Differential regulation of the transcriptional repressor *NRG1* accounts for altered host cell interactions in *Candida albicans* and *Candida dubliniensis*

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Summary

Candida dubliniensis **is genetically closely related to** ***C. albicans,*** **but causes fewer infections in humans and exhibits reduced virulence and filamentation in animal models of infection. We investigated the role of the** ***C. dubliniensis*** **transcriptional repressor-encoding gene** ***CdNRG1*** **in regulating this phenotype. Deletion of both copies of** ***CdNRG1*** **increased the formation of true hyphae by** ***C. dubliniensis*** **in response to serum, exogenous cAMP and CO_2. In addition, deletion of** ***CdNRG1*** **greatly enhanced filamentation and survival of** ***C. dubliniensis*** **in co-culture with murine macrophages. In the reconstituted human oral epithelium (RHE) infection model, the** ***nrg1*****Δ mutant caused increased tissue damage relative to the wild-type strain. However, deletion of** ***CdNRG1*** **did not change the virulence of** ***C. dubliniensis*** **in the systemic mouse model of infection. The increased rate of hypha formation in** ***C. albicans*** **relative to** ***C. dubliniensis*** **in response to phagocytosis by macrophages and serum was associated with rapid downregulation of** ***NRG1*** **expression in** ***C. albicans*****. This study demonstrates that the reduced virulence of** ***C. dubliniensis*** **is due to the inability of this species to modulate** ***NRG1*** **expression in response to the same environmental signals that promote filamentation in** ***C. albicans*****.**

Introduction

Candida dubliniensis is closely related to the pathogenic yeast *Candida albicans* and shares many of its characteristic phenotypic traits, such as the ability to produce true hyphae and chlamydospores (Gilfillan *et al.*, 1998; Sullivan *et al.*, 1995). *Candida dubliniensis* was first described in 1995 and is mainly associated with oral candidosis in severely immunocompromised patients, including those with HIV-infection and AIDS (Sullivan *et al.*, 1995; Sullivan *et al.*, 2004). Although numerous studies have subsequently described the isolation of *C. dubliniensis* from various patient groups and anatomical sites, current evidence suggests that its prevalence in the human population is significantly lower than that of *C. albicans* (Sullivan *et al.,* 2004). Strikingly, the incidence of haematogenous candidosis caused by *C. dubliniensis* in England and Wales was reported to be 30-fold lower than that caused by *C. albicans* (Kibbler *et al.,* 2003). The results of similar epidemiological studies throughout the world confirm that *C. dubliniensis* is rarely responsible for systemic candidosis (Sullivan *et al.*, 2004). This lower incidence of infection suggests that *C. dubliniensis* has a reduced ability to cause the type of infections normally associated with *C. albicans* (i.e. mucosal and systemic candidosis). Two studies have shown that mice infected with *C. dubliniensis* isolates via intravenous injection have significantly higher survival rates than those infected with a similar dose of *C. albicans* (Gilfillan *et al.,* 1998; Vilela *et al*., 2002). Recently, Stokes *et al*. (2007) using an infant mouse oral intragastric model of infection showed that *C. dubliniensis* failed to colonise the gastric mucosa and was less successful than *C. albicans* at passing into the bloodstream to cause haematogenous infection. Furthermore, *C. dubliniensis* exhibited less filamentation *in vivo* compared to *C. albicans*, particularly following systemic spread to the kidneys, a phenotype also noted by Vilela *et al.* (2002).

Filamentation is now widely accepted to be one of the major virulence factors of *C. albicans. Candida dubliniensis* produces fewer hyphae than *C. albicans* under most environmental conditions that promote this morphological transition, including growth in serum and following shifts in pH and temperature (Gilfillan *et al.*, 1998; Stokes *et al.*, 2007). In addition, a recent survey of the *C. dubliniensis* genome using DNA microarrays revealed that several hypha-specific virulence-associated genes of *C. albicans* were either absent in *C. dubliniensis* (e.g. the proteinases *SAP5* and *SAP6*) or were significantly divergent at the nucleotide sequence level (e.g. the hypha-specific adhesin *HWP1*) (Moran *et al.*, 2004). These findings suggest that *C. albicans* hyphal structures may be better adapted to colonise or infect certain tissues *in vivo*. We hypothesise that this reduced filamentation phenotype may be one of the major reasons why *C. dubliniensis* is less invasive and, therefore, less pathogenic than *C. albicans*.

Despite these findings, there are specific environmental conditions in which *C. dubliniensis* produces filaments and chlamydospores more efficiently than *C. albicans*, such as on Staib (Niger [*Guizotia abyssinica*] seed creatinine) agar, and on tobacco extract-based media and Pal's agar (Al-Mosaid *et al.*, 2003; Khan *et al.*, 2004; Staib and Morschhauser, 1999). Staib & Morschhäuser (2005) recently identified the molecular basis for this phenotypic difference. They showed that a zinc-finger DNA-binding protein, Nrg1p, is differentially expressed in the two species when grown in Staib medium. The Ca*NRG1* gene is a homologue of the *Saccharomyces cerevisiae NRG1* gene encoding a transcriptional repressor and was first identified in *C. albicans* as a repressor of filamentation (Braun *et al.*, 2001). *Candida albicans* Nrg1p is a sequence-specific DNA-binding protein that targets the Ssn6-Tup1 co-repressor complex to a subset of genes that are transcriptionally

activated during hypha formation. This program of Tup1p-mediated transcriptional repression is controlled at least in part by regulation of the expression of *NRG1* transcription (Murad *et al.*, 2001a; Murad *et al.*, 2001b). Staib & Morschhäuser (2005) showed that the *C. dubliniensis NRG1* gene is specifically downregulated on Staib medium, thus allowing formation of hyphae and chlamydospores on this medium. In contrast, *C. albicans NRG1* is constitutively expressed in Staib medium and cells remain in the buddding yeast phase of growth.

In the present study, we set out to examine the role of hypha formation as a determinant of differential virulence in *C. albicans* and *C. dubliniensis*. We examined the filamentation and virulence of both species during infection of the murine macrophage cell line RAW264.7, and identified the effects of deletion of *CdNRG1* in *C. dubliniensis* on filamentation and virulence in this model and also during infection of the reconstituted human oral epithelial (RHE) cell model and following intravenous inoculation in mice (Marcil *et al.*, 2002; Schaller *et al.*, 1998).

Results

Candida dubliniensis *does not form true hyphae following phagocytosis by RAW264.7 macrophages*

Several studies have demonstrated that under many *in vitro* and *in vivo* conditions *C. dubliniensis* forms hyphae at a reduced rate compared to *C. albicans* (Gilfillan *et al.*, 1998; Stokes *et al.*, 2007). In order to study the effects of reduced filamentation in *C. dubliniensis* on virulence, we used a simple *Candida*-macrophage co-culture model with the murine cell line RAW264.7. Several investigators have demonstrated that filamentation is crucial to survival and proliferation of *C. albicans* during co-culture with murine macrophage cells (Lo *et al.*, 1997; Lorenz and Fink, 2001; Marcil *et al.*, 2002). Infection of monolayers of RAW264.7 macrophages (1.5×10^5 cells) with *C. albicans* or *C. dubliniensis* yeast cells at multiplicities of infection (MOIs) of 1:2 to 1:128 (*Candida*:macrophage) demonstrated that >90% of *Candida* cells of both species were phagocytosed by 1 h. However, within 3 h of inoculation, phagocytosed *C. albicans* cells had begun to form hyphae, which protrude from within the macrophage (Fig. 1A). In contrast, *C. dubliniensis* cells failed to form true hyphae following phagocytosis and failed to escape from the phagolysosome (Fig. 1B). Following a 16 h co-culture with these macrophage cells at MOIs of 1:2 to 1:128, the proliferation of *C. dubliniensis* isolates was greatly reduced relative to macrophage-free growth controls containing the same inoculum (Fig. 1C). The growth of *C. albicans* isolates in macrophage co-culture was significantly greater ($p < 0.001$) compared to growth of *C. dubliniensis* in macrophage co-culture at all MOIs except 1:128 (Fig. 1C**).**

Stimulation of the RAS-cAMP pathway does not promote filamentation in C. dubliniensis

In order to investigate the molecular basis for the significantly reduced filamentation in *C. dubliniensis* relative to *C. albicans*, we examined whether stimulation of the RAS1-cAMP pathway could induce the formation of true hyphae. Preliminary bioinformatics analysis of the *C. dubliniensis* genome (http://www.sanger.ac.uk/sequencing/Candida/dubliniensis/) suggests that the genes encoding the major elements of this pathway are highly conserved in *C. dubliniensis* (data not shown). In particular, *C. dubliniensis RAS1* shares 83% identity at the amino acid sequence level with its *C. albicans* orthologue. We attempted to stimulate the Ras1p-activated cAMP signalling pathway in *C. dubliniensis* by using a dominant active *CaRAS1* allele containing the G13V substitution which has been shown to render Ras1p hyperactive in *C. albicans* and *S. cerevisiae*. We transformed *C. dubliniensis* CDUM4B with the *ARS*-containing plasmid pLJ65, which contains a hyperactive *RAS1* allele (*RAS1*^{G13V}) under the control of the *C. albicans PCK1* promoter (Leberer *et al.*, 2001). In our hands, this promoter could drive expression of *yGFP3* in *C. albicans* and *C. dubliniensis* when grown in medium containing 1% (w/v) casamino acids as the sole carbon source (data not shown). Transformation of *C. albicans* CAI4 with the *RAS1*^{G13V} expression plasmid (pLJ65) yielded highly wrinkled colonies consisting of filamentous cells following growth on selective medium containing 1% (w/v) casamino acids at 30˚C. However, in *C. dubliniensis* CDUM4B, expression of *RAS1*^{G13V} did not result in any significant change in colonial morphology when compared with strains harbouring the empty pVEC vector (data not shown). In liquid culture, when transformants of both species were grown at 30˚C in minimal medium containing 2% (w/v) glucose and transferred to medium containing

1% (w/v) casamino acids, both species initially produced germ-tubes, probably due to changes in carbon source and cell density. However, following 6 h incubation, *C. albicans* harbouring pLJ65 became hyperfilamentous compared to transformants harbouring the empty pVEC vector (Fig. 2A). In contrast, *C. dubliniensis* transformants harbouring pVEC or pLJ65 reverted to budding or pseudohyphal growth (Fig. 2A). Also, the addition of cAMP (10 mM dibutyryl-cAMP) to liquid DMEM medium failed to stimulate production of filaments in *C. dubliniensis* Wü284, whereas addition of cAMP to *C. albicans* SC5314 cells accelerated hypha production (Fig. 2B). These data suggested that activation of filamentation via the Ras1p-cAMP pathway may be under strong negative repression in *C. dubliniensis*.

Deletion of C. dubliniensis NRG1 *increases the rate of filamentation*

Recently, Staib & Morschhäuser (2005) described a homologue of the *C. albicans* filamentation repressor-encoding gene *NRG1* in *C. dubliniensis,* encoding a protein with 86% similarity at the amino acid sequence level to *C. albicans* Nrg1p. We deleted both copies of the *NRG1* gene in the *C. dubliniensis* strain Wü284 using the SAT1-flipper technique, generating the homozygous *CdNRG1* deletion strain CDM10 (Reuss *et al.*, 2004) (Fig. 3). On normal YPD agar at 37˚C, this strain produced wrinkled colonies consisting of elongated yeast cells and pseudohyphae, whereas the wild-type strain formed smooth colonies (Fig. 4A). The mutant also formed highly wrinkled colonies with hyphal fringes on Spider medium and serum-containing medium (Fig. 4A). Introduction of either the *C. albicans* or *C. dubliniensis NRG1* genes under control of their own promoters on an integrative plasmid could restore the wild-type smooth colony phenotype on most media (strains CDM11 and CDM12, Fig. 4A). However, the phenotype on Spider medium was only partially

complemented by introducing a single copy of *CdNRG1* in strain CDM11, as sectors of the colony exhibited irregular edges (Fig. 4A) On Staib medium, wild-type *C. dubliniensis* strains have been shown to downregulate *NRG1*, yielding rough colonies with hyphal fringes. The *nrg1*Δ/*nrg1*Δ mutant CDM10 also produced this phenoytpe, as did the *C. albicans* and *C. dubliniensis NRG1* reintegrant strains CDM11 and CDM12. These data indicate that trans-acting factors in *C. dubliniensis* can downregulate both the *CaNRG1* and *CdNRG1* genes in *C. dubliniensis* when grown on Staib agar. In liquid DMEM medium at 37˚C, wild-type *C. dubliniensis* cells grew as yeasts and pseudohyphae. The *C. dubliniensis nrg1*Δ/*nrg1*Δ mutant CDM10 was predominantly pseudohyphal in this medium, but unlike the wild-type strain, formation of true hyphae could be stimulated by the addition of 10 mM dibutyryl-cAMP or by growth in the presence of 5% (v/v) CO_2 (Fig. 4B). Again, the non-filamentous wild-type phenotype could be restored by introduction of either the *C. albicans* or *C. dubliniensis NRG1* genes. We also compared the rate of true hypha formation in water containing 50 % (v/v) foetal bovine serum (Fig. 4C). The wild-type *C. dubliniensis* strain Wü284 produced true hyphae under these conditions, but the rate of formation was reproducibly higher in the *nrg1*Δ/*nrg1*Δ mutant strain CDM10. The growth rate of the mutant strain in YPD broth at 37˚C was essentially identical to that of the wild-type strain (doubling times of 75.5 min and 76.5 min, respectively). CDM10 did not exhibit increased tolerance to environmental stress, and like the wild-type failed to grow at 42˚C and grew slowly on medium containing 1 M NaCl. We did not observe any difference in susceptibility to oxidative stress tested by growing CDM10 and wild-type cells in H_2O_2 or the nitric oxide-generating molecule dipropylenetriamine NONOate (Hromatka *et al*., 2005).

Differential expression of NRG1 *in* C. dubliniensis *and* C. albicans

Staib & Morschhäuser (2005) showed that the *NRG1* gene is differentially regulated in *C. albicans* and *C. dubliniensis* when cultured on Staib medium (syn. *Guizotia abyssinica* creatinine agar). On this medium, *C. dubliniensis* produces filaments due to downregulation of the *NRG1* gene. We hypothesised that the opposite may be true under conditions when *C. albicans* encounters host cells (e.g. macrophages), or when conditions are similar to those encountered *in vivo* (e.g. serum at 37˚C), that is, that *NRG1* mRNA levels may be downregulated more rapidly in *C. albicans* in response to host signals. Using the *yGFP3* coding sequence (Cormack *et al.*, 1997) fused to the *C. albicans* and *C. dubliniensis NRG1* promoter sequences, we could monitor the activity of this promoter under a variety of experimental conditions. This reporter gave results similar to those reported by Staib & Morschhäuser (2005) in liquid Staib medium in which *C. dubliniensis* formed chlamydospores. Fluorescence of *C. dubliniensis* chlamydospores produced in this medium was greatly reduced compared to yeast cells (data not shown). The activity of the *NRG1* promoter was also monitored during phagocytosis of yeast cells from both species by RAW264.7 macrophages. By 5 h post inoculation of the macrophages, cells of *C. dubliniensis* were still within the phagolysosome and exhibited strong green fluorescence (Fig. 5A). In contrast, *C. albicans* cells were initially fluorescent following phagocytosis, with fluorescence greatly reduced in hyphae following 5 h co-culture (Fig. 5A). By using real-time PCR we directly quantified the level of *NRG1* expression in both species relative to expression of *TEF1* transcript. Following 1 h co-culture with macrophages, expression levels of *NRG1* in *C. albicans* had dropped to less than 15% of that in preculture cells in YEPD. This decrease coincided with the production of true hyphae of *C. albicans* under these conditions. However, in *C. dubliniensis*,

although expression levels initially dropped to approximately 50% of preculture levels, expression then increased to ~70 % of that observed in preculture cells (Fig. 5B). In 10 % (v/v) foetal calf serum, both species produce true hyphae; however, *C. albicans* produces hyphae more rapidly than *C. dubliniensis*. When *NRG1* expression levels were compared under these conditions, we observed a much more rapid repression of *NRG1* expression in *C. albicans* than in *C. dubliniensis* (Fig. 5C). In *C. dubliniensis*, under the same conditions, significant amounts of true hyphae were only observed at 3 h, when *NRG1* levels dropped below 40 % of those in the YEPD preculture.

Deletion of CdNRG1 *enhances virulence of* C. dubliniensis *in a macrophage co-culture model*

The growth and survival of the *nrg1*Δ/*nrg1*Δ mutant CDM10 in co-culture with macrophages was compared to the wild-type strain. In order to have an equal inoculum of yeast cells, both strains were subjected to gentle sonication in a water bath for 5 min prior to inoculation. This treatment broke up any unseparated cells in the *nrg1*Δ mutant. During co-culture with RAW264.7 macrophages, CDM10 was phagocytosed as efficiently as the wild-type *C. dubliniensis* (Fig. 6A). However, while the wild-type *C. dubliniensis* strain failed to produce filaments following phagocytosis, CDM10 produced abundant true hyphae and some pseudohyphae. When the proliferation of CDM10 was compared with the wild-type in the XTT proliferation assay, CDM10 was found to have significantly increased survival and growth compared to the wild type (Fig 6B). Wild-type levels of survival could be restored in CDM10 by reintroduction of the *NRG1* gene from *C. dubliniensis* (strain CDM11) or from C. *albicans* (strain CDM12). These complemented strains formed

some pseudohyphae during co-culture with macrophages, but exhibited greatly reduced growth compared to CDM10 (Fig 6B). The increased proliferation of CDM10 was found to be significantly greater than the *C. dubliniensis* wild-type and reintegrant strains at MOIs of 1:8 and 1:32 ($p < 0.01$). We also examined the virulence of the *C. albicans nrg1*Δ mutant, MMC3, in this model (Murad *et al.*, 2001b). However, this mutant did not exhibit a phenotype that was significantly different from the parental *C. albicans* strain, as both produced hyphae under the conditions of co-culture and proliferated to a similar extent when measured with the XTT assay (data not shown).

Deletion of CdNRG1 *enhances virulence of* C. dubliniensis *during infection of RHE*

Stokes *et al.* (2007) showed that *C. dubliniensis* is significantly less virulent than *C. albicans* following infection of reconstituted human oral epithelium (RHE) (Schaller *et al.*, 1998). In this study, it was confirmed that *C. dubliniensis* isolates failed to filament during the infection, adhered poorly to the epithelial surface and caused less damage to the epithelial tissue. We compared the virulence of Wü284 with the *CdNRG1*-deletion mutant CDM10 in order to determine if the increased rate of filamentation observed in this strain could enhance RHE tissue damage. The morphology of infecting strains was examined in tissue sections and tissue damage was estimated by measuring the levels of the human enzyme, lactate dehydrogenase (LDH), released from infected epithelial cells. Wü284, typical of wild-type *C. dubliniensis* isolates, grew exclusively in the yeast phase following inoculation onto RHE (Fig. 7A) (Stokes *et al.*, 2007). At 24 h post inoculation, levels of LDH released from tissues infected with strain Wü284 were similar to uninfected controls (Fig. 7B). However, at this time point CDM10 had produced filaments (predominantly

pseudohyphae) and had induced greater levels of LDH release from the infected tissues (Fig. 7B). At 48 h, the integrity of the epithelial surface infected by CDM10 exhibited greater disruption, whereas those infected with wild-type and reintegrated strains were still intact (Fig 7A). The levels of LDH released into the culture medium at this timepoint were also significantly greater in tissues infected with the *nrg1*Δ mutant CDM10 compared with those infected with Wü284 or the reintegrated strain CDM11 ($p < 0.001$, Fig. 7A). The morphology of the reintegrant strain CDM11 was similar to that of the wild-type and induced similar levels of LDH release from infected tissues (Fig. 7B).

Virulence of a C. dubliniensis nrg1Δ *mutant in a systemic mouse infection model*

We compared virulence in the systemic mouse model of infection of *C. dubliniensis* Wü284, the *CdNRG1* deleted strain CDM10 and a derivative with a reintegrated copy of the gene (CDM11). The virulence of all strains was significantly less than that of *C. albicans* SC5314. Similar fungal burdens were recovered in all *C. dubliniensis* strains from the brain, spleen and kidney at two days post-infection (data not shown). Following 28 days infection, no significant difference in survival was observed between the *C. dubliniensis* wild type and *nrg1*Δ mutant strains (mean survival times of 17.8±8.8 and 19±7.1 days) (Fig 8A). Mice infected with *C. albicans* SC5314 in parallel experiments died within 2 days of infection. Candidal burdens in the brain and kidney were assessed for each *C. dubliniensis* strain and no significant difference in load was observed. Due to low burdens in kidneys post infection, wild-type *C. dubliniensis* cells were difficult to find in tissue sections. However, cells that were observed were in the yeast phase, and surrounded by infiltrates of inflammatory cells (Fig. 8B). In contrast, the *nrg1* null strain cells were only found as a single large

clump in a blood vessel of the kidney, consisting of filaments and yeast cells (Fig. 8C).

Discussion

Candida albicans has adapted to survive as a commensal organism on human mucosal surfaces (Odds, 1994). However, *C. albicans* is also an opportunistic pathogen, as it can invade and proliferate in many internal organs when the host's defences are significantly compromised. Its closest relative, *C. dubliniensis* is less frequently recovered from the mucosal surfaces favoured by *C. albicans* and causes fewer opportunistic systemic infections (Sullivan *et al.*, 2004). Identifying the molecular basis for these differences in virulence in two such closely related organisms will provide novel insights into candidal pathogenicity. In this study, we examine the role of the transcriptional repressor Nrg1p in regulating filamentation and virulence in *C. dubliniensis*. *NRG1* was selected for analysis in the present study on the basis of two lines of evidence. Firstly, our own data suggested that positive stimulation of the Ras1p-cAMP pathway with a hyperactive *RAS1* allele or cAMP did not induce filamentation in *C. dubliniensis*. Secondly, in an elegant study, Staib & Morschhäuser (2005) demonstrated that *NRG1* was differentially regulated in *C. dubliniensis* and that this was responsible for pseudohyphal growth and chlamydospore formation by *C. dubliniensis* on Staib medium. Data presented in the present study suggests that differential regulation of *NRG1* in response to stimuli that promote the growth of hyphae could account in part for the differences in virulence in these two species.

Regulation of CdNRG1

Braun *et al.* (Braun *et al.*, 2001) first demonstrated that growth of *C. albicans* in serum at 37˚C resulted in downregulation of *NRG1* transcript levels. Lotz *et al.* (Lotz *et al.*, 2004) have also demonstrated pH regulation of *NRG1* transcription. In this study, we demonstrated a 90% reduction in *NRG1* transcript levels in *C. albicans* following 1 h in 10% serum at 37˚C (Fig. 6). This corresponds well with the levels of germ-tube formation observed under these conditions (~85%, Fig. 4). This downregulation was more rapid than that observed by Braun *et al*. (2001), possibly due to culture medium differences. In contrast, *C. dubliniensis*, which filaments at a slower rate in response to serum, downregulates *CdNRG1* less rapidly. Under the same conditions, only ~10% of *C. dubliniensis* cells formed germ-tubes and *CdNRG1* transcript levels only dropped by 50% of preculture levels. In these experiments, it took approximately 5 h before transcript levels in *C. dubliniensis* dropped to below 20% of preculture levels and > 80% of cells had formed germ-tubes. Similarly, following phagocytosis by macrophages, *C. albicans* rapidly downregulated *NRG1* expression whereas in *C. dubliniensis*, *CdNRG1* expression remained constitutively high. These data highlight a fundamental biological difference between *C. albicans* and *C. dubliniensis*, namely the ability of *C. albicans* to rapidly change its morphology via *NRG1* downregulation in response to environmental signals, specifically those encountered within the host (e.g. phagocytosis and serum at 37˚C). *C. albicans* has long been described as a fungus that can adapt to many different environments and host niches, and this rapid modulation of *NRG1* expression in response to environmental cues may partly explain why *C. albicans* is more successful and widespread in the human oral cavity than its relative *C. dubliniensis*. The rate of filamentation in *C. dubliniensis* was greatly increased in the *nrg1*Δ

homozygous deletion strain CDM10, indicating that the slow rate of hypha formation in *C. dubliniensis* is due to *CdNRG1* mediated repression. Deletion of *CdNRG1* also enhanced the rate of filamentation on Spider medium, and in the presence of CO_2, indicating that *CdNRG1* mediated repression is responsible for the lack of filamentation of *C. dubliniensis* under a wide range of environmental conditions tested. Reintroduction of a single copy of *CaNRG1* or *CdNRG1* was sufficient to reverse these phenotypes. Both genes were introduced under the control of their native promoters, indicating that trans-activating factors in the *C. dubliniensis* host strain were responsible for activation of these genes and suppression of the filamentous phenotypes. On Staib medium, both complemented strains formed rough colonies with filamentous fringes, indicating that in this background, the *CaNRG1* and *CdNRG1* genes were regulated similarly.

Role of CdNRG1 *in* in vitro *models of infection*

We have hypothesised previously that a defect in filamentation may be responsible for the reduced virulence of *C. dubliniensis*. The generation of a homozygous *nrg1*Δ strain allowed us to test this hypothesis. We initially examined the virulence of *C. dubliniensis* in co-culture with RAW264.7 macrophages (Marcil *et al.*, 2002). Wild-type *C. dubliniensis* was significantly less able to proliferate in co-culture with these cells in comparison to *C. albicans*, and this was associated with low levels of filamentation following phagocytosis and constitutive expression of *CdNRG1* (Fig. 7). Deletion of *CdNRG1* greatly increased proliferation and filamentation of *C. dubliniensis* in this model. The reason for this increased proliferation may be two-fold. Increased filamentation allows *C. dubliniensis* cells to escape from the phagolysosome in a fashion similar to *C. albicans* cells (Lo *et al.*, 1997; Marcil *et al.*,

2002). A second possibility is that deletion of *CdNRG1* may alter the expression of many stress-response genes. Indeed, Murad *et al.* (2001) demonstrated that the *C. albicans nrg1*Δ strain had increased sensitivity to H_2O_2. However, the *CdNRG1* mutant did not display increased sensitivity to H_2O_2 or nitric oxide *in vitro* in our assays, indicating possible differences in the sets of genes regulated in both species. In a second *in vitro* model, using reconstituted human oral epithelium, the *C. dubliniensis CdNRG1* mutant also displayed increased virulence relative to the wild-type strain. This system has previously been shown to be a sensitive model of virulence, in which filamentation and proteinase secretion play important roles in tissue penetration (Schaller et al., 1999). Following 24 h growth on the tissues, the wild-type *C. dubliniensis* strain had failed to filament and adhered poorly to the tissue, as previously shown by Stokes *et al.* (2007). In addition, the levels of LDH release (a marker for epithelial cell disruption) induced by this wild-type strain were similar to uninfected control tissues. Following 48 h incubation, the *CdNRG1* mutant had formed filaments, and had caused significantly more damage than wild-type *C. dubliniensis*. The role of filamentation in *C. albicans* during oral colonisation and infection has been well established, and this property is associated with strong adherence and tissue penetration (Bernhardt *et al.*, 2001). The lack of adherence and penetration exhibited by the wild-type *C. dubliniensis* strain on RHE offers an explanation for the low incidence of colonisation and infection caused by *C. dubliniensis* on the oral niches favoured by *C. albicans* (i.e. the dorsum of the tongue, the oesophagus and the palate). Secondly, the increased susceptibility of *C. dubliniensis* to killing by macrophages may also prevent this organism invading and penetrating oral tissues to cause infection. Macrophages are an important part of the cell-mediated immune response in the oral cavity, and this phenotype may explain

why wild-type *C. dubliniensis* mainly causes infection in patients with severe defects in cell-mediated immunity (Challacombe and Sweet, 2002; Sullivan *et al.*, 2004).

Role of CdNRG1 *during systemic infection*

The standard model of systemic virulence in *Candida* spp. involves intravenous inoculation of the organism into mice (MacCallum and Odds, 2005). It has been established that *C. dubliniensis* requires a higher inoculum than *C. albicans* to establish infection in this model ($1x10^5$ cfu/g body weight per mouse; D. MacCallum, unpublished data). This level of inoculum led to the death of all *C. albicans*-infected mice within two days, whereas one third of *C. dubliniensis*-infected mice were still alive by day 28. Infection with the *C. dubliniensis CdNRG1* mutant did not lead to any significant change in survival or organ burdens compared to wild-type. This is a complex model of infection compared to the *in vitro* models used previously in this study. In order to establish infection in this model, the organism must evade neutrophil killing, penetrate the endothelium and escape from the bloodstream and then penetrate and invade target organs such as the kidney and the brain (MacCallum and Odds, 2005). The low virulence of wild-type *C. dubliniensis* in this model suggests that there may be a fundamental virulence defect in one of these processes that cannot be overcome by simply increasing the rate of filamentation. Recent comparative genomic studies have highlighted other possible reasons for the reduced virulence of *C. dubliniensis* in this model (Moran *et al.*, 2004). A significant number of *C. albicans* genes present on Eurogentec DNA microarrays (~4%) appear to have either no orthologue in *C. dubliniensis*, or one with significant divergence at the nucleotide sequence level (Moran *et al.*, 2004). In particular, these studies have shown that *C. dubliniensis* possesses only one orthologous gene of the *SAP4,5,6* subfamily of

secreted aspartyl proteinases, namely *CdSAP4*. These proteinases are expressed in the hyphae of *C. albicans*, and deletion of *SAP6* reduced invasion of parenchymal organs and deletion of all three genes renders *C. albicans* attenuated in the systemic model of infection (Felk *et al.*, 2002; Sanglard *et al.*, 1997). We are currently carrying out heterologous expression studies in *C. dubliniensis* in order to determine the significance of this defect.

Conclusion

C. dubliniensis is the closest known relative of *C. albicans*, yet is clinically less significant and has reduced virulence in models of infection. We have demonstrated that the greater virulence of *C. albicans* relative to *C. dubliniensis* is partly due to its ability to rapidly downregulate *NRG1* in response to environmental signals encountered in vivo. Expression of the *CdNRG1* repressor is responsible for the lack of filamentation of *C. dubliniensis* under many conditions, and that deletion of this repressor can increase filamentation and virulence in co-culture with macrophages and in a model of epithelial tissue invasion. The increased morphological plasticity of *C. albicans* may allow this species to colonise a wider range of mucosal sites. In addition, this may indicate that *C. dubliniensis* has specialised to inhabit a unique niche where filamentation is not required, or may perhaps even be disadvantageous. Along with comparative molecular and genomic approaches, discovery of the preferred niche of *C. dubliniensis* should go a long way to explaining the reason for the phenotypic differences between these two species.

Experimental Procedures

Candida *Strains and growth conditions*

All *Candida* strains were routinely cultured on yeast extract-peptone-dextrose (YEPD) agar, at 37°C. For liquid culture, cells were grown shaking (200 r.p.m.) in YEPD broth, also at 37°C (Gallagher *et al.*, 1992). Genotypes of strains used in this study are listed in Table 1. Several solid media were used to induce filamentation. Spider medium and synthetic low dextrose (SLD) medium were prepared as described (Lui *et al.*, 1994). Tobacco medium was prepared as described (Khan *et al.*, 2004). Induction of filamentation in liquid cultures was carried out with cells from overnight YEPD broth cultures (37˚C), which were washed twice with sterile Milli-Q water and added to prewarmed hyphal-induction medium to a density of 2 x 10^6 cells/ml. Induction media used here include Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich Ireland Ltd) and sterile Milli-Q (Millipore Ireland B.V., Co. Cork, Ireland) water supplemented with 10% (v/v) or 50% (v/v) foetal bovine serum (Sigma-Aldrich). The proportion of unconstricted germ-tubes in each culture was assessed at 30 min intervals by microscopic examination with a Nikon Eclipse 600 microscope (Nikon U.K., Surrey, U.K.).

Macrophage cell culture and infection with Candida

The murine macrophage-like cell line RAW264.7 was obtained from the American Type Tissue Culture Collection (ATCC). The complete medium used to maintain the cell line consisted of DMEM supplemented with 10% (v/v) heat-inactivated foetal bovine serum (Sigma-Aldrich) and 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich). Cells were grown at 37˚C under an atmosphere with 5% (v/v) CO_2 in untreated 90 mm diameter plastic dishes (Sarstedt, Co. Wexford, Ireland).

Co-culture experiments with *Candida* and RAW264.7 macrophages were carried out in flat-bottomed 96-well dishes (Sarstedt). For each experiment, a macrophage suspension of 1.5 x 10^6 cells/ml was prepared in complete medium. For each *Candida* strain to be tested, 16 wells (4 x 4) were dispensed with 100 μl of this suspension and cultured overnight (16 h) to form confluent monolayers. Prior to experiments, confluent monolayers were washed with fresh complete medium and a final volume of 150 μl medium was added to each well. *Candida* strains were cultured overnight in 2 ml YEPD broth in a 25 ml tube at 37˚C with shaking. Cells were washed twice in Dulbecco's modified phosphate buffered saline (DPBS, Sigma-Aldrich) and a suspension of 1.5 x 10^6 cells/ml was prepared in complete medium. A 50 μl aliquot of this suspension was added to quadruplicate wells containing confluent washed monolayers and quadruplicate wells containing 150 μl of complete medium alone. This yielded 7.5 x 10^4 *Candida* cells per well, giving a multiplicity of infection (MOI) of 1:2 (*Candida*:macrophages). Cells from this well (50 μl) were then serially diluted 1:4 in the three adjacent wells to yield MOIs of 1:8, 1:32 and 1:128, respectively. Wells containing complete medium alone acted as macrophage-free growth controls in all experiments. The inoculated plates were incubated at 37˚C in 5% (v/v) CO_2 for 16 h. Following incubation, the survival of the *Candida* cells was assessed by comparing their growth to the macrophage free control wells using an XTT (Sigma-Aldrich) dye reduction assay. Briefly, each well was washed three times with sterile water which resulted in lysis of the macrophages in those wells containing monolayers. Cells were then incubated with 200 μl of a 400 μg/ml XTT solution containing co-enzyme Q (50 μg/ml) for 45 min. Following incubation, 100 μl of the reduced dye solution was removed and the absorbance measured at 480 nm in a Tecan Genios microplate reader (Tecan U.K. Ltd., Reading, U.K.). Absorbance values were

corrected against a cell-free blank and the percentage growth of each *Candida* strains was determined relative to the positive growth control (100%) for that MOI. Each experiment was performed on at least four occasions. Proliferation curves were analysed with 2-way ANOVA to determine if strains exhibited significant differences in proliferation at each MOI, using the Prism 4.0 software package (GraphPad Software, San Diego, CA). Staining of *Candida*-infected macrophages with acridine orange was carried out with infected macrophage monolayers grown on sterile glass coverslips. The medium was removed and a solution of acridine orange (10 μg/ml) in DPBS was added to duplicate wells and incubated for 10 min. The stain was then removed and the cells washed once in DPBS. Fluorescence was detected using a Nikon Eclipse 600 microscope (Nikon) fitted with a super high power mercury lamp (Nikon) and the GFP specific filter set (Endow GFP Bandpass Emission (FGP®-BP) filter combination), (Nikon).

Infection of Reconstituted human oral epithelial (RHE) tissues

RHE tissues were purchased from Skinethic Laboratories (Nice, France) and used as described previously (Schaller *et al.*, 1999; Stokes *et al.*, 2007). Reconstituted epithelium samples (0.5 cm^2) were inoculated with 2 x 10^6 yeast cells in 50 μl of PBS and controls were inoculated with 50 μl of PBS. Inoculated cultures were incubated at 37˚C, 5% (v/v) CO_2 at 100% humidity for 6 h, 12 h, 24h and 48 h. The release of lactate dehydrogenase (LDH) from epithelial cells into the cell-culture medium was measured to quantify the extent of epithelial cell damage. The CytoTox 96® non-radioactive cytotoxicity assay (Promega Corp., Madison, WI) was used to measure the amount of LDH in each sample. The reaction was assayed at 480 nm using a Genios plate reader (Tecan U.K. Ltd.). One unit of LDH activity is equivalent to 1 μM

formazan formed per reaction. The statistical significance of differences in LDH release induced by each strain at each time-point was examined by 2-way ANOVA. Sectioning and staining of tissues for light microscopy was carried out as described by Stokes *et al.* (2007). Tissues were examined using a Nikon Eclipse 600 microscope. Infections were carried out on two separate occasions.

Candida transformation

Transformation of *C. albicans* and *C. dubliniensis* was performed by electroporation as described (Moran *et al.*, 2002). The *URA3* deleted strains CAI4 and CDUM4B were transformed with the *Candida ARS*-containing plasmids pVEC and pLJ65 and transformants were selected on selective minimal medium containing 1% (w/v) casamino acids, as described (Leberer *et al.*, 2001). Deletion of the *C. dubliniensis NRG1* gene in strain Wü284 was carried out using the *SAT1*-flipper described by (Reuss *et al.*, 2004). A deletion construct was created by PCR by amplifying the flanking 5' and 3' regions of the *CdNRG1* gene with the primer pairs NRGKF/NRGXR and NRGSCIIF/NRGSIR, respectively (Table 2). These 5' and 3' fragments were cloned into the *Kpn*I/*Xho*I and *Sac*II/*Sac*I sites, respectively, flanking the SAT1-flipper in plasmid pSFS2A to yield pGM142. The entire deletion construct was released from plasmid pGM142 on a *Kpn*I-*Sac*II fragment and used to transform *C. dubliniensis* Wü284 as described previously (Moran *et al.,* 2002). Nourseothricin-resistant recombinant derivatives were selected on YEPD agar plates containing 100 μg/ml nourseothricin. Integration of the construct at the correct locus was initially confirmed by PCR with the primer pair NRGUP (which bound to upstream chromosomal sequences, Table 2) and FLP1 (which bound to *SAT1* cassette sequences), which yield a product of approximately 1,400 bp in transformants

containing the cassette at the *CdNRG1* locus. The marker was excised and recycled for a second round of transformation as described by Reuss *et al.* (2004). This resulted in the generation of strain CDM10, which contained a deletion between nucleotides -74 and +949 (where the first A of the ATG start codon is +1) in both copies of the *CdNRG1* gene. This was confirmed by Southern blot analysis (Figure 3). Reintroduction of the wild-type *CdNRG1* gene was achieved by PCR amplification of regions –3849 to +1423 of the *NRG1* gene, which was ligated into *Not*I and *Sac*II sites of the *C. dubliniensis* integrating vector pCDRI to yield pCdNRG1. Plasmid pCDRI consists of plasmid pBluescript II containing the *SAT1* resistance marker from pSFS2A (contained on an *Xba*I/*Spe*I fragment) and regions +1800 to +2488 of the *CdCDR1* pseudogene. Targeted integration of pNRG1 into the *CdCDR1* pseudogene in strain CDM10 was achieved by linearisation of the plasmids within the *CdCDR1* region by digestion with *Nco*I and transformation into CDM10 by electroporation, yielding the *CdNRG1*-complemented strain CDM11. The correct genomic integration was confirmed by PCR, using a primer that annealed within the cassette (M13 reverse primer) and a primer that annealed within the *CdCDR1* sequences (TAGR) (Moran *et al.*, 2002). The presence of the whole *CdNRG1* gene within the integrated cassette was also confirmed by PCR with the NRGKF and NRGSCIR primer pair. The homozygous *nrg1*Δ strain CDM10 was also transformed with the empty vector pCDRI for use in parallel experiments with the reintegrant CDM11.

Real-time PCR analysis of gene expression

Cells were harvested from *Candida*-macrophage co-culture experiments or liquid hypha induction medium and frozen directly in liquid nitrogen for RNA preparation. RNA was prepared using TRI-reagent (Sigma) as described (Stokes *et al.*, 2007).

RNA samples were rendered DNA free by incubation with Turbo-RNAfree reagent (Ambion, Austin, TX). RNA concentrations were determined using the Ribo-green kit (Molecular Probes). Reverse transcription was carried out with 1 μg of total RNA with an oligo-dT primer (Promega) and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). Amplification was carried out using the primers pairs QRTEF1F/R (Green *et al.*, 2005) and the primers NRG1A/B (Table 2). NRG1A/B primers were designed using Primer Express software v1.5 (Applied Bisosytems, Foster City, CA) and were homologous to *C. albicans* and *C. dubliniensis NRG1* ORFs. These primers yielded single, specific amplimers from genomic DNA and cDNA templates from both *C. albicans* and *C. dubliniensis*. Real-time detection of *TEF1* and *NRG1* amplimers was carried out using the QuantiTect Sybr green PCR kit (Qiagen, West Sussex, U.K.) and the ABI 7700 sequence detector, performing separate reactions for each gene. *NRG1* gene expression levels were normalised against the expression levels of the constitutively expressed *TEF1* gene in the same cDNA sample.

Construction of GFP reporter strains

C. albicans and *C. dubliniensis* strains were constructed with one allele of the *NRG1*-coding sequence replaced by the yeast-optimised *GFP* (*yGFP3*) coding region using cassettes containing *yGFP3* and the *SAT1* marker gene (Cormack *et al.*, 1997; Reuss *et al.*, 2004). In order to create these constructs, the *yGFP3* coding region was amplified from plasmid pCC1 (Stokes *et al.*, 2007) using primers GFPHF and GFPHR (Table 2), digested with *Hin*dIII and *Eco*RV and ligated into *Hin*dIII/*Eco*RV-digested pBluescript II. The *ACT1* terminator region was released from pCC1 by *Eco*RI digestion and this fragment was subcloned in the *Eco*RI site downstream of the

GFP ORF to create pGM160. The *SAT1*-resistance marker was released from pSFS2A by digestion with *Spe*I and *Xba*I and this fragment ligated to *Spe*I/*Xba*I-digested pGM160 to create pGM161. In order to create *CaNRG1* and *CdNRG1* GFP-promoter fusion cassettes, upstream 5' regions of the *CaNRG1* and *CdNRG1* genes were amplified using the primer pairs PCaNRGF/R and PCdNRGF/R, respectively (Table 2). These primers were similar to primers pairs NRG1P1/P2 and CdNRG1P1/P2 described by Staib & Morschhäuser (2005), except that the internal restriction endonuclease recognition sequences were changed. These fragments were digested with *Kpn*I and *Xho*I and inserted into *Kpn*I/*Xho*I-digested pGM161 to create separate *C. albicans* and *C. dubliniensis NRG1* promoter-GFP fusions. The 3' non-coding regions of the *CaNRG1* and *CdNRG1* genes were then amplified with the primer pairs CaNRG3F/R and CdNRG3F/R respectively (Table 2) and these products were ligated into the corresponding *Sac*II/*Sac*I-digested CaNRG1 or CdNRG1 construct to create plasmids pCaNRG1GFP and pCdNRG1GFP. The cassettes were released by digestion with *Kpn*I and *Sac*I and were used to replace one copy of the *NRG1* gene by targeted gene replacement in *C. dubliniensis* Wü284 and *C. albicans* SC5314. PCR confirmation of the correct allelic replacement was obtained using a primer binding to the *NRG1* promoter (NRGKF, Table 2) and a primer binding within the cassette (ACTTR, Stokes *et al.,* 2007)

Mouse model of systemic candidosis

For virulence testing of strains, immunocompetent 6-8 week old female BALB/c mice (Harlan Sera-Lab Ltd., Loughborough, UK) were challenged intravenously. Fungal strains were grown with shaking for 18–24 h in NGY medium (0.1% [w/v] neopeptone, 0.4% [w/v] glucose, and 0.1% [w/v] yeast extract) at 30°C. Cells were

sonicated prior to inoculum preparation to disrupt clumps, washed twice with sterile water and resuspended in physiological saline. Groups of eight mice were inoculated intravenously with each strain at 1 x 10^5 cfu/g of mouse body weight. Two mice were sampled at two days post-infection to determine organ burdens at this time point. The remaining 6 mice per group were monitored over 28 days when the experiment was terminated. Any mice showing signs of severe illness were terminated prior to the end of the experiment. For each mouse, the kidneys and brains were aseptically removed post mortem, divided in half, and one half homogenized in 0.5 ml of water, and *Candida* tissue burdens determined by viable counting. Survival curves were compared using the logrank test (Prism 4.0, GraphPad software). For histology, the other half of each organ was preserved in formalin. Tissues were paraffin-embedded and 5 μm sections cut. Sections were deparaffinised and stained with Periodic Acid-Schiff, prior to staining with haematoxylin.

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Table 1. *Candida albicans* and *C. dubliniensis* strains used in this study

Strain	Description	Reference
C. albicans		
SC5314	Wild type	(Gillum *et al.*, 1984)
CAI4	*ura3::λ imm434/ura3:: λimm434*	(Fonzi and Irwin, 1993)
MMC3	*ura3::λ imm434/ura3:: λimm434, nrg1::hisG-URA3-hisG/nrg1::hisG*	(Murad *et al.*, 2001b)
NCPF3153	Wild type	(Hasenclever and Mitchell, 1961)
NCPF3824	Wild type	
132A	Wild type	(Gallagher et al., 1992)
KJ	Wild type	This study
JP10	Wild type	(Gee *et al.*, 2002)
C. dubliniensis		
Wü284	Wild type, genotype I	(Morschhauser et al., 1999)
CDUM4B	*ura3::FRT/ura3::FRT*	(Staib *et al.*, 2001)
CDM7	*nrg1::SAT1/NRG1*	This study
CDM8	*nrg1::FRT/NRG1*	This study
CDM9	*nrg1::FRT/nrg1::SAT1*	This study
CDM10	*nrg1::FRT/nrg1::FRT*	This study
CDM11	*nrg1::FRT/nrg1::FRT, CDR1/cdr1::pCdNRG1*	This study
CDM12	*nrg1::FRT/nrg1::FRT, CDR1/cdr1::pCaNRG1*	This study
CD36	Wild type, genotype I	(Sullivan *et al.*, 1995)
CD33	Wild type, genotype I	(Sullivan *et al.*, 1995)
CM1	Wild type, genotype I	(Sullivan *et al.*, 1995)
CBS8500	Wild type, genotype I	(Gee *et al.*, 2002)
CD506	Wild type, genotype II	(Gee *et al.*, 2002)
CAN6	Wild type, genotype II	(Gee *et al.*, 2002)
CD539	Wild type, genotype II	(Gee *et al.*, 2002)
CD5004	Wild type, genotype II	(Gee *et al.*, 2002)

Table 2. Oligonucleotide primers used in this study. Restriction endonuclease sites are underlined.

Name	Sequence 5'-3'	Site
NRGKF	GGCCGGTACCATCTAACTAGTGGTGGATCC	*Kpn*I
NRGXR	GGCCCTCGAGATAATACCTGGTTCAGATTC	*Xho*I
NRGSIIF	GGCCCCGCGGGCTTAGTATAGATATGATCG	*Sac*II
NRGSIR	GGCCGAGCTCTATTTGGAAGATTATACTTG	*Sac*I
NRGUP	CAAGTAAGTTTCCACTATAC	-
FLP1	TTCCGTTATGTGTAATCATCC	-
NRGF	GCATGCGGCCGCAGCACATGTTCTGCCAACC	*Not*I
NRGR	GCATCCGCGGCAGGAAGGAACACCAATAGC	*Sac*II
CaNRGF	GCATGCGGCCGCTAGCCATGGGTATCGGAAAC	*Not*I
CaNRGR	GCATCCGCGGATGGCAATGCAAGAAAATGC	*Sac*II
GFPHF	ATGCAAGCTTTTATTAAAATGTCTAAAGGTG	*Hin*dIII
GFPHR	ATGCGATATCCTTATTTGTACAATTCATCCAT	*Eco*RV
PCdNRGF	TTATCACCTAGGTACCTATTTACAACTAAGAA	*Kpn*I
PCdNRGR	TATACTCGAGGAGTATGAGAATCTAATGAAACTAG	*Xho*I
CdNRG3F	GATCAGACCATCCGCGGGATTAATCACT	*Sac*II
CdNRG3R	GTTGCAACGTGTGGAGCTCCCACATTCCTG	*Sac*I
PCaNRGF	CCTTGCTTTCAGGTACCTTGTCACAGACG	*Kpn*I
PCaNRGR	GTTGCAACGTGTGGAGCTCCCACATTCCTG	*Xho*I
CaNRG3F	ATCAGACCATCCGCGGGGCTAATTAC	*Sac*II
CaNRG3R	TTCCAACATGTGAGAGCTCAAAGTCCTGTGTTG	*Sac*I
NRG1a	GTCTGCAAAGTGTGTTCGAG	-
NRG1b	GACGAGCAAAACGGGCTTCA	-
QRTTEF1R	CCACTGAAGTCAAGTCCGTTGA	-
QRTTEF1F	CACCTTCAGCCAATTGTTCGT	-

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Figure Legends

Fig. 1. Phenotypes of *C. albicans* (A) and *C. dubliniensis* (B) following 3 h co-culture with murine macrophage cell line RAW264.7. Macophage cells were grown on cover-slips and infected with *Candida* spp. at an MOI of 1:2 for 3 h, fixed in methanol and stained with Giemsa. The black scale bar represents 10 μm (C) Proliferation of *C. albicans* isolates (n=6) and *C. dubliniensis* genotype I isolates (n=5) and genotype II isolates (n=4) in co-culture with RAW264.6 macrophages. Proliferation of *Candida* isolates alone (control) and in co-culture was estimated following 16 h incubation using an XTT dye reduction assay. Percentage growth of co-cultured *Candida* was estimated relative to the macrophage-free control cultures. MOIs where *C. albicans* isolates yielded significantly greater levels of proliferation are indicated indicated by '*' ($P < 0.001$).

Fig. 2. Photomicrographs of *C. albicans* CAI4 and *C. dubliniensis* CDUM4B cells transformed with pVEC and pLJ65 (A) and grown in the presence of cAMP (B). (A) *C. albicans* CAI4 and *C. dubliniensis* CDUM4B were transformed with the *CaARS* plasmid pVEC and a derivative harbouring a hyperactive *RAS1* allele (pLJ65). Cells were grown in 1% (w/v) casamino acids to induce expression of *RAS1* from the *PCK1* promoter. (B) *C. albicans* SC5314 and *C. dubliniensis* Wü284 were grown in DMEM with and without 10 mM dibutyryl-cAMP.

Fig. 3. Creation of a homozygous deletion in *CdNRG1* in strain Wü284. Sequential disruptions in both alleles were created with the SAT1-flipper. Genomic DNA from transformants was isolated as described (Gallagher *et al.*, 1992) and digested with *Eco*RI and fragments were resolved on a 1% (w/v) agarose gel. Southern-blotted

DNA was hybridised with a fragment corresponding to the 5' end of the *CdNRG1* gene, amplified with primers NRGUP and NRGXR and labelled by random priming with [α-^{32}P]dATP (6,000 Ci/mmol; 222 TBq/mmol, Amersham International Plc).

Fig. 4. Phenotypic analysis of the *CdNRG1* homozygous deletant strain CDM10. (A) Colonial morphologies of *C. albicans* SC5314, *C. dubliniensis* Wü284 and CDM10 (*nrg1*Δ*/nrg1*Δ) on solid media. (B) Photomicrographs of *C. albicans* SC5314, *C. dubliniensis* Wü284 and CDM10 (*nrg1*Δ*/nrg1*Δ) grown in liquid DMEM in the presence of 5% (v/v) CO_2. (C) Rate of true hypha formation in *C. albicans* SC5314, *C. dubliniensis* Wü284 and CDM10 (*nrg1*Δ*/nrg1*Δ) in water containing 50% (v/v) foetal bovine serum.

Fig. 5. (A) Analysis of *NRG1* promoter activity using *NRG1* promoter-GFP fusion strains of *C. albicans* SC5314 and *C. dubliniensis* Wü284. Strains were co-cultured with RAW264.7 macrophages and fluorescence was examined over time with a Nikon Eclipse 600 microscope (Nikon) fitted with a GFP specific filter set (Endow GFP Bandpass Emission (FGP®-BP) filter combination), (Nikon). (B) Analysis of *NRG1* transcript levels in *C. albicans* SC5314 and *C. dubliniensis* Wü284 during co-culture with Raw264.7 macrophages by real-time PCR, using the Quantitect SYBR Green PCR system (Qiagen) (C) Analysis of *NRG1* transcript levels in *C. albicans* SC5314 and *C. dubliniensis* Wü284 during growth in 10% foetal calf serum.

Fig. 6. Analysis of the effects of deletion of *CdNRG1* during co-culture with macrophages. (A) Photomicrographs of *C. albicans* SC5314, *C. dubliniensis* Wü284 and CDM10 (*nrg1*Δ*/nrg1*Δ) during co-culture with murine macrophages over 5 h

following staining with acridine orange. The white scale bar represents 10 μm (B) Proliferation of *C. dubliniensis* Wü284, CDM10 (*nrg1*Δ*/nrg1*Δ) and the complemented mutant strain CDM11 in the presence of RAW264.7 macrophages. Proliferation was estimated in an XTT dye reduction assay. Points marked '*' indicate MOIs where the mutant strain proliferated to a significantly greater extent than wild-type and reintegrant strains ($P < 0.01$).

Fig. 7. (A) Photomicrograph of *C. albicans* SC5314, *C. dubliniensis* Wü284, CDM10 (*nrg1*Δ*/nrg1*Δ) and CDM11 (*nrg1*Δ*/nrg1*Δ*,* pNRG1) after 24 h growth on reconstituted human oral epithelium (RHE; Skinethic, France). Histological sections were stained with Periodic-Acid Schiff reagent. The black scale bar represents 40 μm (B) Evaluation of tissue damage caused during infection of RHE by *C. albicans* SC5314, *C. dubliniensis* Wü284 and CDM10 (*nrg1*Δ*/nrg1*Δ) and CDM11 (*nrg1*Δ*/nrg1*Δ*,* pNRG1) measured as LDH activity release into culture medium. Tissues infected with the *nrg1*Δ mutant strain CDM10 released significantly higher levels of LDH relative to wild-type and reintegrant strains at 48 h ($P < 0.001$, indicated as *).

Fig. 8. (A) Survival curves for mice infected intravenously with *C. albicans* SC5314, *C. dubliniensis* Wü284, CDM10 (*nrg1*Δ*/nrg1*Δ) and CDM11 (*nrg1*Δ*/nrg1*Δ*,* pNRG1). The experiment was terminated at day 28. (B and C) Histological sections of kidneys stained with periodic-acid Schiff, and post-stained with haematoxylin after infection with *C. dubliniensis* Wü284 (B) and CDM10 (*nrg1*Δ*/nrg1*Δ) (C). Yeast cells in (B) are marked with arrowheads. Scale bar represents 20 μm.

Fig. 1

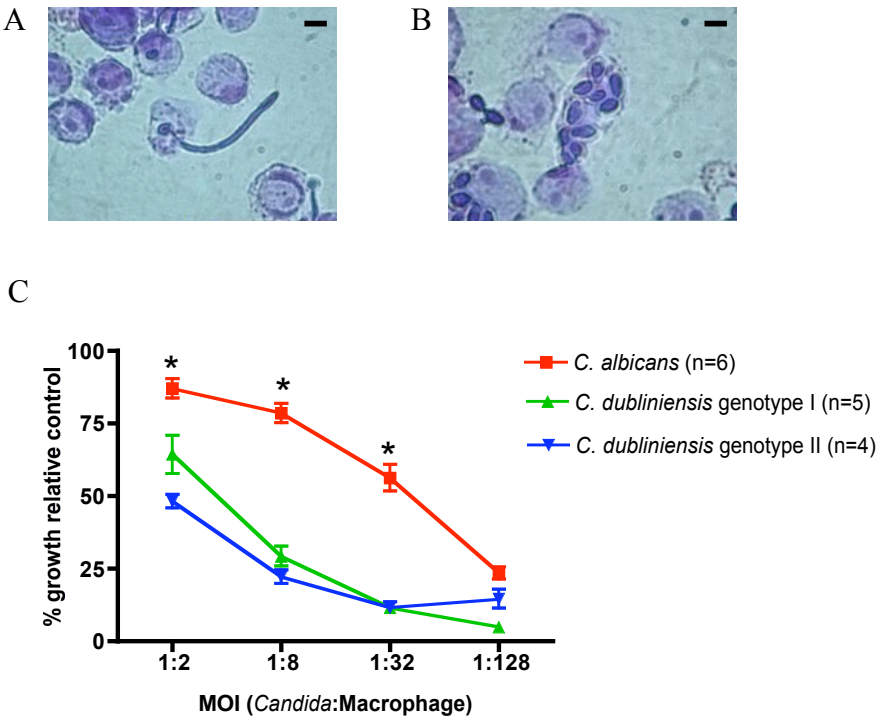
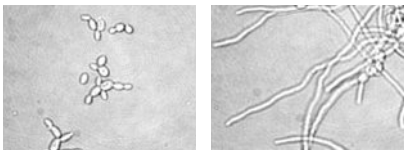

A
B
C
100
75
50
25
0
% growth relative control
*
*
*
1:2
1:8
1:32
1:128
MOI (Candida:Macrophage)
C. albicans (n=6)
C. dubliniensis genotype I (n=5)
C. dubliniensis genotype II (n=4)

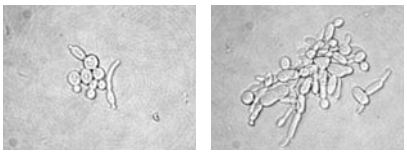
Fig. 2

A

CAI4

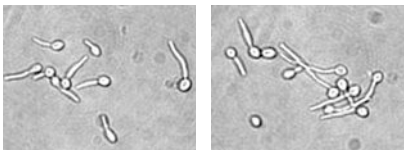


CDUM4B



B

SC5314



Wü284

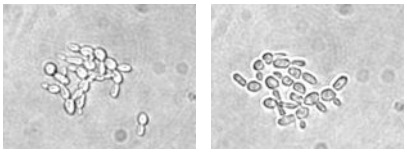


Fig. 3.

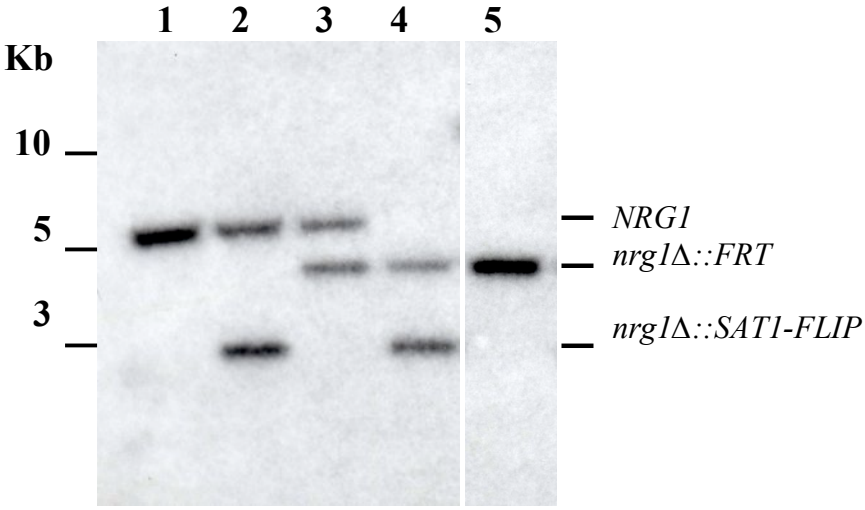


Fig. 4

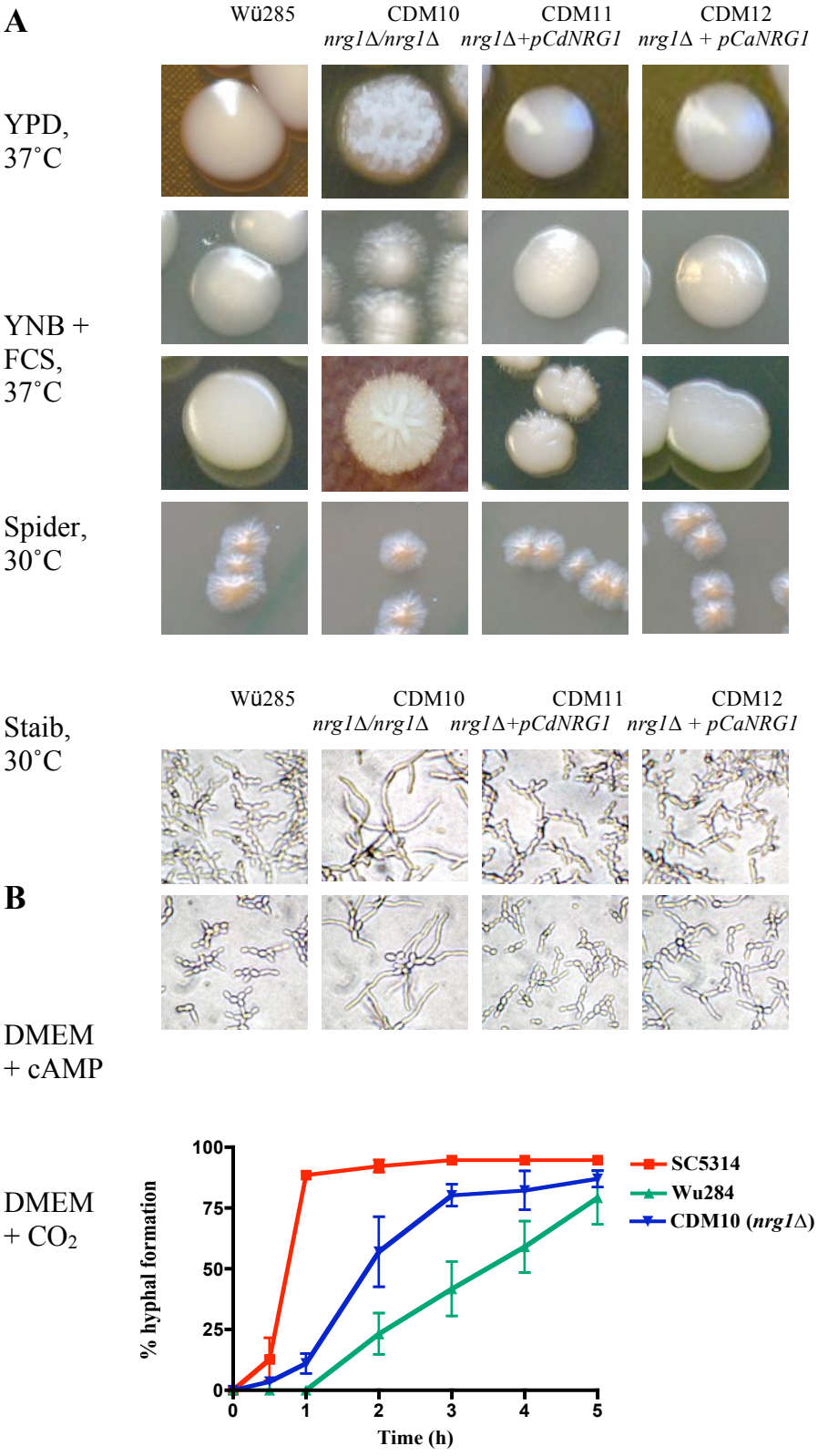

A
Wü285
CDM10
nrg1Δ/nrg1Δ
CDM11
nrg1Δ+pCdNRG1
CDM12
nrg1Δ + pCaNRG1
YPD, 37˚C
YNB + FCS, 37˚C
Spider, 30˚C
Staib, 30˚C
B
DMEM + cAMP
DMEM + CO2
% hyphal formation
Time (h)
SC5314
Wu284
CDM10 (nrg1Δ)

Fig. 5

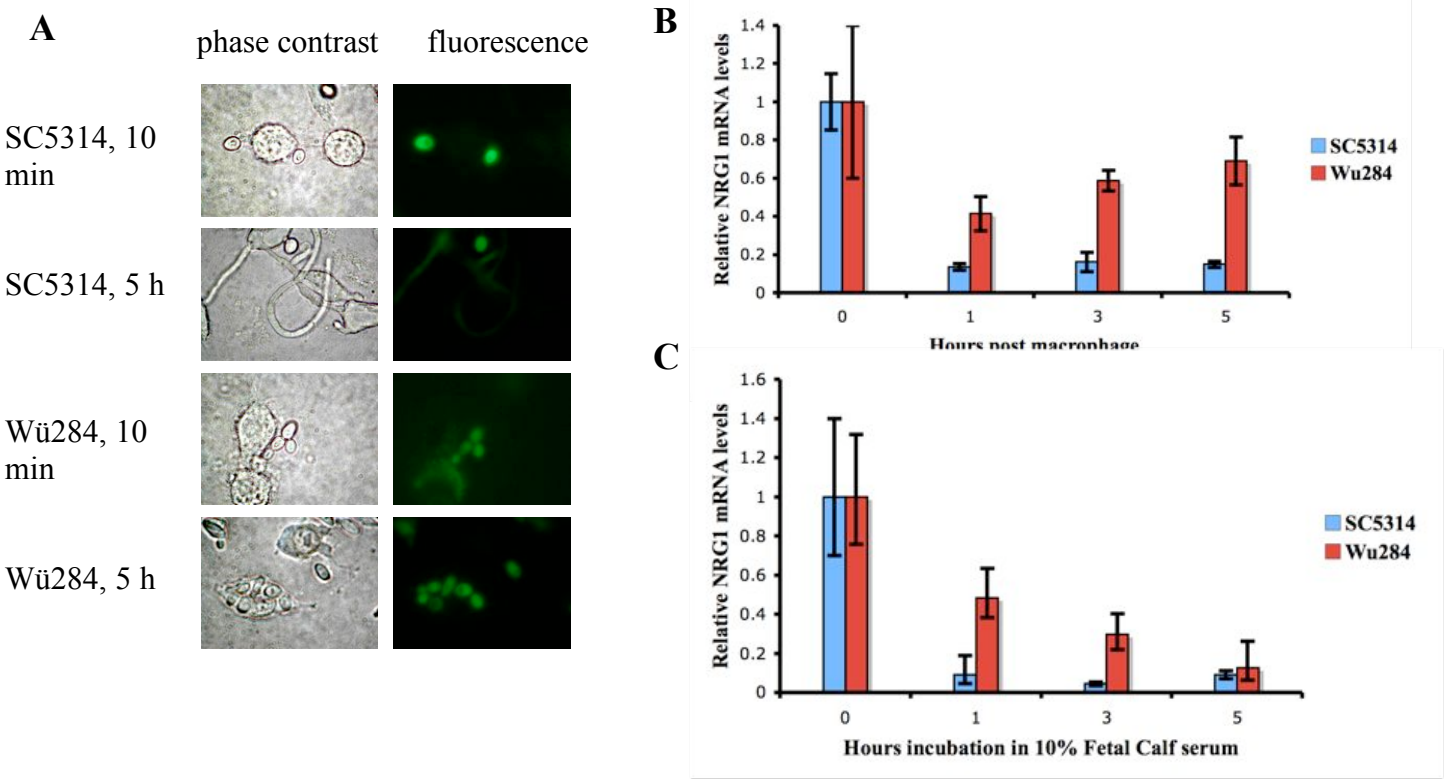
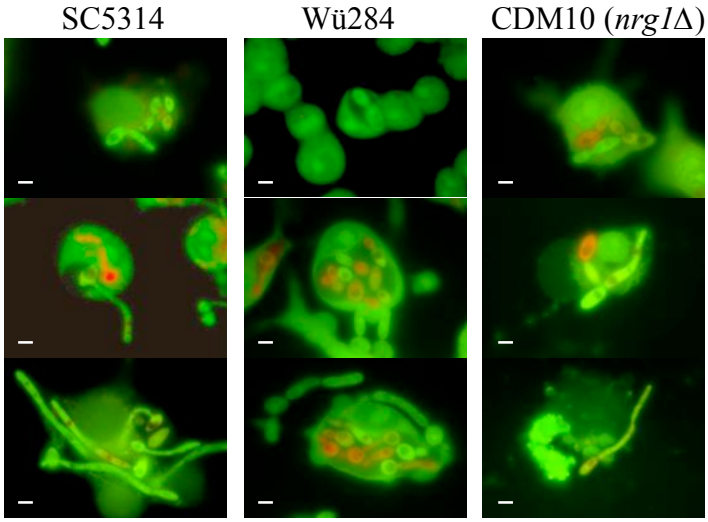

A
phase contrast
fluorescence
SC5314, 10 min
SC5314, 5 h
Wü284, 10 min
Wü284, 5 h
B
Relative NRG1 mRNA levels
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1
3
5
SC5314
Wu284
C
Relative NRG1 mRNA levels
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
1
3
5
SC5314
Wu284
Hours incubation in 10% Fetal Calf serum

Fig. 6

A


SC5314
Wü284
CDM10 (*nrg1*Δ)
0.5 h
3 h
5 h

B

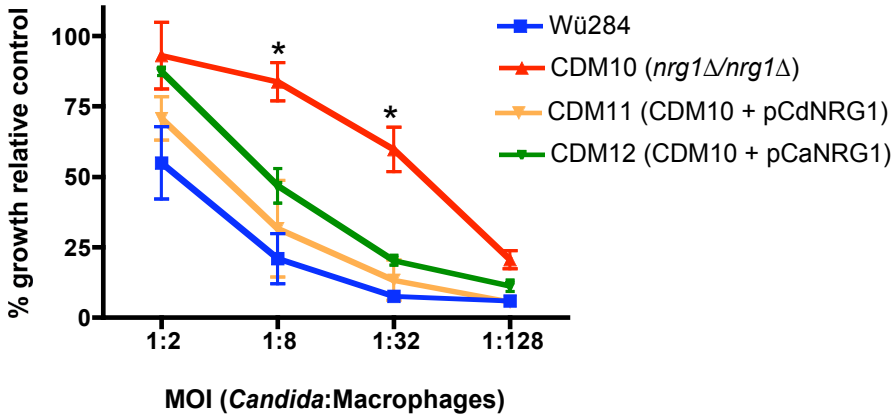
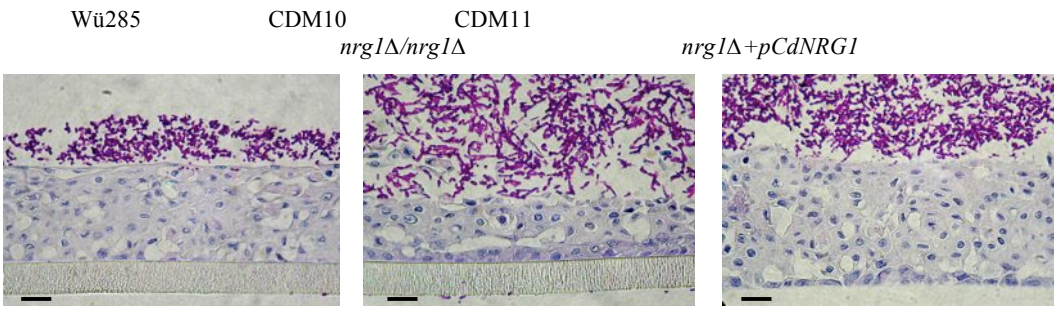

% growth relative control
100
75
50
25
0
1:2
1:8
1:32
1:128
MOI (*Candida*:Macrophages)
*
*
Wü284
CDM10 (*nrg1*Δ/*nrg1*Δ)
CDM11 (CDM10 + pCdNRG1)
CDM12 (CDM10 + pCaNRG1)

Fig. 7

A


Wü285
CDM10
nrg1Δ/nrg1Δ
CDM11
nrg1Δ+pCdNRG1

B

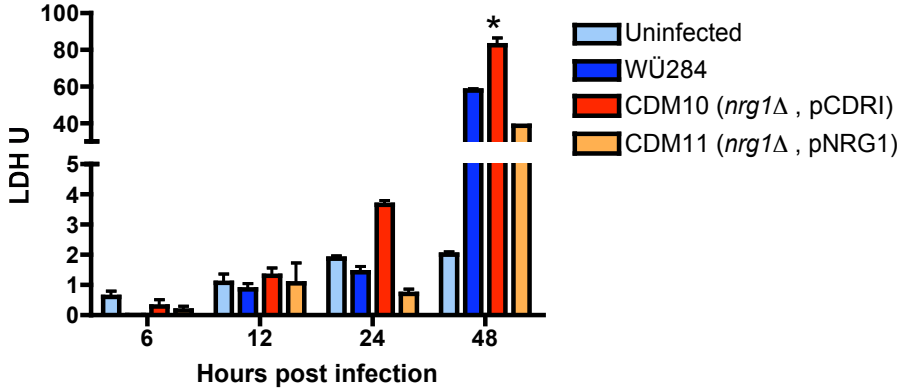
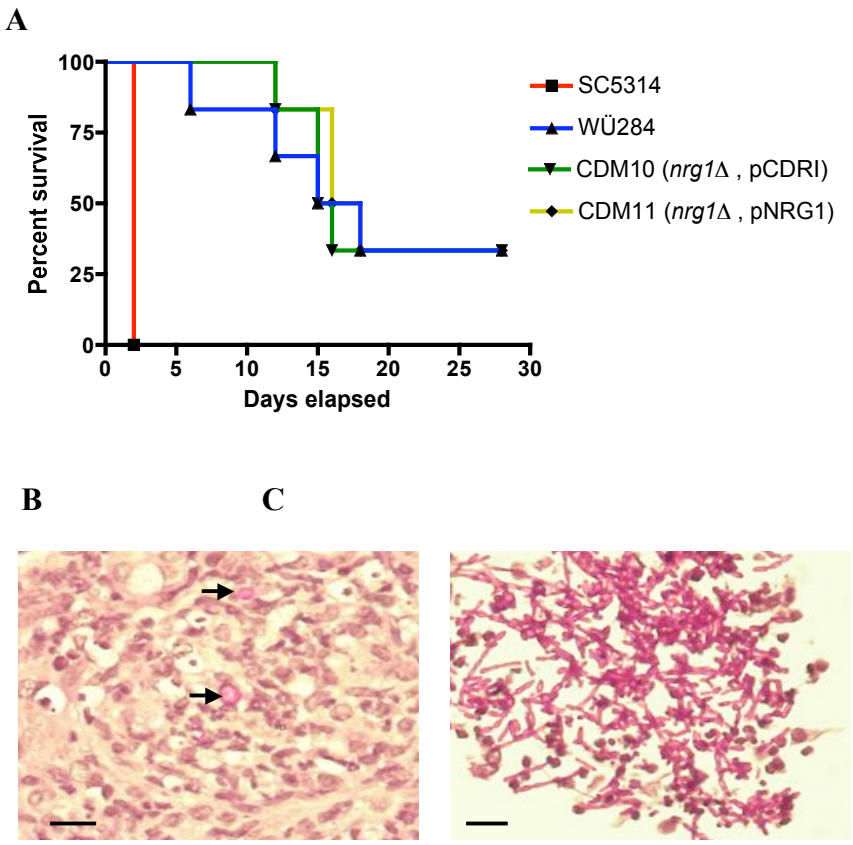

LDH U
100
80
60
40
5
4
3
2
1
0
6
12
24
48
Hours post infection
*
Uninfected
WÜ284
CDM10 (nrg1Δ , pCDRI)
CDM11 (nrg1Δ , pNRG1)

Fig. 8.


A
100
75
50
25
0
Percent survival
0
5
10
15
20
25
30
Days elapsed
SC5314
WÜ284
CDM10 (nrg1Δ , pCDRI)
CDM11 (nrg1Δ , pNRG1)
B
C