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Correlation between virulence of *Candida albicans* mutants in mice and *Galleria mellonella* larvae

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Abstract

Candida albicans is a dimorphic human pathogen in which the yeast to hyphal switch may be an important factor in virulence in mammals. This pathogen has recently been shown to also kill insects such as the Greater Wax Moth *Galleria mellonella* when injected into the haemocoel of the insect larvae. We have investigated the effect of previously characterised *C. albicans* mutations that influence the yeast to hyphal transition on virulence in *G. mellonella* larvae. There is a good correlation between the virulence of these mutants in the insect host and the virulence measured through systemic infection of mice. Although the predominant cellular species detected in *G. mellonella* infections is the yeast form of *C. albicans*, mutations that influence the hyphal transition also reduce pathogenicity in the insect. The correlation with virulence measured in the mouse infection system suggests that *Galleria* may provide a convenient and inexpensive model for the in vivo screening of mutants of *C. albicans*.

Keywords: Immunity; Pathogenicity; Virulence testing; *Candida*; *Galleria*

1. Introduction

The dimorphic human pathogen *Candida albicans* is a significant cause of mortality among immuno-compromised patients [1,2]. *C. albicans* has several morphogenetic forms shifting between a yeast and a hyphal form of growth in response to environmental signals. Although our understanding of the signalling pathways and transcriptional networks underlying these transitions is still rudimentary [3], the ability to undergo these transitions has been implicated in *C. albicans* virulence [4]. Mice infected systemically with *C. albicans* strains defective in genes involved in the yeast to hyphal transition show reduced mortality relative to mice infected with wild-type strains of the pathogen [5,6].

Conventional in vivo assays to determine the relative pathogenicity of yeast isolates and mutants have relied upon the use of a range of mammalian species [7]. These assays have been of great value in elucidating the immune mechanism(s) involved in the host's response to fungal pathogens, but they are expensive, time consuming and involve extensive monitoring of the infected animals. Alternative systems that could provide comparable data without the need to use mammals for in vivo testing would be an useful addition to the analysis of host–pathogen interactions.

Recent evidence suggests that the innate mammalian immune responses are involved in the defence against fungal pathogens [8]. These responses are evolutionarily ancient, existing in both higher and lower animals [9]. Components of the innate immune response are conserved between mammals and insects [10,11], and thus analysis of insect responses to fungal pathogens can provide general insights into the process of host defence against fungi [10,12]. The innate defences of insects like those of mammals consist of structural and passive barriers as well as humoral and cellular responses within the haemolymph

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[13]. The latter are often activated by signal transduction systems comparable to mice [11]. The insect cuticle is the first line of defence and, as well as acting as a physical barrier to the entry of microbes, contains anti-microbial agents that prevent or retard the entry of pathogens into the host's haemocoel. Six types of haemocytes have been identified in lepidopterous (e.g. *Galleria mellonella*). The plasmatocytes and granulocytes participate in phagocytosis, encapsulation and nodule formation [14] that are important elements of the insect's cellular defences against bacteria and unicellular fungi [15]. Larger parasites are encapsulated and nodule formation occurs in response to a number of invading micro-organisms [16]. Humoral factors involved in insect immunity to infection include lysozyme, lectins and the prophenoloxidase cascade [17] and serine proteases and carbohydrases [18]. The insect immune response to micro-organisms has been shown to involve a change in the circulating haemocyte population and synthesis of new haemolymph proteins [19]. Although insects do not produce antibodies they are capable of generating a series of proteins which confer a degree of non-specific immunity to a range of micro-organisms. In addition, the ability of *G. mellonella* larvae to detect differences in the pathogenicity of lipopolysaccharide-deficient mutants of *Pseudomonas aeruginosa* has been demonstrated [17], and a good correlation exists between the virulence of *P. aeruginosa* in *Galleria* larvae and in mice [20]. Larvae of *G. mellonella* have recently been used to determine the relative virulence of *C. albicans* isolates and to differentiate between pathogenic and non-pathogenic yeast species [21].

The work presented in this paper describes the virulence of a series of well-defined *C. albicans* mutants infected into larvae of the wax moth *G. mellonella* through haemocoel injection. The virulence of the *C. albicans* mutants in *G. mellonella* correlated with that determined for systemic infection models in mice. The use of *G. mellonella* larvae may thus represent a means of measuring alterations in fungal virulence without the need to resort to mammalian testing as a first resort.

2. Materials and methods

2.1. Insect larvae

Larvae of the sixth developmental stage of *G. mellonella* (Lepidoptera: Pyralidae, the Greater Wax Moth) were obtained from the MealWorm Company, Sheffield, England. Larvae were maintained at 15°C in wood shavings and stored in the dark. Larvae between 0.2 and 0.4 g in weight were employed in all assays. Ten randomly chosen larvae of the required weight were used per treatment in each experiment. All assays were performed on three independent occasions and the results are the mean percentage survival ± standard deviation.

2.2. Candida strains and culture conditions

Candida strains used were derivatives of CAI4 [22] generated by site-directed deletion of various genes, or the *URA3*+ wild-type SC5314 [22] (see Table 1). *C. albicans* MEN (serotype B, wild-type originally isolated from an eye infection, a gift from Dr D. Kerridge, Cambridge, UK) was employed as a positive control in a number of assays. *Saccharomyces cerevisiae* YJM128 is a clinical isolate [23] (a kind gift from Dr K. Clemons, Santa Clara Valley Medical Centre, San Jose, CA, USA) which was employed in a number of assays as it had a negligible effect on *Galleria* viability.

Yeast isolates were grown to stationary phase (approximate concentration of 3×10^8 cells ml^{-1}) over 15 h in 50 ml of YEPD broth (2% (w/v) glucose (Sigma Chemical Co., Dublin, Ireland), 2% (w/v) bactopeptone (Oxoid Ltd., Basingstoke, England) and (1% (w/v) yeast extract (Oxoid Ltd.)) in 100-ml conical flasks at 30°C and 200 rpm in an orbital incubator. Yeast cultures were maintained on YEPD agar (as above but with the addition of 2% (w/v) agar (Difco, Detroit, MI, USA) at 4°C and sub-cultured every 2 weeks.

2.3. Inoculation of G. mellonella with C. albicans

The yeast cell concentration was assessed using an haemocytometer following dilution of a 100-μl aliquot of culture in phosphate-buffered saline (PBS, pH 7.2). Yeast cells were washed twice with PBS and resuspended in sterile PBS, following harvesting by centrifugation at $2220\times g$ for 5 min in a Beckmann GS-6 centrifuge. Larvae were injected with 1×10^6 cells in 100 μl PBS into the haemocoel through the last left pro-leg as described previously [17] using an SGE 1-ml gas-tight syringe (SGE Scientific Party Ltd., Melbourne, Australia). Three types of controls were used in each assay: (1) The first was the untouched control in which the larvae were not handled and incubated at the same temperature as larvae to be inoculated. (2) The second set of controls consisted of larvae that had

Table 1
Yeast strains used in this study

C. albicans strain	Relevant genotype/mutation
CA LJ3	*Δcacla4/Δcacla4*
SC 5314	Clinical isolate; Ura^+ parent of CAI4
CDH10	*Δhst7/Δhst7*
CDH22	*Δcst20/Δcst20*
CDH107	*Δcaras1/Δcaras1*
CP29-1-7	*Δcpp1/Δcpp1*
CK43B-16	*Δcek1/Δcek1*
CR216	*Δcacdc35/Δcacdc35*
HLC 67	*Δefg1/Δefg1*
HLC 69	*Δcph1/Δcph1*; *Δefg1/Δefg1*
JKC 19	*Δcph1/Δcph1*
MEN	Clinical isolate
S. cerevisiae YJM 128	Clinical isolate

a sterile syringe inserted into the last left pro-leg but no yeast or PBS was injected. (3) The PBS control consisted of larvae injected with 20 μl of sterile PBS through the last left pro-leg. All groups of insects were placed in a static incubator in the dark at 30°C, the optimum temperature for insect growth and development. Ten larvae were used per treatment and assays were performed on three independent occasions. Results represent the mean percentage survival of larvae from all assays.

2.4. Murine challenge

For systemic infections, 8–10-week-old female BALB/c mice were analysed as described [24,25]. Adenylyl cyclase mutants were inoculated at higher cell densities to compensate for the slower growth [26], while standard conditions were used for the analysis of the *Ras1* mutant strain [27].

3. Results

3.1. Determination of pathogenicity of SC5314 and derivatives

Previous studies have focussed on the ability of larvae of *G. mellonella* to differentiate between pathogenic and non-pathogenic yeast species [21] and a correlation between the virulence of bacteria as determined in *Galleria* and mice has been established [20]. In the work presented here we sought to establish whether a similar correlation existed between the virulence of a number of *C. albicans* mutants as determined in insect larvae and in murine systemic infection models. Larvae were inoculated as described and monitored over a 48-h period.

Recent molecular studies of *C. albicans* have focused on the strain background SC5314 and its *ura3* mutant derivative CAI4 [22] and several mutant derivatives of the SC5314 background have been tested in systemic and superficial mammalian models of virulence [7]. We have investigated the pathogenicity of several of these strains (Table 1) using the *G. mellonella* larval virulence model [21]. The strains CR216 (deleted for the adenylyl cyclase gene *CACDC35*) and CALJ3 (deleted for the *CACLA4* gene) had minimal effects on larval mortality (Table 2). None of the larvae infected with the adenylyl cyclase mutant, and only 10% infected with the *cla4* mutant died after 24 h. Larvae infected with the cyclase mutant were all still alive after 48 h. This was in marked contrast to the wild-type SC5314 strain that killed 85% of the infected larvae after 24 h, and all the larvae after a 48 h incubation.

The strains mutated in the *RAS1*, *CPP1*, *CEK1* and *EFG1* genes, as well as the strain containing mutations in both the *EFG1* and *CPH1* genes were more successful in killing the *G. mellonella* larvae (Table 2). In the case of the *ras1* mutant strain 50% of the larvae were killed at 24 h and 90% were killed after 48 h. This was less than that observed for the wild-type *C. albicans* strain SC5314, but more than for the *cacla4* and *cacdc35* mutant strains. Similarly, the double *cph1 efg1* mutant strain was more virulent than the *cacdc35* strain, but less effective at killing the insect larvae than the wild-type strain. The *cpp1*, *cek1*, *efg1* and *cst20* mutant strains were marginally less virulent than the wild-type strain, while the *hst7* and *cph1* defective strains appeared at least as virulent as the wild-type strain. These results were compared with those determined through systemic mouse infection studies. Previous work had established that the *cacla4* mutant strain [5], the *cph1 efg1* strain [6] and the cyclase-defective strain [26] were avirulent in mouse infection models, and these mutants showed little or reduced pathogenicity in the *Galleria* model (Table 2). In contrast, the *hst7*, *cst20* [27] and *cph1* [28] mutant strains were as virulent as the wild-type in the systemic mouse model. The *cpp1*, *cek1* and *efg1* mutants were able to kill mice in the systemic infection models, but their virulence was reduced relative to the wild-type strain [24,25,6]. These mutants also showed intermediate killing levels in the *Galleria* infection model, as did the strain defective in *ras1* function [29]. There was a significant correlation in yeast virulence between the two models ($r = 0.899$, $P < 0.01$).

Table 2
Survival (%) of insect larvae following challenge with yeast isolates

Strain	24 h viability	48 h viability	Mouse viability
Untouched	100	100	control
Injected	100	100	control
PBS	100	97	control
CaLJ3 (cla4)	90	80	avirulent [5]
CR216 (cdc35)	100	100	avirulent [26]
CDH107 (ras1)	50	10	reduced [29]
CP29-1-7 (cpp1)	20	10	reduced [24]
CK43B-16 (cek1)	20	0	reduced [25]
CDH10 (hst7)	10	0	virulent [27]
CDH22 (cst20)	20	0	virulent [27]
HLC67 (efg1)	20	10	reduced [6]
HLC69 (cph1efg1)	33	10	avirulent [6]
JKC19 (cph1)	0	0	virulent [6]
SC5314 (WT)	15	0	virulent (all studies)
MEN (WT)	22	0	positive control
YJM128 (*S. cerevisiae*)	90	90	negative control

All values are the mean percentage survival. Assays were performed on three independent occasions with 10 larvae being employed per yeast strain per assay. In all cases the standard deviation is less than ±5% of the mean.

4. Discussion

Virulence studies in mouse models have correlated the ability of *C. albicans* to undergo the yeast-to-hyphal transition with the pathogenicity of the fungus [5,6]. However, the mutations that disrupt this transition are pleiotropic,

and thus the reduction in pathogenicity could be due to loss of other virulence factors in the strains with compromised hyphal formation [30]. In this study we have determined that a series of mutations which reduce *C. albicans* hyphal formation and virulence in mouse models also reduce their virulence in an insect infection model.

The wax moth *G. mellonella* has previously been used to test the virulence of clinical isolates of *C. albicans* [21], and to correlate the virulence of a variety of *P. aeruginosa* mutants with mice [20]. In this study we found a broad correlation in virulence in the comparison between the mouse infection studies and the *Galleria* infections for a series of defined isogenic single mutants of the SC5314 strain of *C. albicans*. CR216 is deleted for adenylyl cyclase; this strain is totally avirulent in mice [26] and gives 100% survival when tested in *G. mellonella*. Two other *C. albicans* mutants that were avirulent in mice were also examined; *cacla4* [5] and the *efg1 cph1* double mutant [6]. The *cacla4* mutant showed very little killing in *Galleria*, with 90% of the larvae surviving after 24 h. The *efg1 cph1* double mutant was also less virulent than the wild-type strain, with 33% larval survival after 24 h. Thus all these mutants significantly reduce killing in both the murine and insect models.

The *RAS1*-deleted mutant strain is also defective in killing mice, but is more virulent in the systemic murine infection model than the adenylyl cyclase mutant or the *cacla4* mutant. There is 25–50% survival in mice after 35 days of infection [29]. This *ras1* mutant generates 50% killing after 24 h and 90% killing after 48 h in *G. mellonella*. Thus the *Galleria* model places *ras1* mutants at a virulence level similar to that of the double transcription factor mutant *efg1 cph1*, while the murine model suggests the *ras1* mutants retain some level of virulence. This shows that while the correlation between the insect and murine virulence models is good it is not perfect; in particular it appears the loss of the Efg1p and Cph1p transcription factors may be less significant in blocking virulence in insects than in mice.

Cpp1p, Cek1p, Hst7p and Cst20p are components of a MAP kinase pathway that plays a role in fungal virulence in mice. *Cpp1* and *cek1* mutants killed about 50% of infected mice after 35 days [24,25], while the wild-type *C. albicans* strain killed all the infected mice within 7–12 days. These mutants were also slightly less virulent in the *G. mellonella* model, as 20% of the larvae survived infection of either mutant after 24 h, while only 15% of the wild-type infected larvae survived. The *HST7*-deleted strain was found to be wild-type in terms of virulence in a mouse study [27], and only 10% of the *Galleria* larvae infected survived after 24 h, so both assay systems determined the loss of Hst7p function to have no influence on virulence. The *cst20* mutant was marginally less virulent in mice in the same study, as 100% of the mice were killed after 6 days with the wild-type and *hst7* mutant strains, and after 10 days with the *cst20* mutant strain [27]. In *Galleria*, the *cst20* mutant killed 80% of the larvae in 24 h, so in both the *Galleria* and mouse models *cst20* mutants were somewhat less virulent than the wild-type and *hst7* mutant strains. A similar correspondence in the virulence of *C. albicans* mutants in the mouse and *Galleria* models was found for mutants in transcription factors influencing hyphal development. Mutants in the Cph1p transcription factor were as virulent as wild-type strains in mice [6], and killed 100% of infected larvae after 24 h. The *efg1* mutant strain showed reduced virulence in mice [6] and killed only 80% of infected *Galleria* after 24 h. Finally, as noted, the double *efg1 cph1* transcription factor mutant was avirulent in mice and permitted 33% survival of *Galleria* larvae after 24 h.

The work presented here demonstrates that differences in the virulence of hyphal-deficient mutants detected in *Galleria* are similar to those observed in mice. While the insect and mammalian immune systems differ they share a high degree of similarity in terms of their innate immune responses [9] which are important defenses against fungal infections [8]. The response of insects to infection shows strong similarities to that found in mammals [20,21,31] and opens the possibility of reducing the need to use mammals for testing the virulence of mutants by employing *Galleria* larvae as a primary screening mechanism. The ability to screen a large number of mutants in a short time period using *G. mellonella* would have a number of cost and labour advantages compared to the use of conventional vertebrate models.

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