not surprising considering the nature and range of disease caused by *S. aureus* in humans. The development of infection in *D. melanogaster* has previously been observed using GFP-expressing *P. aeruginosa* (Fauvarque *et al.*, 2002) and *M. marinum* (Dionne *et al.*, 2003); infection is characterized by bacterial growth at the site of infection followed by systemic infection and fly death.

Levels of *S. aureus* gene expression *in vivo*

In order to quantify bacterial gene expression within individual flies, three representative *lacZ* reporter gene

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Fig. 5. Growth of *S. aureus* *pheP* (a) and *perR* (b) mutants within wild-type flies. Batches of ten flies were infected with inocula of $2.8 \times 10^8$ c.f.u. ml$^{-1}$ for SJF1385 (*pheP*) or $1.7 \times 10^8$ c.f.u. ml$^{-1}$ for MHK7 (*perR*). At intervals following infection a batch of flies was taken and c.f.u. per fly was determined (○); mean c.f.u. per fly (horizontal black bars) was calculated. The results are representative of two independent experiments.
fusions (hla, agr [RNA III] and mntA) were used. Early after infection low bacterial numbers prohibit accurate assessment of expression. All three fusions were found to be expressed within living flies (Fig. 7). Expression of agr is greatest, at approximately 50-fold higher than hla. Expression levels of hla are comparable to levels seen in vitro, calculated from previous data (Horsburgh et al., 2002a). However, levels of agr are approximately 100-fold higher compared with in vitro levels when calculated per c.f.u. Also, expression of agr shows a decline as cells reach post-exponential phase, whereas in vitro this is when greatest agr expression occurs in SH1000 (Horsburgh et al., 2002a). Such a negative correlation between agr levels and course of infection has been previously noted in a guinea pig model of device-related infection (Goerke et al., 2001). Similarly the use of a GFP transcriptional fusion with sarA in a rabbit endocarditis model (Cheung et al., 1998) has shown a disparity between in vivo and in vitro expression levels from particular promoters. Thus the complex environment of the host involves multiple signals that may contribute to gene expression profiles different from those found in vitro.

**S. aureus infection in flies can be cured with antibiotics**

Silkworm larvae have previously been developed as a high-throughput screen for antibiotics against *S. aureus* (Kaito et al., 2002). Infected silkworm larvae were rescued by the injection of clinically effective antibiotics. In order to
Fig. 7. In vivo lacZ expression analysis of agr (RNAIII) (●), hla (■) and mntA (▲). Batches of five flies were infected with a single lacZ fusion strain. Inocula for each strain were as follows: JLA341 (agr), 1.4 × 10^8 c.f.u. ml^-1; JLA513 (hla), 2.9 × 10^8 c.f.u. ml^-1; SJF987 (mntA), 1.7 × 10^8 c.f.u. ml^-1. At intervals following infection a batch of flies was taken, and β-galactosidase activity and c.f.u. per fly were determined. Background fluorescence was deducted from all samples before calculation of β-galactosidase activity (one unit corresponds to production of 1 pmol MU min^-1 per c.f.u.). The results shown are representative of two independent experiments which showed less than 20% variability. Growth of S. aureus SH1000 is also shown (○).

determine if the D. melanogaster model may be used as a screen for novel antibiotics, following infection with wild-type S. aureus SH1000, flies were fed 10% (w/v) sucrose containing the antibiotics methicillin or tetracycline. Infected flies were rescued in a dose-dependent manner (Fig. 8). For methicillin, the IC_{50} was approximately 1 mg ml^-1. IC_{50} is defined as the concentration of antibiotic in the sucrose solution required for the survival of 50% of flies 48 h following infection. As a control, flies were also inoculated with the methicillin-resistant strain COL and fed methicillin at 2 mg ml^-1 in the sucrose solution. The flies infected with COL were not rescued with methicillin (Fig. 8a). For tetracycline, the IC_{50} was found to be approximately 100 µg ml^-1 (Fig. 8b). Flies infected with a tetracycline-resistant strain (JLA513) were not rescued by treatment with tetracycline (200 µg ml^-1). In contrast to the silkworm larva model, antibiotics are administered in the fly food. This provides a convenient high-throughput screen for novel antibiotics within an in vivo model.

D. melanogaster is killed by several Gram-positive bacterial species

Bacillus megaterium, Bacillus subtilis, Enterococcus faecalis, Listeria monocytogenes, Staphylococcus epidermidis, Streptococcus agalactiae, Streptococcus pneumoniae and Streptococcus pyogenes were all found to kill D. melanogaster using the needle-prick model with comparable inocula (Fig. 9).

The growth of apparently non-pathogenic bacteria (such as B. subtilis) in vivo also causes fly death. Death may occur due to the accumulation of bacterial products within the fly or degradation of host components.

Conclusions

D. melanogaster forms a convenient high-throughput model of S. aureus infection with a defined end point. It is versatile in that microbial status in terms of growth rate and gene expression can be easily measured. Whilst the model is engineered, and it is unlikely that D. melanogaster can be viewed as a natural host for S. aureus, it has the capability of identifying novel virulence determinants. This is important, as the role of a large number of genes in S. aureus is unknown. The D. melanogaster model provides
an initial screen for virulence determinants whose role in mammalian infection can subsequently be analysed. The model can also be used to assay gene expression throughout the course of infection and allows high-throughput in vivo screening for the action of novel antimicrobials. Finally, analysis of the host innate response to *S. aureus* in this defined model will allow host–pathogen interaction to be determined at the molecular level.

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REFERENCES


