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Transcript profiling reveals rewiring of iron assimilation gene expression in *Candida albicans* and *C. dubliniensis*

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Abstract

Hyphal growth is repressed in *Candida albicans* and *C. dubliniensis* by the transcription factor Nrg1. Transcript profiling of a *C. dubliniensis NRG1* mutant identified a common group of 28 *NRG1* repressed genes in both species, including the hypha-specific genes *HWP1, ECE1* and the regulator of cell elongation *UME6*. Unexpectedly, *C. dubliniensis NRG1* was required for wild-type levels of expression of 10 genes required for iron uptake including 7 ferric reductases, *SIT1, FTR1* and *RBT5*. However, at alkaline pH and during filamentous growth in 10% serum most of these genes were highly induced in *C. dubliniensis*. Conversely *RBT5, PGA10, FRE10* and *FRP1* did not exhibit induction during hyphal growth when *NRG1* is downregulated, indicating that in *C. dubliniensis NRG1* is also required for optimal expression of these genes in alkaline environments. In iron depleted medium at pH4.5, reduced growth of the *NRG1* mutant relative to wild-type was observed, however growth was restored to wild-type levels or greater at pH 6.5, indicating that alkaline induction of iron assimilation gene expression could rescue this phenotype. These data indicate that transcriptional control of iron assimilation and pseudohypha formation have been separated in *C. albicans*, perhaps promoting growth in a wider range of niches.

Candida dubliniensis is an opportunistic fungal pathogen that was first identified as a common cause of oral candidosis in HIV-infected patients (Sullivan et al. 2005). *C. dubliniensis* is closely related to *Candida albicans*, the major fungal pathogen of humans. However, *C. albicans* is far more prevalent as a pathogen in the human population, particularly in the case of systemic fungal infection where *C. dubliniensis* is responsible for fewer than 3% of infections (Moran et al. 2012). Recent comparative genomic studies have revealed that several well characterised virulence factors are *C. albicans*-specific (e.g. *ALS3, HYR1, SAP4, SAP5*; Jackson et al. 2009). Murine virulence studies have also associated the reduced capacity of *C. dubliniensis* to establish infection with a reduced ability to undergo the yeast to hypha transition *in vivo* (Stokes et al. 2007). Models of in vitro infection support this finding; *C. dubliniensis* remains in the yeast phase when inoculated on reconstituted human epithelium (RHE) and filaments less efficiently than *C. albicans* following phagocytosis by murine macrophages (Spiering et al. 2010; Moran et al. 2007). *In vitro* studies show that in *C. dubliniensis* serum induced hyphal growth can be repressed by nutrients, especially peptone (O'Connor et al. 2010). The addition of rapamycin to nutrient rich media can stimulate filamentation, indicating a Tor1 dependent method of nutrient repression (Sullivan & Moran 2011). Repression of filamentation is also partly mediated by the transcriptional repressor Nrg1 (Moran et al. 2007; Staib & Morschhäuser 2005; Saville et al. 2003). Deletion of *NRG1* enhances pseudohypha formation in *C. dubliniensis*, however an *NRG1* mutant still requires nutrient depletion to form true hyphae at alkaline pH (O'Connor et al. 2010). A preliminary analysis of the *NRG1* regulated gene set in *C. dubliniensis* indicated that many serum responsive genes are *NRG1* repressed (O'Connor et al. 2010). In this study, the transcriptional profile of a *C. dubliniensis NRG1* mutant was further examined in order to identify genes involved in pseudohypha formation and to compare the regulon with that of the more virulent species *C. albicans*.

A *C. dubliniensis* microarray representing 5,999 orfs from the *C. dubliniensis* genome were used as described (O'Connor et al. 2010). Total RNA was isolated from *C. dubliniensis* strain Wü284 and the *nrg1Δ* derivative CDM10 grown in YPD broth at 30˚C to $OD600_{nm}$ 1.0. These conditions were chosen as they were similar to those used in previous analysis of *C. albicans nrg1Δ* mutants (i.e. mid-exponential YPD cultures) (Kadosh & Johnson 2005; García-Sánchez et al. 2005). Four biological

replicate experiments were performed, including two dye swap experiments. Data was normalized in GeneSpring GX11 using Loess normalization. A *t* test was performed on each data set using the variance derived from replicate spots. Those genes with a *p* value ≤ 0.05 that passed the Benjamini-Hochberg multiple correction test to remove false differential gene expression were selected for analysis. Results from these microarrays have been submitted to the GEO archive (Accession: GSE20537). From this analysis we identified a set of 198 genes that were significantly upregulated in the *nrg1Δ* derivative more than 2-fold compared to the wild-type strain (supplementary table 1). Comparison of this *C. dubliniensis NRG1* repressed gene set with the respective *C. albicans NRG1* regulated gene sets (García-Sánchez et al. 2005; Kadosh & Johnson 2005) identified a group of 28 common *NRG1* repressed genes (Fig 1a). This conserved core *NRG1* response was shown to be significantly enriched with genes encoding cell surface proteins with putative roles in adhesion and pathogenesis (Table). This included the putative adhesins *ALS1, HWP1, EAP1, RBT1, PGA13*, the filamentous growth regulator *UME6* and the hypha-specific gene *ECE1*. The glutathione-s-transferase encoding gene *GST2* was shown to be upregulated in both the *C. albicans* and *C. dubliniensis nrg1* mutants and in addition, the *C. dubliniensis* regulated gene set also contained *GST3* and the orthologues of the glutathione peroxidase encoding genes *GPX1* and *GPX2*. As a result of this finding, susceptibility to the reactive oxygen species generating molecule menadione was examined and the *C. dubliniensis nrg1Δ* mutant was shown to exhibit increased resistance this stress relative to wild-type (Fig 1b). We observed a similar phenotype in the *C. albicans nrg1* mutant MMC3 in the presence of 0.5 mM menadione (Fig 1b). Distinct differences in the *C. albicans* and *C. dubliniensis* Nrg1 regulated gene sets could also be identified. Unlike the published *C. albicans* gene sets, *C. dubliniensis* exhibited increased expression of several genes encoding putative oligopeptide transporters (*OPT4, IFC1, IFC3* and *OPT7;* Table), suggesting a link between nutrient acquisition and pseudohypha formation in *C. dubliniensis*. Further differences could be identified following analysis of the set of 142 downregulated genes (supplementary table 2). This set was enriched with genes encoding iron ion binding proteins (9/142 genes; Table). The latter group included three genes with homology to *C. albicans* plasma membrane ferric reductases (*CFL1, CFL4, CFL5*), the FRP ferric-reductases (*FRP1* and *FRP2*) and the major cell-surface ferric-reductase, *FRE10* and its paralogue,

FRE3. In addition we detected downregulation of genes encoding orthologues of the haem-binding protein Rbt5, the siderophore transporter Sit1 and the high affinity iron permease Ftr1. We selected *RBT5*, *FRP2* and *CFL4* for confirmatory real-time PCR analysis using gene-specific primers (supplementary table 3). This analysis confirmed that these genes were downregulated in the *C. dubliniensis nrg1Δ* mutant (data not shown). Unexpectedly, several genes shown to be upregulated in a *C. albicans nrg1Δ* background were in fact downregulated in the *C. dubliniensis* mutant including the orthologues of *RBT5*, *FRP1* and orf19.6736. These data indicate that *NRG1* is required for optimal expression of genes involved in iron uptake in *C. dubliniensis*.

In *C. albicans*, many genes involved in iron uptake are induced at alkaline pH to promote uptake of insoluble ferric iron compounds (Bensen et al. 2004). In order to determine if the *NRG1*-regulated iron assimilation genes identified in the current study were also alkaline induced in *C. dubliniensis*, we interrogated a previous microarray experiment carried out on *C. dubliniensis* Wü284 grown in Lee's medium pH 7.4 (O'Connor et al. 2010). Several of the *NRG1*-regulated ferric reductases were found to be alkaline induced including *FRP2, FRE3, FRE7, CFL2, CFL4, CFL5,* the siderophore transporter encoding *SIT1* and the high affinity transporter *FTR2* (Fig. 2a). In order to determine if this alkaline induction could occur in the absence of significant *NRG1* expression, we analysed microarray data for Wü284 generated during hyphal growth in alkaline 10% (v/v) serum (O'Connor et al. 2010) (Fig 2a). In this microarray experiment, *NRG1* expression was reduced by 90%, thus allowing us to determine whether *NRG1* is required for expression of these genes during alkaline induced filamentation. Many alkaline-induced ferric reductases remained highly expressed in 10% (v/v) serum despite the reduced *NRG1* expression (Fig. 2a). However, under these conditions, *FRE10, FRP1, RBT5* and *PGA10*, which require *NRG1* for expression in YPD medium, remained downregulated, indicating that *NRG1* is required for optimal expression of these genes in both alkaline and acidic environments. We compared the expression of profile of *RBT5* in both species during filamentation in water plus 10% calf serum (Fig. 2b). Under these conditions, *RBT5* was induced 12-fold (+/- 1.69 standard deviation) in *C. albicans* but exhibited a 4-fold (+/- 0.017 standard deviation) drop in expression in *C. dubliniensis* (Fig. 2b). These data indicate that both *NRG1* and the pH response pathway control the pattern of iron assimilation gene expression during hypha formation in *C. dubliniensis*.

In order to determine if the *C. dubliniensis nrg1Δ* mutant exhibited defective iron assimilating capabilities relative to wild-type, we examined their growth in low iron medium (LIM; Eide & Guarente 1992). To assess iron assimilation, cultures were first depleted of iron by overnight growth in LIM without iron (LIM0) at 37˚C to $OD600_{nm}$ 2.0 ± 0.15. Iron depleted cultures were diluted 1/1000 in fresh LIM pH4.5 supplemented with $FeCl_3$ (1, 10 or 100 μM) and incubated for 24 h at 37˚C with shaking (Fig 3a). Under these conditions, CDM10 exhibited reduced growth following iron depletion relative to Wü284 at all $FeCl_3$ concentrations tested (Fig. 3a). Differences in growth between Wü284 and CDM10 at 100 μM $FeCl_3$ (pH 4.5) were significant (ANOVA, p value < 0.05). In order to determine if alkaline pH could restore expression of iron assimilating capabilities in the *nrg1Δ* mutant, growth was similarly examined in LIM pH 6.5 supplemented with $FeCl_3$ (1, 10 or 100 μM). As iron is less soluble at pH 6.5, cultures were incubated for 48 h to allow sufficient recovery. At pH 6.5 the growth of the *nrg1Δ* mutant was not significantly different from Wü284 in LIM10 and LIM100, suggesting that alkaline induction of iron assimilation genes could restore growth of the mutant to wild-type levels (Fig. 3a). Unexpectedly, in LIM1 pH 6.5, the *nrg1Δ* mutant appeared to grow significantly better than wild-type (Fig. 3a). A similar defect in iron assimilation in the *nrg1Δ* mutant was observed when haemoglobin was used in place of $FeCl_3$. We observed reduced growth of the *nrg1Δ* mutant at pH 4.5 with bovine haemoglobin (0.1 μM and 1 μM) as the sole iron source relative to wild-type (Fig. 3b). Similarly, growth was restored to wild-type levels at pH 6.5 (Fig. 3b). Overall, these data show a defect in iron assimilation in *C. dubliniensis* following deletion of *NRG1*, however alkaline induced expression of ferric-reductases can restore growth to at least wild-type levels at pH 6.5. The enhanced growth of CDM10 at LIM1 pH 6.5 may be due to enhanced induction of some alkaline induced ferric-reductases, but further experiments would be required to determine this.

In contrast to two previous independent analyses of *C. albicans NRG1* mutants, this study revealed a requirement for *NRG1* to maintain expression of iron uptake genes in *C. dubliniensis* (Kadosh & Johnson 2005; García-Sánchez et al. 2005). This resulted in a reduced capacity of the *C. dubliniensis nrg1* mutant to recover from iron starvation. It is not possible from the current data to determine whether this is a direct or indirect affect of *NRG1*, however the mutant did not exhibit any significant

changes in expression of known regulators of iron assimilation such as *SEF1*, *SFU1* or *HAP43* (Baek et al. 2008; Lan et al. 2004; Baek et al. 2008; Chen et al. 2011) These data show that in *C. dubliniensis,* induction of the transcriptional programme regulating filamentous growth has a negative impact on the expression of iron acquisition genes, at least in strain Wü284. In contrast, many *C. albicans* genes are filament induced or Nrg1 repressed including *RBT5, FRP1* and *ALS3* encoding a species-specific ferritin binding protein (Almeida et al. 2008). Although the exact reason for this difference is not clear from the current studies, this dichotomy may contribute to the greater ability of *C. albicans* to colonise different niches relative to *C. dubliniensis*. *C. albicans* forms hyphae in a range of alkaline and acidic environments and optimum expression of iron uptake genes during filamentous growth is likely required for full virulence (Cleary & Saville 2010). In *C. albicans*, repression of iron assimilation gene expression when *NRG1* is downregulated would inhibit growth, particularly in acidic niches such the vaginal and gastric mucosa. Unexpectedly, in *C. dubliniensis RBT5* and *PGA10* remained down regulated in serum, suggesting a possible defect in haemoglobin utilization *in vivo*, although further studies would be required to confirm this.

In summary, our data show strong conservation of the *NRG1* regulated transcriptomes in *C. albicans* and *C. dubliniensis*. However, subtle transcriptional rewiring events can be discerned in the *NRG1* regulated gene sets of both species, particularly with regard to iron assimilation gene expression. The ability of *C. albicans* to express these iron assimilation genes independently of *NRG1* levels supports the notion that this organism is morphologically more flexible than its close relative and may enable it to colonise niches unavailable to *C. dubliniensis*.

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Table. Grouping of genes significantly regulated (>2-fold) in the *C. dubliniensis nrg1Δ* mutant CDM10 compared to wild-type Wü284, identified by transcript profile analysis. Analysis was carried out using the CGD Gene Ontology Term Finder (http://www.candidagenome.org/cgi-bin/GO/goTermFinder).

GO_term	Expression*	Gene(s) annotated to the term
Cell wall	increase	*CDC3, EBP1, HWP1, RBT1, EAP1, SOD5, DDR48, ECM331, CSH1, ALS1, PGA13, XYL2*
Oxidoreductase activity	increase	*ALK8, EBP1, orf19.1365, IFR1, LYS1, SOD5, DFG10, ARA1, orf19.2446, SLD1, ADH4, GRE2, OYE22, orf19.3442, OYE2, orf19.3515, orf19.4287, GRP2, GRE3, CSH1, HMX1, orf19.6143, orf19.6758, ALD6, LYS9, YHB4, GPX2, GPX1*
NADPH dehydrogenase activity	increase	*EBP1, orf19.3234, orf19.3442, OYE2*
Oligopeptide transporter activity	increase	*OPT4, IFC1, IFC3, OPT7*
Iron ion binding	decrease	*CFL1, FRE3, FRE10, CFL5, CFL4, FRP1, RBT5, FRP2, FTR1*
Transcription regulator activity	decrease	*WAR1, orf19.1150, orf19.1253, SNF2, orf19.1574, RRN3, CRZ2, orf19.3035, GAL1, orf19.4342, RME1, SFL1, ZCF30, orf19.5938, ACE2, CUP9, FCR1, orf19.7067, SPT6, STP4*
ncRNA processing	decrease	*UTP22, orf19.1646, MPP10, orf19.2090, orf19.2319, orf19.2320, orf19.2404, HCA4, orf19.4160, RAT1, MDN1, orf19.48, orf19.5160, RRP6, SEN1, orf19.6736, orf19.7067, orf19.7215, orf19.7291, NSA2, SPB1*

* Refers to increase or decrease in expression observed in *C. dubliniensis nrg1Δ* mutant CDM10 compared to wild-type Wü284 in YPD medium

Fig. 1

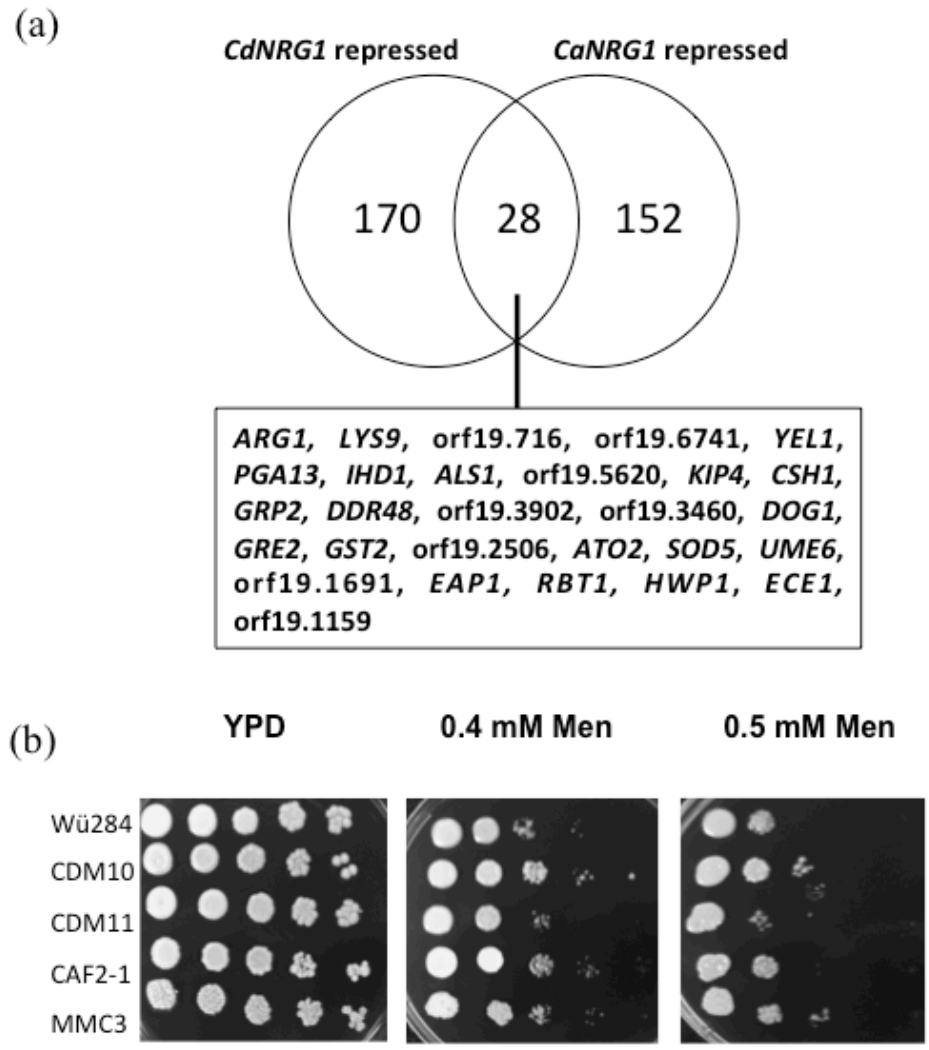

Figure 1. (a) Venn diagram highlighting the common *NRG1* repressed genes in *C. albicans* and *C. dubliniensis*. (b) Growth of *C. dubliniensis* Wü284, CDM10 (*nrg1Δ*), CDM11 (*nrg1Δ* + *NRG1*) and *C. albicans* CAF2-1 and MMC3 (*nrg1Δ*) on YPD medium containing menadione (Men) at 30˚C. A 10 μl aliquot of serially diluted cells (10^7 to 10^3 per ml) were spotted on each plate.

Fig. 2

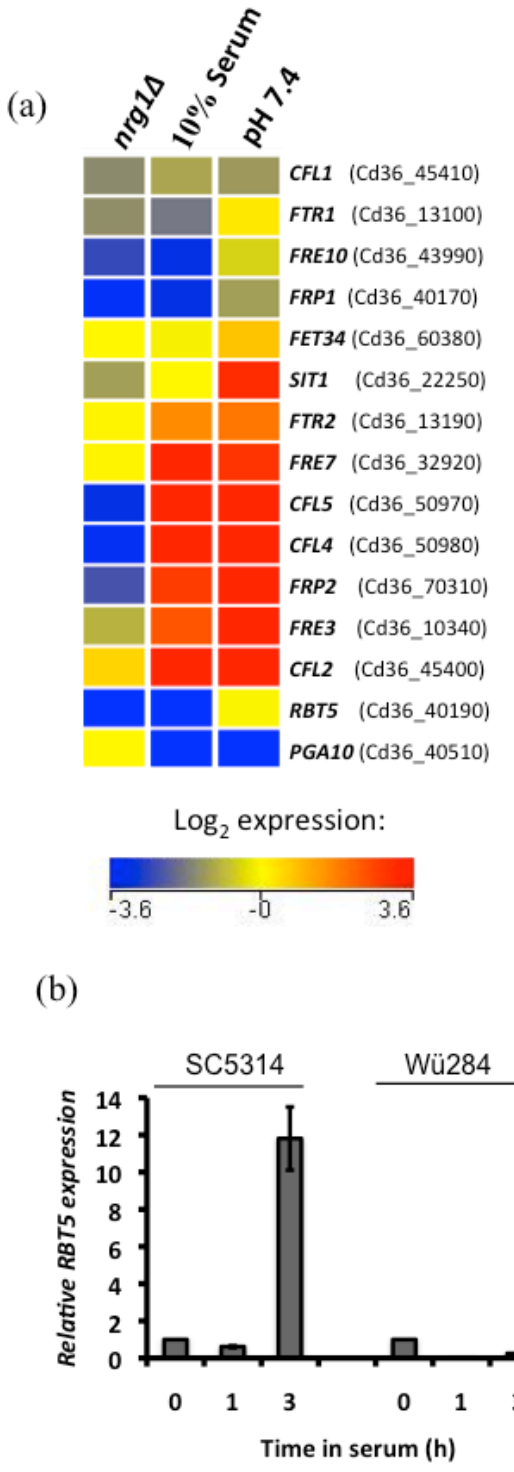

Figure 2. (a) Relative expression of genes involved in iron uptake regulated in the *C. dubliniensis nrg1Δ* mutant (left column), during filamentation of Wü284 in 10% serum (middle column) or in Wü284 in Lee's medium at pH7.4 (right column). (b) Expression of *RBT5* in *C. albicans* SC5314 and *C. dubliniensis* Wü284 during hyphal growth in 10% serum. Expression was monitored by QRT-PCR as described by O'Connor et al. (2010) using the primers described in supplementary table 3.

Fig. 3

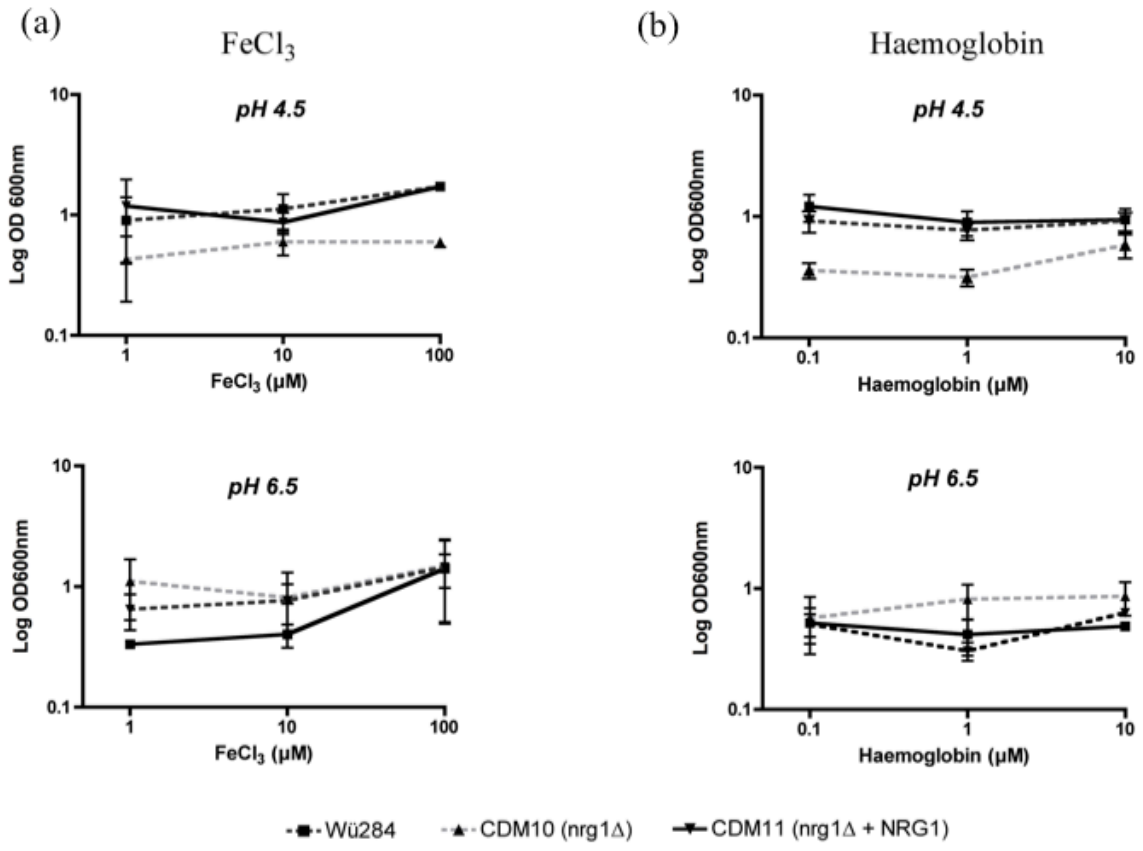

Figure 3. Growth recovery of iron starved cells of *C. dubliniensis* Wü284, CDM10 (*nrg1Δ*) and CDM11 (*nrg1Δ + NRG1*). Cells were starved by overnight growth in low iron medium (LIM) and inoculated in fresh medium supplemented with $FeCl_3$ (a) or bovine haemoglobin (b) at pH 4.5 or pH 6.5, as indicated. Cell densities were determined at 24 h (pH 4.5) or 48 h (pH 6.5) with a spectrophotometer and are the average of three separate experiments. Error bars correspond to standard deviations. Differences in growth between Wü284 and CDM10 at 100 μM $FeCl_3$ (pH 4.5) and 0.1 and 1 μM haemoglobin (pH 4.5) were significant (2-way ANOVA, p value < 0.05).