Wild-type *Drosophila melanogaster* as an alternative model system for investigating the pathogenicity of *Candida albicans*

Marcus T. Glittenberg¹,*, Sukrit Silas¹,²,*, Donna M. MacCallum³, Neil A. R. Gow³ and Petros Ligoxygakis¹,‡

**SUMMARY**

*Candida* spp. are opportunistic pathogens in humans, and their systemic infections display upwards of 30% mortality in immunocompromised patients. Current mammalian model systems have certain disadvantages in that obtaining results is time consuming owing to the relatively long life spans and these results have low statistical resolution because sample sizes are usually small. We have therefore evaluated the potential of *Drosophila melanogaster* as an additional model system with which to dissect the host-pathogen interactions that occur during *Candida albicans* systemic infection. To do this, we monitored the survival of wild-type flies infected with various *C. albicans* clinical isolates that were previously ranked for murine virulence. From our lifetime data we computed two metrics of virulence for each isolate. These correlated significantly with murine systemic infection. Our analysis reveals wild-type *D. melanogaster* as a sensitive and relevant model system; one that offers immense genetic tractability (having an extensive RNA interference library that enables tissue-specific gene silencing), and that is easy to manipulate and culture. Undoubtedly, it will prove to be a valuable addition to the model systems currently used to study *C. albicans* infection.

**INTRODUCTION**

*Candida albicans* is a common diploid, polymorphic fungal organism that often colonises mucosal surfaces in healthy human adults without incidence of disease. However, impairment of host defence mechanisms owing to underlying immune deficiencies, HIV infection, prolonged chemotherapy, major surgery or immunosuppressant treatments can predispose individuals to infections ranging from mucocutaneous forms of candidiasis, to systemic infections such as candidaemia and invasive candidiasis (Enoch et al., 2006; Netea et al., 2008). *Candida* has thus risen to be the fourth most common form of bloodstream infections in the USA over the past three decades (Wisplinghoff et al., 2004). Such systemic infections exhibit an associated mortality rate estimated at >30% in the USA, at >25% in the UK and at >35% in mainland Europe; of the reported deaths, roughly 30-50% are directly attributable to candidaemia. *C. albicans* is responsible for the vast majority of life-threatening disseminated *Candida* infections (up to 80%) and, indeed, is the most common fungal pathogen in humans (Edmond et al., 1999; Kibbler et al., 2003; Gudlaugsson et al., 2003; Wisplinghoff et al., 2004; Tortorano et al., 2006).

Considering the above, it is highly desirable to develop a relevant model system with which to study the host-pathogen interactions taking place during a *Candida* infection. Ideally, this would be one that combines predictive power with genetic tractability, one that is easy to manipulate, and also, one that is available in large numbers. Although mammalian models offer much, they are time consuming, expensive and labour intensive (Ashman et al., 1996; Navarro-Garcia et al., 2001; MacCallum et al., 2009) (for a review, see de Repentigny, 2004). Furthermore, typical sample sizes are too small for in-depth statistical analysis or modelling of lifetime data; this therefore precludes predictions concerning the outcome of an infection. With the realisation that innate immune responses constitute the prototypical host defence that is conserved in metazoans came the usage of genetically tractable invertebrate model systems, such as *Caenorhabditis elegans* and *Drosophila melanogaster*, as more practical alternatives for analysing fungal pathogenesis (for a review, see Mylonakis et al., 2007). Although *C. elegans* has been used very successfully for high-throughput screening of antifungal compounds (Tampakakis et al., 2008; Okoli et al., 2009), the nematode innate immune system was seen to be quite different to that of mammals and insects (for a review, see Irazogui et al., 2010), and this therefore raises issues concerning its suitability as a model system for dissecting the mammalian host defence response. By contrast, studies with *D. melanogaster* have revealed extensive conservation between the mechanisms of mammalian and insect innate immunity (for a review, see Lemaitre and Hoffmann, 2007); however, *D. melanogaster* is yet to be fully exploited as a model system for analysing *Candida* infection. Indeed, the limited studies available were performed using immune-deficient flies (Alarco et al., 2004; Chamilos et al., 2006; Chamilos et al., 2010).

**1** Genes and Development Laboratory, Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, UK

**2** Oxford-Princeton Undergraduate Exchange Program, Department of Molecular Biology, Princeton University, Princeton, NJ 08544, USA

**3** Aberdeen Fungal Group, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen, AB25 2ZZ, UK

*These authors contributed equally to this work

Author for correspondence (petros.ligoxygakis@bioch.ox.ac.uk)

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et al., 2009). In these studies, immunodeficient flies were consistently extremely sensitive to all strains of *Candida* tested and so resolving differences in virulence was not pursued. Consequently, and from a statistical point of view, there was no robust assessment of suitability for analysing host-pathogen interactions.

*D. melanogaster* mounts an efficient systemic innate immune response against disseminated infection, the hallmark of which is the production by the fat body and release into the blood of a battery of potent antimicrobial peptides (AMPs) (see Lemaitre and Hoffmann, 2007). This antimicrobial activity can persist for several days and is specific according to the broad category of the invading pathogen. The signalling pathways that control the production of AMPs are activated by the interaction of host pattern recognition receptors (PRRs) with molecules on the surface of fungi and bacteria, or by secreted virulence factors. PRR families include the peptidoglycan recognition proteins (PGRPs) and the glucan binding proteins (GNBPs). Fungal recognition in particular is attributable to the detection of fungal cell wall by GNBPs, and to activation of the host protease Persephone by secreted fungal proteases (Gottar et al., 2006; El Chamy et al., 2008). Pathogen detection by either mechanism triggers proteolytic cascades that converge on the protease Grass (El Chamy et al., 2008), which then activates Spätzle-processing enzyme (SPE) (Jang et al., 2006). As the terminal protease of the cascade, SPE is responsible for the proteolytic activation of the cytokine Spätzle (Spz), which in turn acts as a ligand for the transmembrane Toll receptor (Weber et al., 2003). This activates the Toll signalling pathway, culminating in the activation of the cytokine Spätzle (Spz), which in turn acts as a ligand for the transmembrane Toll receptor (Weber et al., 2003). This activates the Toll signalling pathway, culminating in the activation of the cytokine Spätzle (Spz), which in turn acts as a ligand for the transmembrane Toll receptor (Weber et al., 2003).

**RESULTS**

Systemic infection with various *C. albicans* clinical isolates results in differential survival of wild-type *D. melanogaster*

In order to assess whether *D. melanogaster* would make a suitable model organism for studying host-pathogen interactions during systemic *C. albicans* infection, we looked to see whether the survival trend of wild-type mice infected with one of 12 *C. albicans* clinical

Table 1. *C. albicans* clinical isolates and their virulence based on host survival

| Clinical isolate | Source country | Anatomical source | BALB/c mice MST ± s.d. (days) | Clustering solution | Test-statistic | Lifetime value |
|------------------|---------------|-------------------|-------------------------------|--------------------|---------------|--------------|
| J990102          | Belgium       | Vagina            | 6.5 ± 0.8                     | Virulent           |              |              |
| SC5314           | USA           | Blood             | 6.8 ± 1.6                     | Very virulent      |              |              |
| YsU751           | Malaysia      | Urine             | 7.3 ± 4.1                     | Virulent           |              |              |
| AM2003/0191      | UK            | Blood             | 10.7 ± 5.9                    | Somewhat virulent  |              |              |
| b30708/5         | UK            | Blood             | 12.8 ± 8.8                    | Virulent           |              |              |
| FJ9              | Fiji          | Oropharynx        | 16.5 ± 5.5                    | Somewhat virulent  |              |              |
| AM2003/0182      | UK            | Blood             | 22.0 ± 5.4                    | Somewhat virulent  |              |              |
| HUN92            | UK            | Blood             | 23.0 ± 7.8                    | Severely attenuated|              |              |
| AM2003/0069      | UK            | Vagina            | 24.5 ± 4.2                    | Attenuated         |              |              |
| a20175.016       | Israel        | Blood             | 26.2 ± 3.0                    | Attenuated         |              |              |
| AM2003/0074      | UK            | Blood             | 27.3 ± 1.6                    | Attenuated         |              |              |
| IHEM3742         | Zaire         | Blood             | 28.0 ± 0.0                    | Severely attenuated|              |              |
| Capmr1Δ         | –             | –                 | –                             | Severely attenuated|              |              |

Clustering solution to categorise *C. albicans* virulence using the test-statistics (grouping consistent to that when using the range test).

Clustering solution to categorise *C. albicans* virulence using the representative lifetime values.

The higher the value the greater the apparent virulence of the *C. albicans* strain.

*Numbers represent the time up to which 75% of flies are predicted to survive.

The 12 clinical isolates are a representative subset selected from 43 that were originally assayed for virulence in a murine intravenous challenge model (MacCallum et al., 2009). Isolates were selected so as to give a good spread of murine MSTs. SC5314 was chosen because it is considered to be the standard wild-type strain: it is the background for most mutant strains and the genome used to compile the *Candida* Genome Database. *Capmr1Δ* is a severely attenuated cell wall mutant (not a clinical isolate) that was included as a reference for low virulence. Injecting sterile PBS had little adverse effect on fly survival.
isolates (Table 1) could be reproduced in wild-type Oregon R-S (OreR) flies (with survival being a measure of C. albicans virulence in mice) (MacCallum et al., 2009). To determine the conditions of our experiment, we infected OreR flies with a dilution series of the standard wild-type C. albicans strain SC5314. Contrary to previous studies (Alarco et al., 2004; Chamilos et al., 2006), we found that by injecting C. albicans yeast cells directly into the haemolymph of wild-type flies we were capable of causing high-mortality systemic infections. Furthermore, we observed that mortality decreased successively with each sixfold dilution of the initial inoculum, with $\sim 2.1 \times 10^3$ SC5314 yeast cells per fly producing what we considered the most suitable mortality rate (i.e. flies gradually succumbed to infection at each 24-hour interval over the 7-day monitoring period, and to an extent to which differences in survival could be shifted above or below this curve; data not shown). Therefore, for our survival experiments, we infected OreR flies with $\sim 2.1 \times 10^3$ yeast cells per fly for each of the clinical isolates. In parallel, we similarly infected immune-deficient spz$^{-/-}$ flies: these are extremely sensitive to fungal infection and thus served as an additional measure of yeast virulence (Ligoxygakis et al., 2002; Apidianakis et al., 2004; Gottar et al., 2006). To monitor the consequences of the injection procedure per se, we injected sterile PBS.

To visually represent survival trends of OreR and spz$^{-/-}$ flies infected with one of the clinical isolates or a severely attenuated cell wall mutant (Capmr1$\Delta$; used as a reference for low virulence) (Bates et al., 2005), we constructed estimates of survival curves (graphical representations of the cumulative probabilities of survival past any given time) from lifetime data obtained through counting the number of flies surviving post-infection at 24-hour intervals over a 7-day period. Because only right-censored data were present at the end of the study (i.e. the only lifetime data ‘missing’ were of flies that had survived the duration of the experiment), the estimated survival curves were identical to 1 minus the empirical cumulative distribution functions (CDFs) of the censored data. One of the 13 C. albicans strains, or sterile PBS, was injected into OreR or spz$^{-/-}$ flies independently on three occasions, and the three estimated survival curves were compared pairwise using the log-rank test: no significant departures from equality were observed (Fig. 2). By contrast, for spz$^{-/-}$ flies, no informative significant differences were found between the estimated survival curves for the 12 clinical isolates; however, the severely attenuated cell wall mutant Capmr1$\Delta$ was not as virulent to the immune-deficient flies as the other C. albicans strains (Fig. 2, spz$^{-/-}$ flies).

Mathematical modelling of the lifetime data

We also wanted to obtain a metric of C. albicans virulence from the lifetime data itself; that is, one that was independent of any
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assumed reference values. For, although estimated survival curves make it easy to visually assess survival dynamics, they only provide a qualitative assessment, in that it is impossible to compare two survival curves unless either an arbitrary time point is specified, or a statistical technique such as the log-rank test is employed. These techniques, although very robust, often cannot detect subtle qualities of the estimated survival curves, and their exclusive use might potentially result in the loss of such information.

Therefore, we created a mathematical model to fit our lifetime data. As an initial guess to what a good model would be, we chose the Weibull distribution because it is a very commonly used model in the industrial sector for fitting reliability data, i.e. time-to-failure (‘death’ can be considered an example of this). The Weibull distribution was particularly suited to our needs because it is widely considered to be a mathematical expression of the weakest-link concept (Weibull, 1951): Drosophila that is systemically infected with C. albicans might be considered as a stressed system in which the failure of the unit as a whole is locally initiated by the failure of the weakest ‘link’ or vital biological subsystem within the unit. To ascertain that there were no systematic departures of our lifetime data from the Weibull distribution, we plotted the logarithm of the negative log of the surviving proportion (i.e. flies alive after a given time point) in each infected group against the log of time, and found the plots to be linear (data not shown). We therefore fitted a Weibull probability distribution function (see Eqn 1, below) to our lifetime data and recovered the maximum likelihood estimates of the parameters of the fit: \( \rho \) and \( \gamma \) in every case. The model was found to fit the data well in most cases (\( P<0.2 \) for all ‘good’ fits; \( \chi^2 \) goodness-of-fit test). The exceptions were data sets in which most of the flies had died within the first 24 hours, as with \( spz^{-/} \) flies, and those in which almost none of the flies had died by the end of the observation period. Representative graphs of 1 minus the Weibull cumulative distribution function (Eqn 2, below) superimposed on the corresponding estimated survival curve are shown for OreR flies infected with SC5314 and s20175.016 (Fig. 3). The parameters of the fit (\( \rho \) and \( \gamma \)) were then used to compute the 0.75 quantiles of the model survival curves \( \{S(t)\} \) for each strain, as the ‘representative lifetimes’ (Eqn 3, below; \( \tau \), time up to which 75% of the flies are predicted to survive) of the infected OreR flies (Table 1; supplementary material Table S1). We picked \( P=0.75 \) as the cumulative probability of survival at which to obtain the representative lifetimes of the infected flies because it was accessible within the range of our data and did not require undue extrapolation of the model. Higher cumulative probability values, although easily accessible within our data sets, might not have been representative of the survival dynamics of the infections with more virulent strains.

\[
P(t) = \frac{\gamma}{\rho} t^{\gamma-1} e^{-\left(\frac{t}{\rho}\right)^{\gamma}}
\]

(1)

\[
S(t) = 1 - \text{Weibull CDF} = 1 - \left(1 - e^{-\left(\frac{t}{\rho}\right)^{\gamma}}\right) = e^{-\left(\frac{t}{\rho}\right)^{\gamma}}
\]

(2)

\[
\tau = \rho \left(\ln p\right)^{\frac{1}{\gamma}}
\]

(3)

The representative lifetime values obtained were in good agreement with the log-rank representations of C. albicans virulence (Spearman’s rho, \( r=-0.941 \) and \( P<0.01 \); Table 1).

**Grouping C. albicans strains on the basis of virulence**

We wanted to classify the C. albicans strains on the basis of their virulence to the fly independent of rank, because although ranking revealed a statistically significant correlation for clinical isolate virulence between mice and flies, it did not provide an instructive way with which to look at the data. We therefore used grouping methods, which account for the relative differences between strains, to cluster the log-rank test-statistics or the representative lifetime values.

We compared pairwise the OreR estimated survival curve for a C. albicans strain to those for the 12 other strains and to that for the sterile-PBS-injected flies using the log-rank test, and arranged the test-statistic values into a vector for that strain. The 14 vectors (i.e. 13 strains and 1 PBS) were then grouped using a K-means approach.

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**Fig. 2. Assessment of C. albicans strain virulence in flies, using the log-rank test.** The estimated survival curves for flies infected with one of 13 C. albicans strains were compared individually to the curve for those flies injected with PBS, using the log-rank test. The resulting test-statistic values (shown with 95% confidence intervals) can be regarded as a metric of virulence: the log-rank test generates a bigger test-statistic when differences between the compared are greater, and, therefore, a higher column implies a greater C. albicans virulence. The test-statistic columns are arranged according to increasing murine MST (Table 1). Based on these values, there is a significant correlation between the virulence of the C. albicans clinical isolates to mice and OreR flies (Spearman’s rho, \( r_i = -0.876 \) and \( P<0.01 \)), but not with the immune-deficient \( spz^{-/} \) flies (\( r_i = 0.539 \) and \( P>0.05 \)).
clustering algorithm that employed the squared Euclidean method to calculate distances between them. Several different algorithms as well as cluster sizes were tested in order to arrive at an optimal and informative clustering solution; the algorithm was iterated several times to prevent against non-global minima. To assess the robustness of the clustering solution, a silhouette plot was generated (supplementary material Fig. S2): the solution had two silhouette values below the 0.6 threshold and a mean silhouette value of ~0.75. (Note that a value of 1 for a clustered object means it is in the centre of a cluster and thus well separated from neighbouring clusters.) Furthermore, the test-statistics were clustered in accordance with the K-means solution when grouped at the 95% level using a range test. Confidence bounds were computed for log lifetime values using the covariance matrices of the parameters of the Weibull fits, and the strains were subsequently grouped together if they were concordant at the 95% level (supplementary material Table S1). The resultant grouping was in general accordance with that for the test-statistics (Table 1).

Predicting the virulence of *C. albicans* clinical isolates in murine models of infection

To assess whether our approach was sufficient to allow a meaningful prediction regarding the murine virulence of uncharacterised *C. albicans* clinical isolates, we performed a blind experiment. Using identical methods, we generated virulence metrics for *OreR* flies infected with one of nine clinical isolates [labelled 1–9, from the MacCallum et al. study (MacCallum et al., 2009)] and categorised them based on our previously defined parameters. For the test-statistic clustering solutions, two isolates (AM2003/0191 and HUN92) were seen to deviate somewhat, both being relatively less virulent to the flies than they were to mice (Table 1); however, considering also the variation around the murine MSTs, it seemed reasonable to consider that *C. albicans* clinical isolates classified by test-statistic clustering as (very) virulent to wild-type flies, somewhat virulent, or (severely) attenuated, will give murine MSTs of around ≤13 days, ≥14 to ≤22 days and ≥23 days, respectively. We see that only clinical isolate 3 deviates from this; having predicted murine survival of ≥23 days for this isolate, mice actually succumbed to infection after around 12.5 days (supplementary material Table S2). Furthermore, for the complete set of 21 clinical isolates tested in this study, there was a significant correlation between the murine MSTs and test-statistics (Spearman’s rho, \( r_s = -0.709 \) and \( P < 0.01 \)).

*Drs* expression profiles of *OreR* flies infected with the various *C. albicans* clinical isolates are not statistically separable

The finding that differences between the survival of immune-deficient spz\(~/-\) flies infected with the different *C. albicans* clinical isolates were not informative suggested the resolvable virulence of the clinical isolates might actually be a consequence of Toll signalling in the host, because Spz is required to activate the Toll pathway (Lemaitre and Hoffmann, 2007). Therefore, we hypothesised that a correlation might exist between the virulence of the strains and the intensity of Toll signalling. To test this, we quantified *Drs* mRNA levels from infected *OreR* flies, with *Drs* being a downstream target of Toll signalling (Lemaitre and Hoffmann, 2007).

*OreR* flies were injected with one of the 13 *C. albicans* strains (Table 1) or with sterile PBS and, at time intervals of 0, 6, 12, 24 and 48 hours post-infection, five flies of each gender were removed and the pooled *Drs* mRNA levels quantified by qPCR. The fly innate immune response is considered to be regulated principally at the level of transcription (for a review, see Lemaitre and Hoffmann, 2007) and, as such, increases in *Drs* mRNA will predominantly reflect enhanced transcription of the *Drs* gene. The expression profiles for the 13 *C. albicans* strains were similar, mostly showing an increase that peaked at 24 hours post-infection, which thereafter started to decline (Fig. 4). At each time point, a one-way ANOVA was used to look for strain differences: at 0, 6 and 12 hours post-
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Fig. 4. Drs expression profiles for OreR flies infected with the various C. albicans strains. The C. albicans strains or sterile PBS were injected into OreR flies, and the pooled Drs mRNA levels – normalised to the non-immune ribosomal gene RP49 – from ten flies (five for each gender) were determined at 0, 6, 12, 24 and 48 hours post-infection via qPCR. The Drs mRNA levels induced by each strain were expressed as fold change relative to the PBS injection (comparative ΔΔCt method); owing to low-level Drs expression in response to the physical act of injection per se, fold changes at 6 and 12 hours might be slightly dampened. The given values represent the mean for three independent sets of injection (n=3), and the error bars show 95% confidence intervals. At each time point the data followed a normal distribution with equal variance, and a one-way ANOVA was therefore used to look for strain differences. Significant differences were found at 24 and 48 hours post-infection: 95% Tukey HSD intervals revealed that these were between only the cell wall Capmr1Δ mutant and some of the clinical isolates (six at 24 hours, and 11 at 48 hours post-infection), not between any of the isolates themselves.

DISCUSSION

C. albicans is the most important fungal pathogen of humans (Edmond et al., 1999; Kibbler et al., 2003; Gudlaugsson et al., 2003; Wisplinghoff et al., 2004; Tortorano et al., 2006; Wilson et al., 2009). Consequently, various mammalian and invertebrate model systems have been developed to evaluate C. albicans infection in vivo (for reviews, see de Repentigny, 2004; Mylonakis et al., 2007; Mylonakis, 2008). However, although each system offers certain benefits, each has distinct disadvantages. For example, invertebrate studies using Galleria mellonella and C. elegans seem to be somewhat limited with respect to dissecting C. albicans pathogenesis from the perspective of the host (Mylonakis et al., 2007; Mylonakis, 2008). Also, although mammalian systems offer a highly relevant and genetically tractable host, they are expensive, time consuming (e.g. require extensive monitoring of infected animals), restricted to small sample sizes and their use raises complex ethical issues. Considering the above, we evaluated the suitability of D. melanogaster as an additional model system in which to study C. albicans infection; to date, there has not been a robust assessment of this highly genetically tractable and manageable organism regarding such studies.

Our criterion for suitability was that differences in the virulence of 12 C. albicans clinical isolates, which were assessed recently in wild-type BALB/c mice using an intravenous challenge model (MacCallum et al., 2009), should be predictably resolved in wild-type flies. From analysing lifetime data generated through infecting wild-type flies, it seemed reasonable to consider that C. albicans clinical isolates classified as (very) virulent to wild-type flies, somewhat virulent, or (severely) attenuated, will give murine MSTs of around ≤13 days, ≥14 to ≤22 days and ≥23 days, respectively. Using these MST ranges to classify the 43 clinical isolates tested in mice (MacCallum et al., 2009),
2009), of which the 12 we analysed were a subset, we found that 17 are classified as virulent to mice, 11 as somewhat virulent and 15 as attenuated; this supports the predictive potential of our classifications. Indeed, we found that the parameters defined by our initial grouping analysis (which are consistent when the set of 21 isolates is grouped) were sufficient to reasonably predict the murine virulence of an additional set of _C. albicans_ clinical isolates. Although there are some notable deviations between the virulence of an isolate towards the two species, for example, clinical isolates 3 and AM2003/0191, overall, there is a strong correlation between the 21 virulence metrics and the murine MSTs. Furthermore, if we consider isolate 3 as an outlier and exclude it from the analysis, we see that the correlations between murine MSTs and our virulence metrics are consistent to those when only the initial 12 isolates were correlated (Spearman’s rho for the 20 clinical isolates: MSTs correlated with test-statistics, \( r_s = -0.840 \) and \( P < 0.01 \); MSTs correlated with lifetime values, \( r_s = 0.784 \) and \( P < 0.01 \)). For the weakest isolates, i.e. those with an MST of 28 (±0) days, we observed a broad range of our virulence metrics; a plausible explanation for this is that murine survival was not monitored beyond 28 days; thus, it is quite possible differences in survival would predictably resolve at later time points.

Because representative lifetime values were generated through mathematical modelling of the lifetime data, they can take values other than those at time of observation (e.g. intervals of 24 hours post-infection, as is the norm for _D. melanogaster_ survival studies) and are therefore analogous to the median lifetime values recorded in murine models of infection, where data is usually taken at the actual time of death. This means that a large number of flies can be infected (>1000 flies can be comfortably infected by a single person per day) and scored at convenient time points to return a relevant metric with which to compare the virulence of different _C. albicans_ strains, or their virulence under different host conditions.

Using criteria identical to that for performing the OreR survival experiments, we also assessed the survival of infected _spz−/−_ flies; these are very sensitive to yeast infection and thus served as an additional measure of virulence (Apidianakis et al., 2004; Gottar et al., 2006). However, in nearly all cases, _spz−/−_ flies injected with one of the clinical isolates succumbed to infection within 48 hours, with the majority not surviving beyond 24 hours. Furthermore, the survival curves did not resolve coherently. Only the Capmr1Δ mutant was statistically separable from all the other strains, with the _spz−/−_ flies being more likely to survive infection with this strain than with the other strains; this suggests that Capmr1Δ is an intrinsically weak strain of _C. albicans_. In agreement, with OreR flies it was the only strain that decreased in number (measured by colony forming units) during the first 24 hours of infection (data not shown), indicating that wild-type flies are somewhat capable of resisting Capmr1Δ infection (Read et al., 2008); this might also explain the weak Drs profile for the strain.

Our survival analysis suggested that Spz activity might actually be necessary to predictably resolve differences in the survival of infected OreR flies, raising the possibility that the differential virulence of the clinical isolates is translated via Toll signalling in the host. We therefore measured the levels of _Drs_ expression over time in OreR flies infected with the various _C. albicans_ clinical isolates, assuming, as is common to do so, that _Drs_ expression is representative of Toll signalling as a whole. However, we did not find evidence to support our hypothesis; furthermore, we failed even to find statistically significant differences between the _Drs_ expression profiles. Although this suggests that an obvious relationship between the differential virulence of the clinical isolates and intensity of Toll signalling is unlikely to exist, it does not exclude a requirement for the pathway. For example, the upregulation of infection-responsive _D. melanogaster_ genes involved in iron sequestration and/or transport is in part dependent on Toll signalling (DeGregorio et al., 2001; DeGregorio et al., 2002); because the acquisition of iron by pathogens (including _C. albicans_ is a crucial factor regarding their pathogenicity (Almeida et al., 2009), sequestration of iron by the host is an important defence response to infection (Latunde-Dada, 2009). Therefore, a _C. albicans_ strain that is highly efficient at acquiring iron from the host, under the conditions of an equivalent Toll defence response, might exhibit greater virulence than one not so competent; however, in a Toll mutant, in which host iron might be more readily available and the benefit of efficient iron acquisition therefore not so pronounced, the two strains will exhibit closer virulence. Many scenarios are plausible: activation of Toll signalling upon infection promotes a large transcriptional shift in the fly (DeGregorio et al., 2001; DeGregorio et al., 2002; Pal et al., 2008) (however, genome-wide transcriptional profiling of flies infected with _C. albicans_ is yet to be published) and affects physiology. For example, Toll signalling also stimulates haemocyte proliferation (Zettervall et al., 2004), and recently it was shown to non-autonomously attenuate insulin signalling when specifically activated in fat body tissue (DiAngelo, 2009). Notably, suppressing or enhancing insulin signalling alters the survival dynamics of flies infected with certain bacteria (Dionne et al., 2006; Libert et al., 2008). Furthermore, in diabetic individuals, _C. albicans_ colonises the oral cavity at higher densities than in non-diabetic individuals, suggesting reduced resistance to the pathogen in the former (Goncalves et al., 2006).

An alternative scenario is that the differential virulence of the clinical isolates comes from the isolates per se; that is, from their ability to overwhelm the host, rather than how they are contained by host defence-response strategies, although the two are likely to be mutually inclusive. Considered virulence factors of _C. albicans_ include (for reviews, see Calderone and Fonzi, 2001; Naglik et al., 2003; Whiteway and Oberholzer, 2004; Brown et al., 2007; Wilson et al., 2009): adhesins, which promote adherence of the pathogen to host tissues; proteins required for the transition from the yeast to the hyphal form; and secreted aspartyl proteinases (SAPs) and other secreted hydrolases, which contribute to the provision of nutrients and penetration of host barriers. It might be that pathogen virulence evolved as a countermeasure to environmental predation by small invertebrates (Mylonakis et al., 2007). For example, the natural host of the bacterium _Legionella pneumophila_ is believed to be amoebae and, notably, the replication strategy of this bacterium – the cause of its pathogenicity – is extremely similar in amoebae and in human macrophages (Farbrother et al., 2006; Zhiru et al., 2009). Thus, it might be that an ancient protein interaction(s) conserved during evolution underlies the similar virulence trend observed between flies and mice infected with the _C. albicans_ clinical isolates, and that the complexity of the mammalian immune system (or general physiology) above this, fundamentally, becomes redundant.
Previous studies using *D. melanogaster* have focused successfully on resolving the pathogenicity of *C. albicans* mutants in a *Toll* mutant fly, noting the resistance or tolerance of wild-type flies to infection (Alarcó et al., 2004; Chamilos et al., 2006). We found that immune-deficient flies are of limited use for resolving the pathogenicity of *C. albicans* clinical isolates and, furthermore, that *Toll* signalling might actually be necessary in translating the *C. albicans* infection and, importantly, that this system will have mammalian relevance regarding integrated host defence mechanisms of resistance or tolerance towards the infection (Dionne and Schneider, 2008). Alternate invertebrate model systems seem somewhat limited with regards to the latter. For example, *C. elegans* has only a single *Toll*-like receptor (and seems not to encode NFκB-like homologues), and this receptor does not have a central role in the innate immune response (Irazoiqui et al., 2010). Although a comparative study with *G. mellonella* larvae and mice revealed a significant correlation regarding *C. albicans* virulence, the yeast strains used were mutant (Brennan et al., 2002). Thus, the observed differences in virulence might be intrinsic properties of the mutants per se (as seen with Capmr1Δ in this study), rather than as a consequence of host-pathogen interactions; whether differences in clinical isolate virulence can be predictably resolved in *G. mellonella* remains to be tested. In addition, there is not a sequenced and annotated genome for *G. mellonella* and, consequently, microarray technology and RNA interference libraries are present in an available (Mylonakis et al., 2007; Mylonakis, 2008); also, isolating allelic mutants is problematic. Thus, this makes it difficult to identify host factors required to combat or control an invading pathogen (Kim et al., 2010); however, the host response to infection can be monitored through physiology and protein-orientated studies (Mowlids and Kavanagh, 2008; Mowlids et al., 2009). Taking into consideration the model systems that are currently available to study *C. albicans* infection, we feel that *D. melanogaster* will be a valuable addition to these, being one that offers good resolution of clinical isolate virulence, relevance to mammalian systems (exemplified by this study), high statistical resolution, and genetic tractability, e.g. tissue-specific gene silencing using RNA interference libraries.

**METHODS**

**C. albicans strains**

The *C. albicans* strains used throughout this study were supplied by Donna MacCallum (MacCallum et al., 2009) and are listed in Table 1: the 12 clinical isolates were isolated from various tissues and selected so as to give a good spread of murine mean survival times. An additional strain (*Capmr1Δ*) that was severely attenuated in a mouse model of virulence was included as a control for low pathogenicity: *Capmr1Δ* has a weakened cell wall due to defects in O- and N-glycosylation (Bates et al., 2005). In all cases (unless stated otherwise), strains were cultured from a single colony in 10 ml Sabouraud broth (within a 50 ml Universal tube) for 18-21 hours, shaking at 200 rpm in a 30°C incubator; at 30°C, the *C. albicans* strains grow as yeast cells rather than filamentous forms (e.g. hyphal forms), and are therefore easier to count and inject. Upon receiving, all strains were grown over night in Drop-Out[URA–] media and plated on Sabouraud agar. From these a single colony was cultured over night in Sabouraud broth, and 25% glycerol stocks made; these were stored at −80°C. Working subcultures were grown as isolated colonies on Sabouraud agar 90-mm plates and kept at 4°C; fresh subcultures were plated from the −80°C stocks every 2 weeks.

**Drosophila stocks**

We used isogenised *OreR* flies as our wild-type background (Bloomington *Drosophila* Stock Center #4269) and *Drs-GFP*; *spz* (spz−/−) flies as our immune-deficient background: *spz* is an EMS-induced hypomorphic allele of the Toll receptor ligand Spz (Lindsay and Zimm, 1992), and *Drs-GFP* is an infection-responsive Toll-dependent reporter that expresses GFP via upstream promoter regions of the *Drs* gene (Ferrandon et al., 1998). *spz−/−* flies were obtained at the time of injection from an isogenised *CyO-GFP/Drs-GFP*, *TM3/spz* stock by selecting against the *CyO-GFP* and *TM3* balancers. All flies were maintained on maize malt molasses food in bottles and reared pre-infection at 25°C; standard procedures were followed for storing and manipulating flies, with the exception of feeding them *Saccharomyces cerevisiae* yeast.

**In vivo infection assays**

Flies were infected at the same time of day with a standardised amount of *C. albicans* yeast cells administered by the same person. For inocula: *C. albicans* strains were cultured from a single colony as outlined above; cells were harvested by centrifugation (1160 g for 3 minutes) and washed in 1× phosphate buffered saline (1× PBS; at all times of application this was sterile); washed cells were again centrifuged and re-suspended in 1× PBS to an OD600 of 0.95-1.05 (readings taken with a Thermo Scientific NanoDrop 1000 spectrophotometer). Haemocytometer counts established that these inoculum suspensions contained approximately 2×10⁸ yeast cells/ml; the one exception being *Capmr1Δ*, which tends to form large cell aggregates (Bates et al., 2005). The number of viable cells/ml of these inocula also approximated to 2×10⁸ yeast cells/ml, indicating that the yeast were generally not affected by the inoculum preparation process, consistent with that seen by MacCallum et al. (MacCallum et al., 2009).

To infect flies, an inoculant was further diluted threefold in 1× PBS, and ∼32 nl injected directly into the haemolymph (this approximates to 2.1×10³ *C. albicans* yeast cells/fly); delivery was via a hand-held micro-injector (Drummond Nanoject II coupled to a fine glass needle) and the entry point was a dorsolateral region of the thorax below the wing hinge. 1× PBS was administered in an identical fashion (note that the term ‘post-infection’ includes the 1× PBS injection). When applied to *Drs-GFP* flies, this infection procedure strongly activated the infection-responsive reporter in fat body tissue of the head, thorax and abdomen, indicating a robust systemic immune response. Conversely, with infected *spz−/−* flies, the *Drs-GFP* reporter was not activated in any tissue, indicating the lack of a systemic (Toll-dependent) immune response.

For collecting survival data, sets of 30 (15 males and 15 females) CO₂-anaesthetised *OreR* or *spz−/−* flies, aged 2-4 days at 25°C, were inoculated with one of the 13 *C. albicans* strains or injected with only 1× PBS (to control for the physical injury caused by injection per se); males and females from each set were separated into different vials so as to remove potential physiological effects of
mating on survival dynamics. Flies were kept at 30°C post-infection – a compromise between C. albicans growth conditions and fly resistance to temperature – and transferred to fresh vials every 2 days; notably, C. albicans strains have been shown to form extensive hyphae in flies at 29°C (Alarco et al., 2004; Chamilos et al., 2006). For scoring survival, the number of dead flies was recorded every 24 hours by visual inspection of the vials over a 7-day period. However, flies that had died <2 hours post-infection (<3%) were excluded from the survival counts, being considered to have died as a result of the physical act of injecting. Injection of the 13 C. albicans strains or 1× PBS was repeated independently three times for both OreR and spz−/− flies, using fresh yeast plated from the ~80°C stocks and successive generations of the original isogenic fly stocks.

**Measuring Drs mRNA levels using qPCR**

Injections to determine Drs mRNA levels were carried out independently from those for collecting survival data, but the procedure was identical up to just beyond the point of injection: a set of 60 flies (30 males and 30 females) were injected, and five flies of each sex removed to a 1.5 ml Eppendorf at 0, 6, 12, 24 and 48 hours post-infection, and stored immediately at ~80°C.

From these flies, total RNA was extracted (within 7 days of freezing) using TRIzol reagent (Invitrogen) and chloroform, precipitated sequentially in isopropanol and 70% ethanol, and re-suspended in autoclaved Milli-Q water; 5 μg of this was treated with RNase-free recombinant DNase I (Roche Applied Science) to remove genomic contaminants, subsequently cleaned with phenol-chloroform/ethanol, and again re-suspended in autoclaved Milli-Q water; at this stage total RNA was checked for concentration and purity – it was considered pure if both A260/A230 and A260/A280 were >2 (readings taken with a Thermoscientific NanoDrop 1000 spectrophotometer) – and, if pure, 0.5 μg was reverse-transcribed into cDNA via the AffinityScript Multi Temperature cDNA Synthesis kit (Stratagene Products Division, Agilent Technologies), as recommended by the manufacturer’s protocol. The cDNA was stored at ~20°C.

For quantitative real-time PCR (qPCR) we used previously published primer pairs (Gobert et al., 2003): Drs(+) 5′-CTGAGAACCTTTCAATATGATG-3′; Drs(−) 5′-TCCCAGGACCACCAGCAT-3′; RP49(+) 5′-GACGCTTCAAGGGACAGTATCTG-3′; RP49(−) 5′-AACGGCGGTTCTGCATGAG-3′. Drs was our gene of interest, and Ribosomal Protein 49 (RP49) an internal control gene that did not respond to C. albicans infection (data not shown). qPCR reactions were performed using the Brilliant SYBR Green QPCR Master Mix product (Stratagene Products Division, Agilent Technologies), as outlined in the manufacturer’s instructions. Because both our qPCR targets were <200 bp in length, amplification was performed with 40 cycles: 30 seconds at 95°C; 30 seconds at 60°C; 30 seconds at 72°C. For our qPCR conditions, optimal primer and template concentrations were determined via a set of cDNA template/primer serial dilutions to be: Drs 400 nM, RP49 600 nM, and cDNA template tenfold diluted from the original stocks – primer efficiencies were 98% for Drs and 92% for RP49. Each reaction was carried out in triplicate (i.e. technical repeats) in a QIAGEN Rotor-Gene Q real-time PCR cycler with a 72-well rotor, and preliminary data analysis was carried out with the bundled software (Rotor-Gene Q v1.7.94 for Windows). Negative controls were with an equivalent amount of total RNA or no cDNA template.

Gene Q values for each technical repeat were averaged, and from these Drs mRNA levels normalised to the internal RP49 control (AC_P value). Drs mRNA levels for infected flies were expressed as fold changes relative to those flies injected with PBS, using the comparative Ct method (Schmittgen and Livak, 2008).

**Statistical analyses**

We pooled the survival data (n=87) from our three independent biological repeat sets of injection (i.e. there were no significant departures between repeats; P>0.1, log-rank test) and plotted estimates of survival curves for each C. albicans strain (and sterile PBS) using an empirical cumulative distribution estimation algorithm. Significant differences between survival data were identified using the log-rank test (Lawless, 1982). The lifetime data was modelled using a Weibull distribution (Weibull, 1951) to compute the parameters of the fit; the goodness-of-fit of the models was evaluated using χ² tests. The C. albicans strains were grouped on the basis of their virulence to flies via a K-means clustering algorithm or a range test analogous to the Student-Newman-Keuls
method. A Spearman's rho test (corrected for tied values) was used to assess a correlation of ranks between the murine and fly virulence metrics; a minus sign indicates a negative correlation; P-values were derived from a two-tailed t-distribution.

For qPCR analysis, we had three biologically independent measurements per strain (or PBS) per time point (n=3). At each time point, all normalised Drs mRNA values were expressed relative to one of the PBS injections, and standardised as described by Willems et al. (Willems et al., 2008); analysis of the residuals for the standardised data showed that they followed a normal distribution (Lilliefors test, P=0.12 to >0.5) and had equal variance (Levene's test, P=0.06 to 0.21) at each time point. Thus, mean values were plotted with 95% confidence intervals (determined using a t-statistic of approximately 4.3, i.e. P=0.05, degrees of freedom=2), and one-way ANOVA was used to look for significant differences between the means at each time point. Where a significant difference was found, 95% Tukey HSD intervals were used to locate which mean values were statistically separable.

All statistical analyses were carried out in MATLAB R2007a. We considered P<0.05 to be statistically significant. However, where the number of independent comparisons being made was large, P<0.01 was considered statistically significant.

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AUTHOR CONTRIBUTIONS
M.T.G., N.A.R.G. and P.L. designed the research; M.T.G. and S.S. performed the experiments; M.T.G. and S.S., with input from PL, analysed data; N.A.R.G. and D.M.M. contributed new reagents; M.T.G. and S.S. wrote the paper with input from M.T.G., N.A.R.G. and P.L.

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