



HAL
open science

Genome-wide gene expression profiling and a forward genetic screen show that differential expression of the sodium ion transporter Ena21 contributes to the differential tolerance of *Candida albicans* and *Candida dubliniensis* to osmotic stress

Brice Enjalbert, Gary P Moran, Claire Vaughan, Tim Yeomans, Donna M Maccallum, Janet Quinn, David C Coleman, Alistair J. P. Brown, Derek J Sullivan

► **To cite this version:**

Brice Enjalbert, Gary P Moran, Claire Vaughan, Tim Yeomans, Donna M Maccallum, et al.. Genome-wide gene expression profiling and a forward genetic screen show that differential expression of the sodium ion transporter Ena21 contributes to the differential tolerance of *Candida albicans* and *Candida dubliniensis* to osmotic stress. *Molecular Microbiology*, 2009, 72 (1), pp.216-228. 10.1111/j.1365-2958.2009.06640.x . hal-02146793

HAL Id: hal-02146793

<https://hal.science/hal-02146793>

Submitted on 7 Jun 2019

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Genome-wide gene expression profiling and a forward genetic screen show that differential expression of the sodium ion transporter *Ena21* contributes to the differential tolerance of *Candida albicans* and *Candida dubliniensis* to osmotic stress

Brice Enjalbert,^{1†¶} Gary P. Moran,^{2¶}
Claire Vaughan,^{2‡¶} Tim Yeomans,^{2§}
Donna M. MacCallum,¹ Janet Quinn,³
David C. Coleman,² Alistair J. P. Brown¹ and
Derek J. Sullivan^{2*}

¹Aberdeen Fungal Group, School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Aberdeen AB25 2ZD, UK.

²Microbiology Research Unit, Division of Oral Biosciences, Dublin Dental School and Hospital, University of Dublin, Trinity College Dublin, Dublin 2, Ireland.

³Institute for Cell and Molecular Biosciences, Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne NE2 4HH, UK.

Summary

***Candida albicans* is more pathogenic than *Candida dubliniensis*. However, this disparity in virulence is surprising given the high level of sequence conservation and the wide range of phenotypic traits shared by these two species. Increased sensitivity to environmental stresses has been suggested to be a possible contributory factor to the lower virulence of *C. dubliniensis*. In this study, we investigated, in the first comparison of *C. albicans* and *C. dubliniensis* by transcriptional profiling, global gene expression in each species when grown under conditions in which the two species exhibit differential stress tolerance. The profiles revealed similar core responses to**

stresses in both species, but differences in the amplitude of the general transcriptional responses to thermal, salt and oxidative stress. Differences in the regulation of specific stress genes were observed between the two species. In particular, *ENA21*, encoding a sodium ion transporter, was strongly induced in *C. albicans* but not in *C. dubliniensis*. In addition, *ENA21* was identified in a forward genetic screen for *C. albicans* genomic sequences that increase salt tolerance in *C. dubliniensis*. Introduction of a single copy of *CaENA21* was subsequently shown to be sufficient to confer salt tolerance upon *C. dubliniensis*.

Introduction

Candida albicans is the most pathogenic yeast species. It is a common cause of superficial infections (e.g. vaginitis, diaper rash and oral thrush) and in certain groups of immunocompromised patients it can cause systemic infections with high levels of mortality (Odds, 1988). A number of factors contribute to the virulence of *C. albicans*, including the ability to form filaments, cell–cell and cell–surface adhesion, the production of extracellular proteinases and stress adaptation. *Candida dubliniensis* is closely related to *C. albicans* in evolutionary terms, sharing its properties of commensalism and opportunistic infection (Sullivan *et al.*, 1995). In a recent study it was shown that 95.6% of genes are $\geq 60\%$ homologous in the two species, with the vast majority of genes being approximately 90% homologous (Moran *et al.*, 2004). Given the similarity of the genomes of these two species it is not surprising that they share many phenotypic traits, including the ability to produce hyphae and chlamydo spores. This can present difficulties in accurately discriminating between them in clinical samples. However, despite their phylogenetic relatedness, epidemiological data suggest that *C. albicans* is significantly more pathogenic than *C. dubliniensis* which is rarely identified in cases of systemic disease (i.e. 2% of cases of candidemia) while *C. albicans* is usually

*For correspondence. E-mail Derek.Sullivan@dental.tcd.ie; Tel. (+353) 16127276; Fax (+353) 16127295. Present addresses: †Ingénierie des Systèmes Biologiques et des Procédés, UMR5504, UMR792, CNRS, INRA, INSA, ISBP/INSA, 135 Avenue de Ranguieu, 31077 Toulouse Cedex 4, France; ‡Health Research Board, Baggot Street, Dublin 2, Ireland; §Allergy Standards Ltd, Trinity Enterprise Campus, Dublin 2, Ireland. ¶These authors contributed equally to this work.

responsible for approximately 50–65% of cases. (Kibbler *et al.*, 2003; Sullivan *et al.*, 2004; Odds *et al.*, 2007). This observation has been confirmed using a range of infection models, including the mouse model of systemic infection and the neonatal mouse gastric infection model (Gilfillan *et al.*, 1998; Vilela *et al.*, 2002; Stokes *et al.*, 2007). The molecular basis for the differential virulence of *C. dubliniensis* and *C. albicans* has yet to be established. However, it has recently been shown that *C. dubliniensis* has a lower filamentation rate than *C. albicans*, resulting in a decreased capacity to invade tissues in a reconstituted human epithelial infection model (Stokes *et al.*, 2007). This suggests that a lower capacity to produce hyphae *in vivo* might contribute to the relatively low virulence of *C. dubliniensis*. However, other factors might also contribute, at least in part, to the reduced virulence of *C. dubliniensis* in comparison with *C. albicans*.

Candida albicans can colonize diverse niches within its human host [e.g. the oral cavity, the gastrointestinal tract and the vagina (Odds, 1988)] and has the capacity to respond rapidly to changes in environmental conditions (Enjalbert *et al.*, 2003; Lorenz *et al.*, 2004; Fradin *et al.*, 2005; Tournu *et al.*, 2005). This species is also able to tolerate a wide range of environmental stresses (Smith *et al.*, 2004). The inactivation of key stress-protective enzymes or stress-signalling proteins attenuates the virulence of *C. albicans*, indicating that stress responses contribute to the virulence of this pathogen (Wysong *et al.*, 1998; Alonso-Monge *et al.*, 1999; Hwang *et al.*, 1999; Fradin *et al.*, 2005). Genome-wide analyses of *C. albicans* have revealed major transcriptional reprogramming following exposure to specific stresses. This has provided insights into the mechanisms of stress adaptation in this fungus, and highlighted differences in these stress adaptation mechanisms compared with those found in model yeast species (Gasch *et al.*, 2000; Chen *et al.*, 2003; Enjalbert *et al.*, 2003; 2006). For example, it was shown that *C. albicans* does not mount a core transcriptional response under environmental conditions that stimulate a common stress response in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Instead, *C. albicans* mounts a more specific core stress response, involving the activation of a relatively small set of genes under stress conditions that activate the stress activated protein kinase, Hog1p (Enjalbert *et al.*, 2006). Also, while Msn2-like transcription factors play a key role in the core stress response in *S. cerevisiae*, their role in *C. albicans* has diverged significantly (Nicholls *et al.*, 2004; Ramsdale *et al.*, 2008) suggesting that stress responses have diverged in pathogenic yeasts in comparison with benign model yeasts.

Several studies have shown that *C. dubliniensis* is less resistant than *C. albicans* to elevated temperatures, high salt concentrations and oxidative stress (Pinjon *et al.*, 1998; Alves *et al.*, 2002; Vilela *et al.*, 2002). It has also

recently been reported that the *C. dubliniensis* Cnh1p Na⁺/H⁺ antiporter has far less transport activity than the *C. albicans* Cnh1p protein (Krauke and Sychrova, 2008). Given the importance of stress responses for the virulence of *C. albicans*, we reasoned that the lower stress tolerance of *C. dubliniensis* might help to explain the disparity in the virulence of these two species. Therefore, in this study we have compared the genome-wide transcriptional responses of *C. dubliniensis* and *C. albicans* under environmental conditions that emphasize their differential stress tolerances. In addition, we have performed a genome-wide screen for *C. albicans* sequences that confer increased salt tolerance upon *C. dubliniensis*. Both approaches indicate that differential regulation of the *ENA21* gene, encoding a sodium ion transporter, can account for the different salt tolerance of *C. dubliniensis* and *C. albicans*. However, ectopic expression of *ENA21* in *C. dubliniensis* was insufficient to increase the virulence of this pathogen.

Results

Sensitivity of C. dubliniensis and C. albicans to environmental stresses

Previous studies have suggested that *C. dubliniensis* is less tolerant of environmental stress than *C. albicans* (Pinjon *et al.*, 1998; Alves *et al.*, 2002; Vilela *et al.*, 2002). Given the importance of stress responses in microbial pathogenesis we reasoned that this reduced tolerance to stress might contribute, at least in part, to the reduced virulence of *C. dubliniensis* relative to *C. albicans*. Therefore, before embarking upon a genomic comparison of stress responses in these species, we first confirmed their differential tolerance of the specific stresses used in the expression profiling experiments.

The comparative ability of *C. albicans* SC5314 and *C. dubliniensis* CD36 to tolerate specific stresses was tested (Fig. 1). The data obtained using these strains (whose genomes have been sequenced) were representative of those obtained for a range of strains belonging to both species (data not shown). Both *C. albicans* and *C. dubliniensis* displayed similar tolerance of mild stress conditions (e.g. on media supplemented with 0.6 M NaCl or 1 mM H₂O₂). However, the growth of *C. dubliniensis* was inhibited to a significantly greater extent, compared with the inhibition of *C. albicans* growth, when cells were exposed to more acute stresses (e.g. temperatures $\geq 42^{\circ}\text{C}$; osmotic stresses ≥ 1 M NaCl; oxidative stresses ≥ 5 mM H₂O₂) (Fig. 1). These data indicate that while *C. dubliniensis* is able to adapt to mild stresses it does not have the same capacity as *C. albicans* to adapt to more extreme osmotic, oxidative and temperature stresses.

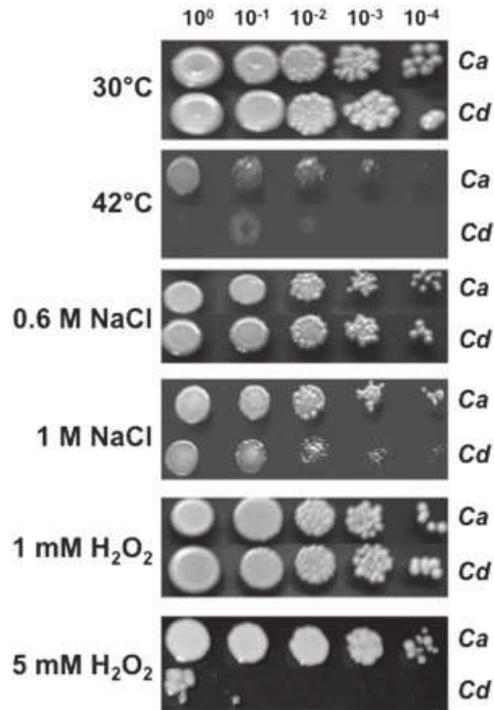


Fig. 1. Comparative growth of *C. albicans* SC5314 (Ca) and *C. dubliniensis* CD36 (Cd) under conditions of heat shock, osmotic stress (NaCl) and oxidative stress (H₂O₂). Ten-fold dilutions of mid-exponential phase cultures were spotted onto YPD plates supplemented with the compounds indicated on the left of the figure, with the exception of the spots incubated at 42°C; all other spots were incubated at 30°C.

Expression profiling of *C. dubliniensis* and *C. albicans* following exposure to acute stresses

Establishing the molecular basis of the differential stress phenotypes of *C. albicans* and *C. dubliniensis* is likely to contribute to our understanding of the role of stress responses in the virulence of *Candida* species. Therefore, we used genome-wide expression profiling to perform an unbiased exploration of the molecular differences between the stress responses of *C. albicans* and *C. dubliniensis*. Exponentially growing *C. dubliniensis* CD36 and *C. albicans* SC5314 cells were exposed to the following stress conditions and transcript profiling performed 30 min later: a shift from 30°C to 42°C; 1.0 M NaCl; and 5 mM H₂O₂. These conditions were chosen because the two *Candida* species displayed differential sensitivities to these relatively acute stresses (Fig. 1). A 30 min time point was used because previous studies have shown that most *C. albicans* stress genes are induced at this point (Enjalbert *et al.*, 2003). Four independent biological replicates (with dye swap) were analysed for each condition, statistically robust changes in expression were identified using SAM (Significance

Analysis of Microarrays) software, and a cut-off of ≥ 1.5 -fold changes was used to identify stress-regulated genes.

A previous microarray analysis in *C. albicans* examined the effects of mild stress conditions (i.e. a shift from 25°C to 37°C; 0.3 M NaCl; and 0.4 mM H₂O₂) upon global gene expression (Enjalbert *et al.*, 2003). These conditions had previously been demonstrated to stimulate broad-ranging core transcriptional responses to stress in model yeasts (Gasch *et al.*, 2000; Chen *et al.*, 2003). However, of the 445 *C. albicans* genes that were induced in response to at least one of these conditions, only one gene was commonly induced by all three conditions (Enjalbert *et al.*, 2003). In the current study, where we have examined more extreme levels of the same stresses, there was a significant increase in the number of stress-induced genes in *C. albicans* (see Table S1 and Fig. S1). A total of 1016 *C. albicans* genes were induced in response to at least one of these conditions (Fig. 2A). Despite this 2.5-fold increase in the number of stress-induced genes, the number of genes that were commonly induced by all three stress conditions remained low. Eleven genes (1.1% of the stress genes) were induced by all three stresses, of which seven have no known function. This finding is consistent with our previous report indicating that *C. albicans* expresses a relatively small core stress response, compared with benign model yeasts (Enjalbert *et al.*, 2006). We also observed that although moderate stresses and acute heat shock decrease the level of expression of

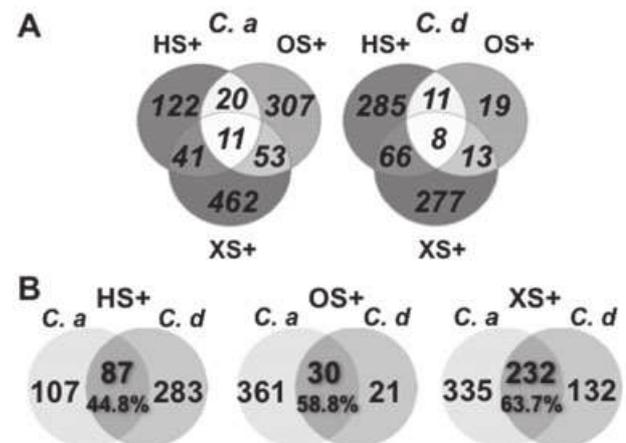


Fig. 2. Global analysis of *C. albicans* and *C. dubliniensis* gene expression exposed to heat shock, osmotic stress and oxidative stress.

A. Venn diagrams indicating the numbers of genes induced in *C. albicans* (C. a) and *C. dubliniensis* (C. d) subjected to the three stress conditions (HS+: heat shock from 30°C to 42°C; OS+: osmotic stress with 1 M NaCl; XS+: oxidative stress with 5 mM H₂O₂).

B. Venn diagrams indicating the numbers of overlapping induced genes in *C. albicans* and *C. dubliniensis* submitted to the same stress condition. The gene lists were obtained using the SAM software to define the statistically significant genes (False Discovery Rate < 10%) and by refining the list with a cut-off of 1.5.

ribosomal genes (Enjalbert *et al.*, 2003; this work), a strong osmotic or oxidative stress does not impact significantly upon these genes. This reinforces the notion that yeast cells mount dose-dependent responses to stresses, modulating their adaptive strategies depending upon the specific nature of the deleterious conditions (Quinn *et al.*, 2002; Ramsdale *et al.*, 2008 and Fig. S2).

A similar observation was made in *C. dubliniensis*. A total of 679 *C. dubliniensis* genes were induced in response to at least one of the three stress conditions examined. Eight (1.2%) of these genes were induced by all three stresses. Of these eight genes, two are in common with the 11 core stress genes from *C. albicans*: *orf19.7085* (unknown function) and *RPN4/orf19.1069* (a putative regulator of proteasome genes). Both of these belong to the previously defined core stress genes of *C. albicans* (Enjalbert *et al.*, 2006). Therefore, like *C. albicans*, *C. dubliniensis* exhibits a relatively small core transcriptional stress response in terms of the numbers of genes involved, compared with model yeasts.

General impact of acute stresses upon the *C. dubliniensis* and *C. albicans* transcriptomes

The transcriptional responses of *C. albicans* and *C. dubliniensis* to the acute heat, osmotic and oxidative stresses displayed a relatively high degree of similarity (Fig. 2B). About 45% of the *C. albicans* genes that were induced in response to the heat shock were also induced in *C. dubliniensis* (Fig. 2B). Approximately 59% of the *C. dubliniensis* genes that were induced by the salt stress were also induced in *C. albicans*. Similarly, about 64% of the *C. dubliniensis* genes that were induced by the oxidative stress were also induced in *C. albicans*. Our comparison of the genes that are regulated in each species suggests that, while there is significant overlap between the transcriptional responses of *C. dubliniensis* and *C. albicans* to each of these stresses, the corresponding stress signalling pathways have some different outputs in each species.

Scatter plots revealed that the overall level of expression of genes in *C. albicans* and *C. dubliniensis* is comparable under each stress condition tested (Fig. S3). However, the number of genes that were induced in each species differed significantly from one condition to another. For example, about twice as many genes were induced in response to heat shock in *C. dubliniensis* (370 genes) than in *C. albicans* (194 genes). In contrast, approximately twice as many genes were induced in response to oxidative stress in *C. albicans* (567 genes) compared with *C. dubliniensis* (364 genes). In the case of the osmotic stress, the response mounted by *C. dubliniensis* to salt osmotic stress was relatively weak, with only 51 genes induced, compared with the 391 genes that were upregulated in *C. albicans*. These differences do not simply reflect

the differential stress sensitivities of these pathogens because in all cases *C. dubliniensis* was more sensitive to these stresses (Fig. 1). Instead the data suggest that the stress signalling pathways might exhibit different levels of responsiveness to these environmental changes as well as some differences in their downstream targets.

Impact of the stresses upon cellular functions in *C. dubliniensis* and *C. albicans*

We examined the behaviour of 'hallmark genes' in the microarray experiments to facilitate the comparison of the two species with regard to the impact of stresses upon relevant cellular functions. Hallmark genes were defined as genes of known function in *S. cerevisiae* that display regulatory responses characteristic of the relevant functional category in that yeast. Having selected these hallmark genes we then examined their fold-regulation in both *C. dubliniensis* and *C. albicans* under each of the three stress conditions analysed (Fig. 3). In general these hallmark genes behaved similarly in both species, although there were some notable differences.

Heat shock genes. Most of the heat shock hallmark genes, many of which encode molecular chaperones, were strongly induced in response to heat shock in both *C. dubliniensis* and *C. albicans* (Fig. 3). Of the eight heat shock hallmark genes examined, five were induced in both species, two were induced in neither species, and *HSP30* was induced only in *C. dubliniensis*. Our previous studies have indicated that *HSP12* expression is induced in *C. albicans* in response to heat, oxidative and osmotic stresses (Enjalbert *et al.*, 2003; Smith *et al.*, 2004). However, *HSP12* upregulation was not observed in this study, possibly because of the different stress conditions examined. In general the heat shock hallmark genes were not induced in response to the other stresses examined, but several were upregulated in response to the oxidative stress in *C. albicans* (*HSP30*, *HSP78*, *HSP104*).

Osmotic stress genes. The majority of the osmotic stress hallmark genes were upregulated in both *C. dubliniensis* and *C. albicans* following exposure to 1.0 M NaCl. For example, *STL1* was strongly induced in both species (Fig. 3). *STL1* encodes a glycerol proton symporter that is transiently induced in response to osmotic shock in *S. cerevisiae* (Ferreira *et al.*, 2005). Also, while the *GPD1* gene was not induced in either species, *CdGPD2* was more strongly induced than *CaGPD2*. *GPD1* and *GPD2* are presumed to encode glycerol 3-phosphate dehydrogenase isoenzymes critical for the biosynthesis of the osmolyte, glycerol. The *ENA21* and *ENA22* genes were upregulated in *C. albicans*, as reported previously in other microarray studies of the osmotic stress response in this

	HS+		OS+		XS+				
	Ca	Cd	Ca	Cd	Ca	Cd			
Heat shock hallmark genes									
HSP10.3	2.5	3.9	0.9	0.8	1.0	0.9	orf19.7215.3	CA5341	Mitochondrial heat shock chaperonin
HSP12	1.0	1.3	1.0	1.5	0.8	1.2	orf19.3160	CA0627	Heat shock protein
HSP30	1.0	3.8	0.9	0.9	3.6	1.1	orf19.4526	CA1507	Heat shock protein
HSP60	4.7	5.2	1.2	1.1	0.7	0.9	orf19.717	CA1239	Heat Shock Protein 60 (HSP60)
HSP78.5f	3.0	10.5	1.1	1.0	2.2	1.5	orf19.882	CA4684	Heat shock protein of clpb family
HSP90	3.4	4.8	1.3	1.2	1.0	1.0	orf19.6515	CA4959	Heat shock protein
HSP104	1.0	1.0	1.0	1.0	1.8	1.7	orf19.6389	CA5135	Heat shock protein, 5-prime end
SSA4	2.1	3.5	1.5	1.6	0.9	0.9	orf19.4980	CA1230	cahsp70 mRNA for heat shock
Osmotic stress hallmark genes									
ENA21.3	1.2	1.1	2.6	1.6	0.9	1.1	orf19.5170	CA4425	P-type ATPase, 3-prime end
ENA22	1.1	0.7	2.8	1.3	1.1	2.3	orf19.6070	CA4929	P-type ATPase involved in Na ⁺ efflux
GPD1	0.5	0.6	0.6	0.6	2.2	1.2	orf19.1756	CA2263	Glycerol-3-phosphate dehydrogenase
GPD2	0.5	0.9	1.9	7.6	2.9	1.8	orf19.691	CA0824	Glycerol 3-phosphate dehydrogenase
STL1	0.3	0.3	14.5	5.6	1.8	1.5	orf19.5753	CA0472	Glycerol proton symporter
Oxidative stress hallmark genes									
CAP1	1.5	1.9	1.5	1.1	1.4	2.2	orf19.1623	CA0183	Transcriptional activator
CTA1	1.1	1.7	1.2	2.1	3.7	6.0	orf19.6229	CA3011	Catalase A, peroxisomal(by homology)
TTR1	1.5	1.9	1.1	1.5	7.1	4.5	orf19.6059	CA4919	Glutaredoxin
TRX1	1.5	3.1	1.0	1.2	2.2	4.1	orf19.7611	CA6010	Thioredoxin
SOD2	0.9	1.5	1.1	1.1	2.8	1.6	orf19.3340	CA2719	Manganese-superoxide dismutase
SOD1.3	1.4	4.8	1.0	1.1	1.2	1.5	orf19.2770.1	CA4120	Cu,Zn-superoxide dismutase
Nitrogen utilization ID:19740 9.1E-05 (14 genes in total)									
NPR1	0.9	1.8	0.4	0.8	1.4	1.0	orf19.6232	CA0576	Nitrogen permease reactivator protein (by homology)
IFC1	0.8	1.1	0.3	0.7	1.0	2.4	orf19.3746	CA3257	Unknown Function
GAT1	0.8	1.1	0.4	1.0	1.0	1.1	orf19.1275	CA1410	Nitrogen regulation (by homology)
Peptide transport ID:15833 1.6E-03 (17 genes in total)									
OPT1	0.3	1.1	0.4	1.1	1.0	1.0	orf19.2602	CA1902	Oligopeptide transporter
OPT2	0.8	1.1	0.3	0.7	1.0	2.4	orf19.3746	CA3257	Oligopeptide transporter
OPT2.53f	1.0	1.1	0.5	1.0	1.0	2.7	orf19.2847.1	CA2870	Oligopeptide transporter
OPT3	1.2	1.0	0.4	0.7	1.1	2.6	orf19.3749	CA2349	Oligopeptide transporter
OPT4	0.9	1.2	0.5	0.8	1.1	2.6	orf19.2292	CA0442	Oligopeptide transporter
OPT5	1.1	1.0	0.8	0.8	1.0	2.5	orf19.5121	CA3919	Oligopeptide transporter
OPT7	0.7	2.9	0.8	1.1	1.1	2.1	orf19.5673	CA4380	Putative oligopeptide transporter
Glucose transport ID:15758 1.0E-04 (20 genes in total)									
HGT5	1.5	1.3	3.0	1.5	0.6	0.8	orf19.6005	CA6062	Putative permease (by homology)
SNF3	1.1	1.0	1.6	0.7	0.4	0.5	orf19.5962	CA6095	High affinity glucose transport protein
HGT12	1.1	0.9	2.7	1.2	1.4	0.9	orf19.3668	CA4038	Hexose transporter
STL1	0.3	0.3	14.5	5.6	1.8	1.5	orf19.5753	CA0472	Sugar transporter (by homology)
HGT11	1.1	1.0	3.2	1.1	1.4	1.0	orf19.4527	CA1506	Hexose transporter
HXK2	0.6	0.9	0.8	0.4	1.0	1.0	orf19.542	CA0127	Hexokinase II (by homology)
HXT62	0.9	1.0	0.9	0.7	1.0	1.0	orf19.2023	CA1067	Sugar transporter
HXT61	0.4	0.6	0.7	0.4	1.0	0.9	orf19.2020	CA1070	Sugar transporter
HXT5.3f	0.9	0.9	1.0	0.6	1.1	1.0	orf19.2021	CA1069	Sugar transporter, 3-prime end
Manganese ion transport ID:6828 1.1E-04 (4 genes in total)									
SMF11	0.9	1.2	1.9	0.9	1.0	0.6	orf19.4690	CA0087	Manganese transporter (by homology)
SMF12	1.0	1.0	2.5	1.0	1.0	0.6	orf19.2270	CA1879	Manganese transporter (by homology)
HIP1	1.7	1.2	2.0	0.8	0.9	2.5	orf19.4940	CA3708	Histidine/manganese transporter
SMF3	1.0	1.0	0.7	1.0	0.4	0.3	orf19.5022	CA5241	Probable manganese transporter (by homology)

Fig. 3. Comparison of expression of specific genes in specific functional categories. These include the three stress hallmark groups as well as other gene groups selected for their specificity to one of the species. For the latter, genes that were induced or repressed ≥ 2 -fold in either species were entered to the GO Term Finder tool (<http://www.candidagenome.org/cgi-bin/GO/goTermFinder>). Four processes (nitrogen utilization, peptide transport, glucose transport and manganese transport) were found to be significant in only one species ($P < 0.01$).

pathogen (Enjalbert *et al.*, 2003; 2006). However, the *C. dubliniensis* *ENA* genes displayed minimal levels of activation. The *C. albicans* *ENA21* gene and its paralogue *ENA22* are orthologues of the *S. cerevisiae* *ENA2* gene that encodes a P-type ATPase sodium pump. *ScENA2* confers salt tolerance upon *S. cerevisiae* through sodium ion efflux (Garcia-deblas *et al.*, 1993). The *C. dubliniensis* genome (http://www.sanger.ac.uk/sequencing/Candida_dubliniensis/) contains two genes with over 90% nucleotide sequence identity to *CaENA21* and *CaENA22* (Cd36_72570/*CdENA21* and Cd36_00320/*CdENA22*). Therefore, the lack of induction of *CdENA21* and *CdENA22* in response to salt stress was surprising. We reasoned that this lack of induction might partially account for the relative sensitivity of *C. dubliniensis* to salt stress (Fig. 1).

Oxidative stress genes. The oxidative stress hallmarks, which include catalase, superoxide dismutase, glutaredoxin and thioredoxin genes, were generally strongly induced in both *C. dubliniensis* and *C. albicans* in response to 5 mM H₂O₂. As described previously for mild stress conditions (0.4 M H₂O₂), the oxidative stress hallmark genes were also upregulated slightly in response to a mild heat-shock (Enjalbert *et al.*, 2003), a phenomenon that seems more pronounced in *C. dubliniensis*.

Other cellular functions. We have established that, with some notable exceptions, most of the stress hallmark genes displayed similar expression patterns in *C. dubliniensis* and *C. albicans*. Using the gene ontology tool we then screened our transcript profiling datasets for functional categories that displayed statistically significant differences in their regulation between these two species. For most functional categories no significant differences were observed. However, a small number of processes did display significant differences between *C. dubliniensis* and *C. albicans* ($P < 0.001$). These were nitrogen utilization, peptide transport, glucose transport and manganese ion transport (Fig. 3). The oligopeptide transporter genes (*OPT*) and the nitrogen utilization genes, which include the regulators *GAT1* and *NPR1* (Boeckstaens *et al.*, 2007; Dabas and Morschhäuser, 2007), were repressed in response to the acute osmotic stress in *C. albicans*, but only to a minor extent in *C. dubliniensis*. Also most *OPT* genes were induced in response to the oxidative stress in *C. dubliniensis*, but not in *C. albicans*. Some genes involved in glucose and manganese ion transport were more strongly induced by osmotic stress in *C. albicans* than in *C. dubliniensis*.

To summarize, our genome-wide expression profiling indicated that *C. albicans* and *C. dubliniensis* generally display similar transcriptional responses to the relatively acute heat, salt and oxidative stresses examined here.

However, some differences were observed between these species with respect to the regulation of genes encoding various transporters, and in particular, the lack of induction of *ENA21/22* sodium ion efflux pumps in *C. dubliniensis* provided a possible explanation for the relative sensitivity of this pathogen to salt stress.

A genetic screen for C. albicans genes that suppress the osmotic sensitivity of C. dubliniensis

Our transcript profiling provided a powerful screen for genes whose stress regulation differs between *C. dubliniensis* and *C. albicans*. In parallel, we performed forward genetic screens for *C. albicans* sequences capable of suppressing the relative sensitivity of *C. dubliniensis* to heat, oxidative or salt stress. We reasoned that these two screens – one based on stress gene regulation, and the other upon stress phenotype – would provide complementary insights into the mechanistic basis for the differential stress sensitivities of these two pathogens.

The genetic screens were performed by transforming *C. dubliniensis* CdUM4B (*ura3/ura3*) with a *C. albicans* genomic library constructed in the plasmid pRMI (*URA3*), and selecting for transformants capable of growth at 42°C, or on medium supplemented with 5 mM H₂O₂ or 1 M NaCl. Several saturating screens yielded no *C. dubliniensis* transformants capable of growth at 42°C or on medium containing 5 mM H₂O₂. This was consistent with the idea that the ability to grow under these conditions is a polygenic trait. However, 11 transformants capable of growth on 1 M NaCl were identified. Of these, four exhibited a stable and reproducible salt-resistant phenotype, tolerating up to 1.2 M NaCl. Sequence analysis revealed that all four *C. dubliniensis* transformants carried plasmids containing the entire coding sequence of the *C. albicans* *ENA21* gene. This strongly suggested that the transformation of *C. dubliniensis* with *CaENA21* was sufficient to increase the salt tolerance of this species, despite the presence of an orthologous gene in *C. dubliniensis* (*CdENA21*) that shares 91% identity.

To test this further, we integrated a single copy of the *CaENA21* gene under the control of its native promoter at the *CDR1* locus in *C. dubliniensis* strain CD36. This had no effect upon the growth of *C. dubliniensis* on YPD medium. However, a single copy of *CaENA21* was sufficient to confer greatly enhanced resistance to 1.0 M and 1.2 M NaCl (Fig. 4). Indeed, the growth of *C. dubliniensis* CD36 expressing *CaENA21* on medium containing 1.0 M NaCl was comparable to that of *C. albicans* SC5314 (see Fig. 1). Similar results were obtained with another reference *C. dubliniensis* strain, Wü284 (Fig. 4). Therefore, the introduction of *CaENA21* was sufficient to confer salt tolerance upon *C. dubliniensis*.

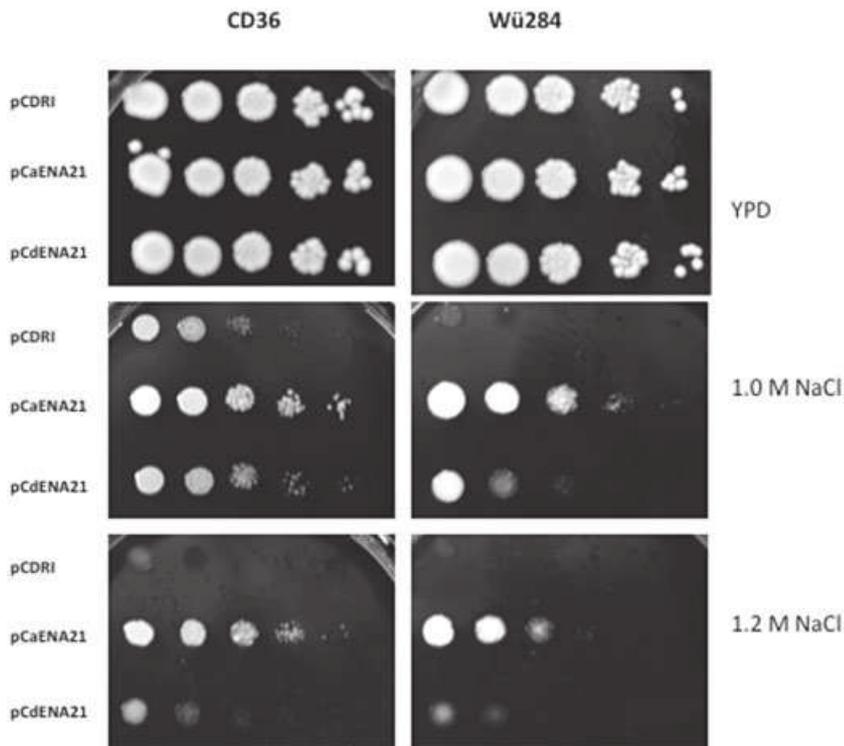


Fig. 4. Growth of derivatives of *C. dubliniensis* strains CD36 and Wü284 harbouring the integrative plasmid pCDRI on YPD agar supplemented with NaCl. Strains were transformed with empty plasmid (pCDRI) or derivatives containing the *C. albicans* (pCaENA21) or *C. dubliniensis* (pCdENA21) genes. Spot plates were prepared as described in the *Experimental procedures*.

To determine whether the native *C. dubliniensis* *ENA21* gene is capable of conferring a similar phenotype, we then integrated the *CdENA21* gene under the control of its native promoter at the *CDR1* locus in *C. dubliniensis* strains CD36 and Wü284. The introduction of one additional single copy of *CdENA21* did enhance the growth of *C. dubliniensis* at 1 M NaCl, but unlike the *C. albicans* *ENA21* gene, *CdENA21* did not support significant growth at 1.2 M NaCl. These data suggest that the CdEna21 protein has the ability to confer salt tolerance but that either differential expression of the *CaENA21* and *CdENA21* genes, or perhaps functional differences in the activity of the encoded proteins, might account for the relatively high salt resistance of *C. albicans* compared with *C. dubliniensis*.

Differential expression of ENA21 in C. albicans and C. dubliniensis

Our microarray experiments suggested that the *ENA21* gene is differentially expressed in *C. dubliniensis* and *C. albicans* in response to 1.0 M NaCl. To test this further we performed real-time PCR (RT-PCR) on the *ENA21* transcript and its paralogue *ENA22* in both species, measuring their expression levels relative to the *TEF1* internal control (Fig. 5A). The basal levels of *ENA21* mRNA were approximately 10-fold higher in *C. albicans* compared with *C. dubliniensis*. By 1 h post exposure to 1 M NaCl *CaENA21* mRNA levels were induced fivefold in

C. albicans, whereas *CdENA21* expression levels did not increase in *C. dubliniensis*. These data were consistent with our transcript profiling data, confirming that while *ENA21* responds to salt stress in *C. albicans*, it does not do so in *C. dubliniensis*.

When *ENA22* mRNA levels were examined it was observed that the basal levels of expression were low in both species (Fig. 5A). *ENA22* expression was induced in both species following exposure to 1 M NaCl, but the levels of *ENA22* induction were lower than the basal levels of *ENA21* mRNA in *C. albicans*. These data reinforced the idea that differential *ENA21* expression levels in *C. dubliniensis* and *C. albicans* contribute to the different salt tolerances of these pathogens.

If differential *ENA21* expression levels do account for the different salt tolerances of *C. dubliniensis* and *C. albicans* one would expect this to be reflected in relatively high *ENA21* mRNA levels in salt-tolerant *C. dubliniensis* transformants. Therefore, we measured *ENA21* mRNA levels in the *C. dubliniensis* CD36 and Wü284 transformants carrying *CaENA21* and *CdENA21* in the presence or absence of 1 M NaCl (Fig. 5B). Once again, *ENA21* mRNA levels were measured by RT-PCR relative to the *TEF1* internal control. *CaENA21* mRNA levels were strongly induced by NaCl in both *C. dubliniensis* CD36 and Wü284. Some induction of the cloned *CdENA21* gene was observed in *C. dubliniensis* CD36 in the presence of NaCl and this may account for the elevated NaCl tolerance exhibited by CD36 transformants relative to Wü284 (Fig. 4). However, no induction of

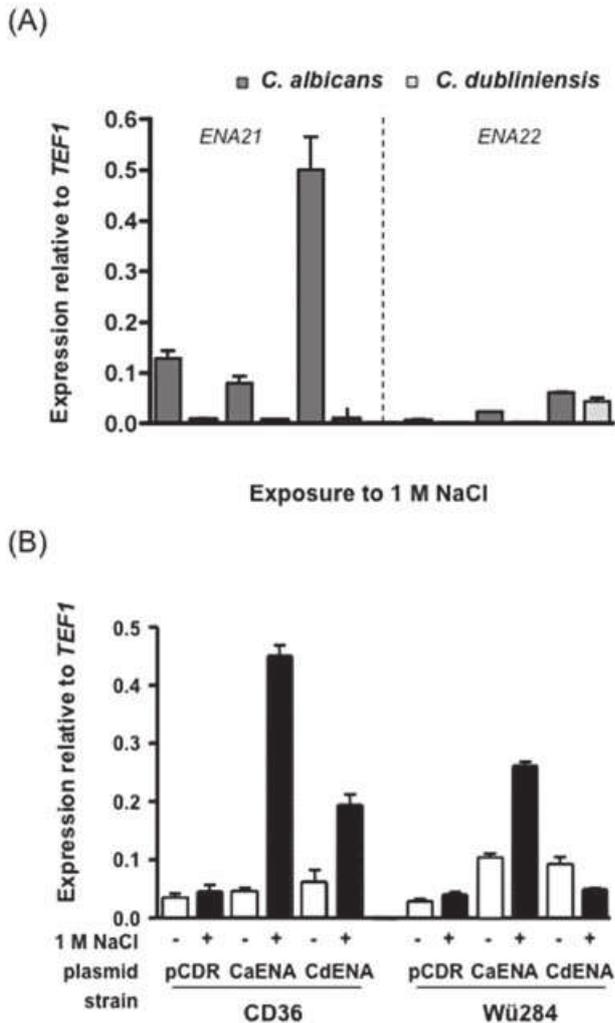


Fig. 5. Real-time PCR analysis of *ENA21* and *ENA22* expression. A. Expression of *ENA21* (left) and *ENA22* (right) was analysed in *C. albicans* (dark grey bars) and *C. dubliniensis* (light grey bars) relative to the expression of *TEF1*. Cells were grown to OD₆₀₀ 1.0 (0 min) before the addition of 1 M NaCl. RNA was isolated 30 and 60 min after the addition of NaCl. B. Expression of *ENA21* in derivatives of *C. dubliniensis* strain CD36 (left) and Wü284 (right) harbouring the integrative plasmid pCDR1 or derivatives of the plasmid containing the cloned *C. albicans* *ENA21* gene (CaENA21) or the cloned *C. dubliniensis* *ENA21* gene (CdENA21).

chromosomal *CdENA21* sequences was observed in *C. dubliniensis* transformants carrying the empty pCDR1 vector, and no *CdENA21* induction in *C. dubliniensis* Wü284 cells carrying pCdENA21. These data reinforce the view that elevated *ENA21* expression levels increase the salt tolerance of *C. dubliniensis*. In an attempt to determine if differences in the activity of the two promoters accounted for the differential expression of *CdENA21* and *CaENA21*, we performed a promoter-swap experiment. To do this we created a fusion between the *CaENA21* promoter and the *CdENA21* open reading frame (ORF) and downstream

sequences. However, when this construct was transformed into *C. dubliniensis* CD36 or Wü284, we did not observe any significant induction of this gene in response to NaCl. Consistent with this, the phenotype on NaCl plates was identical to *C. dubliniensis* transformants that harboured an additional native *CdENA21* gene (data not shown). This suggests that the regulation of *ENA21* transcript levels in *C. albicans* and *C. dubliniensis* may be mediated through changes in transcript stability rather than transcription.

Hog1 activation in *C. dubliniensis*

ENA21 is upregulated in response to osmotic shock by the stress-activated protein kinase Hog1 in *C. albicans* (Enjalbert *et al.*, 2006). To investigate whether the minimal induction of *ENA21* and *ENA22* in *C. dubliniensis* in response to salt stress was due to a lack of Hog1p activation, we examined the phosphorylation status of this protein by Western blot analysis. Following exposure to NaCl, Hog1p was expressed and phosphorylated at similar levels in *C. dubliniensis* and *C. albicans* (Fig. 6). Therefore, the absence of *ENA21* and *ENA22* induction in *C. dubliniensis* is not due to a lack of Hog1 signalling. This view is reinforced by the observation that other Hog1-dependent genes such as *GPD2* and *SLT1* were strongly induced by NaCl (Fig. 3).

Effect of CaENA21 upon the virulence of *C. dubliniensis*

Our data indicated strongly that *CaENA21* confers increased salt stress tolerance upon *C. dubliniensis*. To test whether this influenced the virulence of *C. dubliniensis*, we compared the virulence of *C. dubliniensis* cells containing pCaENA21, pCdENA21 and the control pCDR1 plasmid using the classical mouse model of disseminated

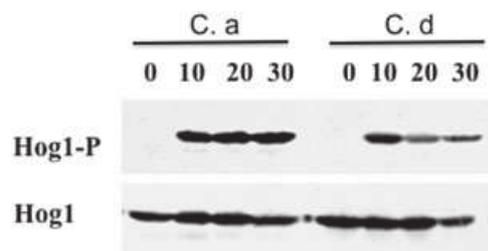


Fig. 6. Phosphorylation of Hog1 in *C. albicans* and *C. dubliniensis* exposed to 1.0 M NaCl. Western blot analysis of Hog1 from *C. albicans* SC5314 (*C. a*) and *C. dubliniensis* CD36 (*C. d*) cells after treatment with 1.0 M NaCl for the times indicated. Western blots were probed with an anti-phospho p38 antibody, which only recognizes the phosphorylated, active form of *C. albicans* and *C. dubliniensis* Hog1 (Hog1-P). Total levels of Hog1 protein were determined by stripping and reprobating the blot with an anti-myc antibody that recognizes both phosphorylated and unphosphorylated forms of Hog1.

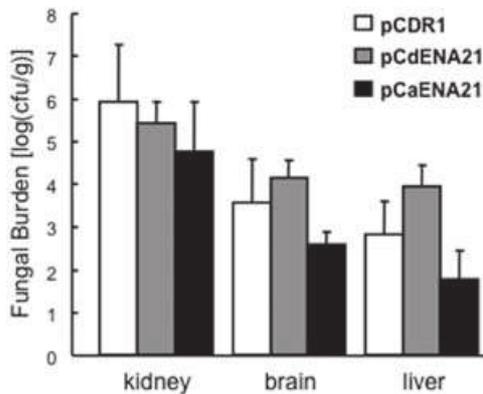


Fig. 7. Effect of heterologous expression of *CaENA21* on virulence. Organ burdens determined for groups of six BALB/c mice infected with the CD36 strains. White bars represent *C. dubliniensis* CD36 containing pCDR1; grey bars, CD36 containing pCdENA21; and black bars, CD36 containing pCaENA21.

candidiasis (MacCallum and Odds, 2005). No significant difference in the survival of mice infected with these isogenic strains was observed (not shown). Furthermore, these strains displayed no significant differences with respect to their fungal burdens in the kidney, brain and liver (Fig. 7). Therefore, the increased salt tolerance of *C. dubliniensis* cells carrying the *CaENA21* gene was not sufficient to increase their virulence. This indicates that increasing the osmotic stress resistance of *C. dubliniensis* is not sufficient to increase the virulence of this species to the virulence levels of *C. albicans*, for example. Therefore, other traits of *C. dubliniensis* cells such as their thermal sensitivity, oxidative stress sensitivity and reduced rates of hyphal development might contribute to their relatively low virulence. Our data are consistent with the idea that the basis of this low virulence phenotype is multifactorial.

Discussion

Epidemiological observations and virulence data from animal models both indicate that, despite the close phylogenetic relationship between *C. albicans* and *C. dubliniensis*, the latter species is less capable of colonizing and infecting humans. Recent studies have confirmed that the genomes of both species are closely related, with only 4.4% of *C. albicans* genes being absent or significantly divergent in the *C. dubliniensis* genome (Moran *et al.*, 2004). Comparison of the *C. albicans* and *C. dubliniensis* genome sequences indicates that the vast majority of gene orthologues in these species are $\geq 90\%$ identical and both species share a high degree of synteny. This close genetic relatedness of the two species results in their sharing many phenotypic traits, to the point where it can be difficult to discriminate between them using phenotypic tests.

One phenotypic difference identified soon after the discovery of *C. dubliniensis* is the reduced capacity of this species to grow at temperatures $\geq 42^\circ\text{C}$, conditions under which the majority of *C. albicans* strains grow (Sullivan *et al.*, 1995). Indeed, comparative growth at 45°C has been recommended as a simple phenotypic test to discriminate between the two species (Pinjon *et al.*, 1998). These findings suggest that *C. albicans* is better able to tolerate thermal stress than *C. dubliniensis*.

Data presented here (Fig. 1) and in other studies (Sullivan *et al.*, 1995; Pinjon *et al.*, 1998; Alves *et al.*, 2002; Vilela *et al.*, 2002) show that *C. dubliniensis* is also less tolerant than *C. albicans* to heat shock, NaCl and H_2O_2 , indicating that *C. dubliniensis* is relatively more susceptible to a range of environmental stresses compared with *C. albicans*. Therefore, *C. dubliniensis* appears less well equipped than *C. albicans* to adapt to the stresses experienced by these pathogens in the diverse environmental niches they occupy in the human body. This might, in part, contribute to the lower virulence of *C. dubliniensis* compared with *C. albicans*.

It was conceivable that a general lack of activation of stress responses in *C. dubliniensis* might have accounted for the relatively low tolerance of this pathogen to environmental stress. Therefore, in the first transcript profiling comparison of the two species, we used genome-wide expression profiling to compare the transcriptional responses of *C. dubliniensis* and *C. albicans* to stresses, focusing on experimental conditions under which *C. dubliniensis* grows relatively poorly (a $30\text{--}42^\circ\text{C}$ heat shock, 1.0 M NaCl and 5 mM H_2O_2). These experiments revealed that, in general, *C. dubliniensis* mounts a similar transcriptional response to *C. albicans* following exposure to these heat, salt and oxidative stresses (Figs 2 and 3). In both *C. dubliniensis* and *C. albicans* a relatively small subset of genes was commonly induced by all three of the stresses examined (about 1% of the set of stress induced genes). This strengthens the view that *C. albicans* (and now *C. dubliniensis*) exhibits a relatively narrow core stress response compared with the benign model yeasts *S. cerevisiae* and *S. pombe* (Enjalbert *et al.*, 2006). The expression of hallmark stress genes was remarkably similar in both species. There were differences between the species with respect to the expression of a variety of transporters involved in peptide and manganese uptake. However, with one notable exception, the transcript profiling data revealed no obvious explanations for the relatively low stress tolerance of *C. dubliniensis* compared with *C. albicans*.

One notable exception was the minimal induction of *ENA21* and *ENA22* in *C. dubliniensis* following exposure to salt stress. This suggested that insufficient expression of the Ena21/22 sodium ion efflux pumps might contribute to the salt sensitivity of this species. This view was

strongly reinforced by the repeated isolation of the *CaENA21* gene in an unbiased genetic screen for *C. albicans* genes that can suppress the salt sensitivity of *C. dubliniensis*. All four of the *C. albicans* genomic clones that were capable of suppressing the salt sensitivity of *C. dubliniensis* carried the *CaENA21* gene. The significance of the *CaENA21* gene in conferring salt resistance upon *C. dubliniensis* was further confirmed by the subsequent integration of this gene into the *C. dubliniensis* genome. Once again, this increased the resistance of *C. dubliniensis* cells to salt stress (Fig. 4). Furthermore, increased *CaENA21* expression in *C. dubliniensis* correlated with elevated salt resistance (Fig. 5). When an additional copy of *CdENA21* was integrated into the *C. dubliniensis* genome, the level of expression observed was significantly lower than that observed for *CaENA21* in *C. albicans*. Nevertheless, an increase in salt tolerance was evident in these *C. dubliniensis* cells, suggesting that *CdEna21p* is functional and can confer salt tolerance. The finding that *CaENA21* and *CdENA21* are expressed at higher levels in CD36 compared with another *C. dubliniensis* strain (i.e. Wü284) also suggests that there may be differences in *ENA21* regulation between different strains of *C. dubliniensis*.

Our data strongly suggest that the relatively low levels of *CdENA21* expression in *C. dubliniensis* contribute to the salt sensitivity of this species, compared with *C. albicans*. These relatively low *CdENA21* expression levels are not due to a lack of Hog1 signalling as the Hog1 stress-activated protein kinase was phosphorylated in response to salt stress in *C. dubliniensis* (Fig. 6). Other osmotic stress genes were also induced in response to the NaCl treatment (Fig. 3). Therefore, differences in the *ENA21* promoters could account for the differential responsiveness of this gene in *C. dubliniensis* and *C. albicans*. Preliminary analysis of the promoters has identified an upstream region (~800 to ~1300) that shares minimal sequence homology between the two species (data not shown). This area contains multiple putative transcription factor binding sites. Differences in these putative transcription factor binding sites might contribute to the differential regulation of the *ENA21* genes in these species. However, the promoter-swap experiment indicated that simply replacing the *CdENA21* promoter with the *CaENA21* promoter is insufficient to restore *C. albicans*-like regulation upon the *CdENA21* gene. Therefore, differences in the post-transcriptional regulation of *ENA21* probably also contribute to the differential regulation of this gene in *C. dubliniensis* and *C. albicans*. This requires further investigation.

High salt levels are found in the medulla of the kidney and it has been shown that *CaENA21* is upregulated almost threefold in mouse renal infections (Walker *et al.*, 2008). Therefore, we tested whether increasing the salt

tolerance of *C. dubliniensis* has an impact upon the virulence of this pathogen. This was done by comparing the survival rates and tissue burdens for mice infected with *CaENA21* transformants and control strains (Fig. 7). *CaENA21* expression did not increase the virulence of *C. dubliniensis*. This suggests that traits in addition to salt sensitivity contribute to the relatively low virulence of this species compared with *C. albicans*. These additional traits might include the thermal and oxidative stress sensitivity of *C. dubliniensis* (Fig. 1) and/or the relatively low rates of hyphal development observed for this species (Gilfillan *et al.*, 1998; Stokes *et al.*, 2007). The role of *CaENA21* in *C. albicans* virulence is currently unclear and could be assessed by deleting this gene; however, it is likely that any phenotypic defect would be masked by the expression of other sodium transporters, such as *Cnh1p* or *Ena22p*.

In summary, our data indicate that the low levels of *ENA21* transcript induced in response to NaCl stress can account for the relative salt sensitivity of *C. dubliniensis* compared with *C. albicans*. The basis for the observed differences in *ENA21* expression in the two species may be due to differences in transcriptional regulatory pathways, to differential post-transcriptional controls, or to a combination of both. Our work shows how a combination of expression profiling and forward genetic screens can provide a powerful experimental approach towards the elucidation of the mechanistic basis for important phenotypic differences between these closely related *Candida* species.

Experimental procedures

Strain and growth conditions

Candida dubliniensis strains CD36 (Sullivan *et al.*, 1995), Wü284 (Morschhäuser *et al.*, 1999) and CdUM4B (*ura3/ura3*: (Staib *et al.*, 2001)) and *C. albicans* SC5314 (Gillum *et al.*, 1984) were used in this study. Strains were routinely grown at 30°C in YPD containing 0.02% adenine and 0.008% uridine or in SD minimal medium (Sherman, 1991).

Stress sensitivity tests

To test the sensitivity of strains to specific stresses, they were grown in YPD broth at 30°C to mid-exponential phase and 10-fold serial dilutions of these cells spotted onto YPD plates and YPD plates supplemented with the appropriate concentration of NaCl or H₂O₂. Growth was monitored after 24 h at 30°C (42°C for the heat-shock assay).

Transcript profiling

Transcript profiling was performed on *C. albicans* SC5314 and *C. dubliniensis* CD36 as described previously (Enjalbert *et al.*, 2006). The strains were cultured to OD₆₀₀ = 1 in YPD supplemented with uridine at 30°C (200 r.p.m.), and then

divided into two parts: one was exposed to a stress condition whereas the other was the control. For the heat shock experiments, cells were transferred from 30°C to 42°C by immersing the sample in a water bath at $t=0$. To impose the hyperosmotic shock, NaCl was added to a final concentration of 1.0 M. To apply the oxidative shock, hydrogen peroxide (H_2O_2) was added to a final concentration of 5 mM. After 30 min, cells were collected by centrifugation, snap frozen in liquid N_2 , disrupted using a microdismembrator (Braun, Melsungen, Germany) and RNA prepared as described previously (Hauser *et al.*, 1998). Cy3- and Cy5-labelled cDNAs were prepared from total RNA, and the probes were hybridized with whole genome microarrays containing c. 6000 *C. albicans* genes (Eurogentec, Seraing, Belgium). Slides were scanned using a ScanArray Lite scanner (PerkinElmer Life Sciences, Beaconsfield, UK) and quantified using QuantArray software (version 2.0). Data normalization and analysis were performed using GeneSpring (Silicon Genetics, Redwood City, CA), and statistical analysis was performed using SAM (Tusher *et al.*, 2001). Expression ratios were calculated by comparing stressed cells with their unstressed control. Data from four independent biological replicates, two of them obtained with a dye swap, were used for each strain and each condition. These datasets are available at ArrayExpress (<http://www.ebi.ac.uk/microarray/> with the experiment code: E-MEXP-1650).

Forward genetic screen in *C. dubliniensis*

A *C. albicans* genomic DNA library constructed in the plasmid pRM1 (Pla *et al.*, 1995) was kindly provided by Dr Federico Navarro-García (Universidad Complutense de Madrid). *C. dubliniensis* CdUM4B cells were transformed with this library by electroporation (Staib *et al.*, 2001) to yield about 40 000 independent transformants. To identify *C. albicans* genes that suppress the inability of *C. dubliniensis* to grow under stressful conditions, 40 pools of transformants were generated and 5×10^5 cells from each pool were spread onto SD plates containing 1.2 M NaCl or 5 mM H_2O_2 and grown at 30°C, or onto SD medium alone and incubated at 42°C. Positive clones were subcultured and the stability of their salt, peroxide or temperature tolerant phenotypes was assessed, and the plasmids rescued from stable *C. dubliniensis* transformants. To ascertain the identity of *C. albicans* ORFs isolated in this screen, the inserts were sequenced and homology searches performed using the *C. albicans* WU-BLAST2 Search facility at the *Candida* genome database (CGD; <http://www.candidagenome.org/>).

Gene manipulations in *C. dubliniensis*

The *C. albicans* *ENA21* (*CaENA21*) and *C. dubliniensis* *ENA21* (*CdENA21*) genes and their promoters were integrated into the *C. dubliniensis* genome in single copy. This was accomplished by PCR amplification of these genes from chromosomal DNA using the Expand Long Template PCR System (Roche Applied Science) using template DNA from *C. albicans* SC5314 and *C. dubliniensis* CD36. The entire *CaENA21* ORF was amplified, including 1385 bp of upstream sequences and 588 bp of downstream sequences. The *CdENA21* gene was

PCR amplified including 2414 bp of upstream sequences and 223 bp of downstream sequences. These PCR products were cloned in pGEM-T (Promega) and sequenced to confirm that no mutations had been introduced. To create a fusion between the *CaENA21* promoter and the *CdENA21* ORF, we removed an ~2 kb HindIII/EcoRV fragment containing the entire *CaENA21* promoter along with exon 1 and the beginning of exon 2 of the coding sequence (encoding the first 49 aa) from the *CaENA21* construct. A similar HindIII/EcoRV fragment was then removed from the *CdENA21* construct and the *CaENA21* HindIII/EcoRV fragment was fused in frame to the *CdENA21* ORF using a HindIII site conserved at the same position in the *CdENA21* ORF. The inserts were then subcloned into pCDRI (Moran *et al.*, 2007) using the NotI and SacII restriction endonuclease sites in pGEM-T, to create the plasmids pCaENA21 and pCdENA21 and pFusion. Targeted integration of these pCDRI-based plasmids at the *CDR1* locus of *C. dubliniensis* strains CD36 and Wü284 was carried out as described by Moran *et al.* (2007). The *ENA21* copy number in these strains was assayed by RT-PCR using the primers ENA21F/R (see next section). Strains carrying pCaENA21 or pCdENA21 were shown to harbour three copies of *ENA21* gene relative to *TEF1*.

Real-time PCR analysis of gene expression

For RNA preparation, cells were harvested from YPD cultures and frozen in liquid nitrogen. RNA was prepared using TRI-reagent (Sigma) as described (Stokes *et al.*, 2007). RNA samples were rendered DNA free by incubation with TurboDNA-free reagent (Ambion, Austin, TX) and RNA concentrations 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 following primers pairs: QRTEF1F/R (Green *et al.*, 2005); ENA21F/R (ENA21F: 5'-GGGCCAACCAATTTTACTTTG-3'; ENA21R: 5'-AATAAAGTACAACCAATTGCAACACC-3'); and ENA22F/R (ENA22F: 5'-GATCCACTGCATTTGCCTCA-3'; ENA22R: 5'-AAATTGGTTAGCCCAAAGTTCAC-3'). Primer sets ENA21F/R and ENA22F/R were designed using Primer Express software v1.5 (Applied Biosystems, Foster City, CA) and were homologous to *C. albicans* and *C. dubliniensis* *ENA21* and *ENA22* ORFs respectively. These primers yielded single, specific amplicons from genomic DNA and cDNA templates from both *C. albicans* and *C. dubliniensis*. Real-time detection of *TEF1* and *ENA* amplicons was carried out using the QuantiTect Sybr green PCR kit (Qiagen, West Sussex, UK) and the ABI 7700 sequence detector. *ENA* gene expression levels were normalized against the expression levels of the constitutively expressed *TEF1* gene in the same cDNA sample.

Hog1 phosphorylation assays

Hog1 phosphorylation was examined by Western blotting (New England Biolabs) as described previously (Smith *et al.*, 2004). Western blots were probed with an anti-phospho p38 antibody, which only recognizes the phosphorylated, active

form of *C. albicans* and *C. dubliniensis* Hog1 (Hog1-P). Total levels of Hog1 protein were determined by stripping and reprobing the blot with an anti-Hog1 antibody (Santa Cruz Biotechnology, Sanata Cruz, CA) that recognizes both phosphorylated and unphosphorylated forms of Hog1.

Virulence assays

Immunocompetent 6- to 8-week-old female BALB/c mice (Harlan Sera-lab, Loughborough, UK) were challenged intravenously with *C. dubliniensis* cells grown with shaking for 18–24 h at 30°C in NGY medium [0.1% (w/v) Neopeptone, 0.4% (w/v) glucose and 0.1% (w/v) Yeast Extract]. Cells were harvested, washed twice, and then resuspended in physiological saline. Groups of five or six mice were inoculated via the lateral tail vein with 1.7×10^5 cfu g⁻¹ body weight (MacCallum and Odds, 2005). Mice were monitored over 28 days and animals showing signs of distress or illness were humanely terminated and deaths recorded as occurring the following day. Kidneys, liver and brain were removed aseptically post mortem, homogenized in 0.5 ml of water, and *C. dubliniensis* tissue burdens determined by viable counting. Organ burdens were compared by Mann–Whitney *U*-test. All experimentation was carried out under the terms of the UK Home Office licenses for research on animals.

Acknowledgements

We thank Dr Federico Navarro-Garcia, of the Universidad Complutense de Madrid for the gift of the *C. albicans* genomic library. B.E. and A.J.B.P. were funded by the European Commission (MRTN-CT-2003–504148) and the Wellcome Trust (080088). A.J.B.P. was also funded by the BBSRC (BB/FO0513X/1). G.P.M., C.V., T.Y., D.C. and D.S. were funded by the Irish Health Research Board (HRB; RP08/2000 and RP/2004/235) and Science Foundation Ireland (SFI; 03IN3B463). J.Q. was supported by the BBSRC (BB/C510383/1).

References

- Alonso-Monge, R., Navarro-Garcia, F., Molero, G., Diez-Orejas, R., Gustin, M., Pla, J., *et al.* (1999) Role of the mitogen-activated protein kinase Hog1p in morphogenesis and virulence of *Candida albicans*. *J Bacteriol* **181**: 3058–3068.
- Alves, S.H., Milan, E.P., de Laet Sant'Ana, P., Oliveira, L.O., Santurio, J.M., and Colombo, A.L. (2002) Hypertonic sabouraud broth as a simple and powerful test for *Candida dubliniensis* screening. *Diagn Microbiol Infect Dis* **43**: 85–86.
- Boeckstaens, M., Andre, B., and Marini, A.M. (2007) The yeast ammonium transport protein Mep2 and its positive regulator, the Npr1 kinase, play an important role in normal and pseudohyphal growth on various nitrogen media through retrieval of excreted ammonium. *Mol Microbiol* **64**: 534–546.
- Chen, D., Toone, W.M., Mata, J., Lyne, R., Burns, G., Kivinen, K., *et al.* (2003) Global transcriptional responses of fission yeast to environmental stress. *Mol Biol Cell* **14**: 214–229.
- Dabas, N., and Morschhäuser, J. (2007) Control of ammonium permease expression and filamentous growth by the GATA transcription factors GLN3 and GAT1 in *Candida albicans*. *Eukaryot Cell* **6**: 875–888.
- Enjalbert, B., Nantel, A., and Whiteway, M. (2003) Stress-induced gene expression in *Candida albicans*: absence of a general stress response. *Mol Biol Cell* **14**: 1460–1467.
- Enjalbert, B., Smith, D.A., Cornell, M.J., Alam, I., Nicholls, S., Brown, A.J.P., and Quinn, J. (2006) Role of the Hog1 stress-activated protein kinase in the global transcriptional response to stress in the fungal pathogen *Candida albicans*. *Mol Biol Cell* **17**: 1018–1032.
- Ferreira, C., van Voorst, F., Martins, A., Neves, L., Oliveira, R., Kiehlbrandt, M.C., Lucas, C., and Brandt, A. (2005) A member of the sugar transporter family, Stilp is the glycerol/H⁺ symporter in *Saccharomyces cerevisiae*. *Mol Biol Cell* **16**: 2068–2076.
- Fradin, C., De Groot, P., MacCallum, D., Schaller, M., Klis, F., Odds, F.C., and Hube, B. (2005) Granulocytes govern the transcriptional response, morphology and proliferation of *Candida albicans* in human blood. *Mol Microbiol* **56**: 397–415.
- Garciadeblas, B., Rubio, F., Quintero, F.J., Bañuelos, M.A., Haro, R., and Rodríguez-Navarro, A. (1993) Differential expression of two genes encoding isoforms of the ATPase involved in sodium efflux in *Saccharomyces cerevisiae*. *Mol Gen Genet* **236**: 363–368.
- Gasch, A.P., Spellman, P.T., Kao, C.M., Carmel-Harel, O., Eisen, M.B., Storz, G., *et al.* (2000) Genomic expression programs in the response of yeast cells to environmental changes. *Mol Biol Cell* **11**: 4241–4257.
- Gillfillan, G.D., Sullivan, D.J., Haynes, K., Parkinson, T., Coleman, D.C., and Gow, N.A.R. (1998) *Candida dubliniensis*: phylogeny and putative virulence factors. *Microbiology* **144**: 829–838.
- Gillum, A.M., Tsay, E.Y., and Kirsch, D.R. (1984) Isolation of the *Candida albicans* gene for orotidine-5'-phosphate decarboxylase by complementation of *S. cerevisiae* *ura3* and *E. coli* *pyrF* mutations. *Mol General Genet* **198**: 179–182.
- Green, C.B., Zhao, X., Yeater, K.M., and Hoyer, L.L. (2005) Construction and real-time RT-PCR validation of *Candida albicans* PALS-GFP reporter strains and their use in flow cytometry analysis of *als* gene expression in budding and filamenting cells. *Microbiology* **151**: 1051–1060.
- Hauser, N.C., Vingron, M., Scheideler, M., Krems, B., Hellmuth, K., Entian, K.D., and Hoheisel, J.D. (1998) Transcriptional profiling on all open reading frames of *Saccharomyces cerevisiae*. *Yeast* **14**: 1209–1221.
- Hwang, C.S., Rhie, G., Kim, S.T., Kim, Y.R., Huh, W.K., Baek, Y.U., and Kang, S.O. (1999) Copper- and zinc-containing superoxide dismutase and its gene from *Candida albicans*. *Biochim Biophys Acta* **1427**: 245–255.
- Kibbler, C.C., Ainscough, S., Barnes, R.A., Gransden, W.R., Holliman, R.E., Johnson, E.M., *et al.* (2003) Management and outcome of blood stream infections due to *Candida* species in England and Wales. *J Hosp Infect* **54**: 18–24.
- Krauke, Y., and Sychrova, H. (2008) Functional comparison of plasma-membrane Na⁺/H⁺ antiporters from two pathogenic *Candida* species. *BMC Microbiol* **8**: 80.

- Lorenz, M.C., Bender, J.A., and Fink, G.R. (2004) Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot Cell* **3**: 1076–1087.
- MacCallum, D., and Odds, F.C. (2005) Temporal events in the intravenous challenge model for experimental *Candida albicans* infections in female mice. *Mycoses* **48**: 151–161.
- Moran, G., Stokes, C., Thewes, S., Hube, B., Coleman, D.C., and Sullivan, D. (2004) Comparative genomics using *Candida albicans* DNA microarrays reveals absence and divergence of virulence-associated genes in *Candida dubliniensis*. *Microbiology* **150**: 3363–3382.
- Moran, G.P., MacCallum, D.M., Spiering, M.J., Coleman, D.C., and Sullivan, D.J. (2007) Differential regulation of the transcriptional repressor Nrg1 accounts for altered host cell interactions in *Candida albicans* and *Candida dubliniensis*. *Mol Microbiol* **66**: 915–929.
- Morschhäuser, J., Ruhnke, M., Michel, S., and Hacker, J. (1999) Identification of CARE-2-negative *Candida albicans* isolates as *Candida dubliniensis*. *Mycoses* **42**: 29–32.
- Nicholls, S., Straffon, M., Enjalbert, B., Nantel, A., Macaskill, S., Whiteway, M., and Brown, A.J.P. (2004) Msn2- and Msn4-like transcription factors play no obvious roles in the stress responses of the fungal pathogen *Candida albicans*. *Eukaryot Cell* **3**: 1111–1123.
- Odds, F.C. (1988) *Candida and Candidosis: A Review and Bibliography*. London: Baillière Tindall.
- Odds, F.C., Hanson, M.F., Davidson, A.D., Jacobsen, M.D., Wright, P., Whyte, J.A., *et al.* (2007) One year prospective survey of *Candida* bloodstream infections in Scotland. *J Med Microbiol* **56**: 1066–1075.
- Pinjon, E., Sullivan, D., Salkin, I., Shanley, D., and Coleman, D. (1998) Simple, inexpensive, reliable method for differentiation of *Candida dubliniensis* from *Candida albicans*. *J Clin Microbiol* **36**: 2093–2095.
- Pla, J., Perez-Diaz, R.M., Navarro-Garcia, F., Sanchez, M., and Nombela, C. (1995) Cloning of the *Candida albicans* HIS1 gene by direct complementation of a *C. albicans* histidine auxotroph using an improved double-ARS shuttle vector. *Gene* **165**: 115–120.
- Quinn, J., Findlay, V.J., Dawson, K., Millar, J.B., Jones, N., Morgan, B.A., and Toone, W.M. (2002) Distinct regulatory proteins control the graded transcriptional response to increasing H₂O₂ levels in fission yeast *Schizosaccharomyces pombe*. *Mol Biol Cell* **13**: 805–816.
- Ramsdale, M., Selway, L., Stead, D., Walker, J., Yin, Z., Nicholls, S.M., *et al.* (2008) Mnl1 regulates weak acid-induced stress responses of the fungal pathogen *Candida albicans*. *Mol Biol Cell* **19**: 4393–4403.
- Sherman, F. (1991) Getting started with yeast. *Methods Enzymol* **193**: 3–21.
- Smith, D.A., Nicholls, S., Morgan, B.A., Brown, A.J.P., and Quinn, J. (2004) A conserved stress-activated protein kinase regulates a core stress response in the human pathogen *Candida albicans*. *Mol Biol Cell* **15**: 4179–4190.
- Staib, P., Moran, G.P., Sullivan, D.J., Coleman, D.C., and Morschhäuser, J. (2001) Isogenic strain construction and gene targeting in *Candida dubliniensis*. *J Bacteriol* **183**: 2859–2865.
- Stokes, C., Moran, G.P., Spiering, M.J., Cole, G.T., Coleman, D.C., and Sullivan, D.J. (2007) Lower filamentation rates of *Candida dubliniensis* contribute to its lower virulence in comparison with *Candida albicans*. *Fungal Genet Biol* **44**: 920–931.
- Sullivan, D.J., Westerneng, T.J., Haynes, K.A., Bennett, D.E., and Coleman, D.C. (1995) *Candida dubliniensis* sp. nov.: phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals. *Microbiology* **141**: 1507–1521.
- Sullivan, D.J., Moran, G.P., Pinjon, E., Al-Mosaied, A., Stokes, C., Vaughan, C., and Coleman, D.C. (2004) Comparison of the epidemiology, drug resistance mechanisms, and virulence of *Candida dubliniensis* and *Candida albicans*. *FEMS Yeast Res* **4**: 369–376.
- Tournu, H., Tripathi, G., Bertram, G., Macaskill, S., Mavor, A., Walker, L., *et al.* (2005) Global role of the protein kinase, Gcn2, in the human pathogen, *Candida albicans*. *Eukaryot Cell* **4**: 1687–1696.
- Tusher, V.G., Tibshirani, R., and Chu, G. (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* **98**: 5116–5121.
- Vilela, M.M., Kamei, K., Sano, A., Tanaka, R., Uno, J., Takahashi, I., *et al.* (2002) Pathogenicity and virulence of *Candida dubliniensis*: comparison with *C. albicans*. *Med Mycol* **40**: 249–257.
- Walker, L.A., MacCallum, D.M., Bertram, G., Gow, N.A.R., Odds, F.C., and Brown, A.J.P. (2008) Genome-wide analysis of *Candida albicans* gene expression patterns during infection of the mammalian kidney. *Fungal Genet Biol* **46**: 210–219.
- Wysong, D.R., Christin, L., Sugar, A.M., Robbins, P.W., and Diamond, R.D. (1998) Cloning and sequencing of a *Candida albicans* catalase gene and effects of disruption of this gene. *Infect Immun* **66**: 1953–1961.