In the generally nonpathogenic yeast *Saccharomyces cerevisiae*, two transcription factors, Ace2p and Swi5p, with 37% identity, regulate the expression of several genes during the late M1 and early G1 stages of the mitotic cell cycle (5, 17). Targets of Ace2p only are predominantly expressed in the daughter cell, and they include chitinase (encoded by *CTSI*) and other proteins required for separation from the mother cell (4, 5, 23). Deletion of *ACE2* results in increased pseudohyphal growth and invasion of agar (11, 16). Swi5p alone regulates the expression of the HO endonuclease, which initiates mating-type switching (5, 15).

Ace2p and Swi5p are both conserved in species closely related to *S. cerevisiae*, including the opportunistic pathogenic yeast *Candida glabrata* (6). The *Candida albicans* genome, however, contains only a single gene orthologue (*C. albicans* *ACE2*), whose product is equidistant from both *S. cerevisiae* proteins (10). Deletion of *ACE2* from *C. albicans* results in gross attenuation of virulence of the fungus in a mouse model of disseminated infection (9). The pathogenic *C. glabrata* *ACE2* mutant did not lead to terminal illness but generated significantly larger brain and lung burdens than those in controls. The *C. albicans* *ace2* null mutant was very slightly attenuated and the *S. cerevisiae* *ace2* and *swi5* null mutants were substantially attenuated relative to their parental control strains. The phenotype of aggressive hypervirulence, unique to disruption of the *C. glabrata* *ACE2* gene among the strains tested, was not seen when the *C. glabrata* *ace2* strain was tested in immunologically intact mice. The different effects seen with these mutants rule out the clumping phenotype as the explanation for hypervirulence in the *C. glabrata* *ace2* mutant. The absence of *C. glabrata* *ace2* hypervirulence in healthy mice may be a tool for definitive future study of host-parasite cross talk in microbial opportunism.

Different Consequences of *ACE2* and *SWI5* Gene Disruptions for Virulence of Pathogenic and Nonpathogenic Yeasts

Donna M. MacCallum,1 Helen Findon,2 Claire C. Kenny,3 Geraldine Butler,3 Ken Haynes,2 and Frank C. Odds1*

Aberdeen Fungal Group, School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Aberdeen,1 and Department of Infectious Diseases, Imperial College London, Du Cane Road, London,2 United Kingdom, and School of Biomolecular and Biomedical Research, Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland3

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Mutants of *Candida albicans*, *Candida glabrata*, and *Saccharomyces cerevisiae* with disruptions in the *ACE2* gene and *C. glabrata* and *S. cerevisiae* *swi5* disruption mutants were tested for virulence in a murine challenge model of disseminated yeast infection. All mutants showed a clumping phenotype, but clumping was minimized in challenge inocula by inclusion of chitinase in the growth medium. In animals rendered temporarily neutropenic by cyclophosphamide treatment, the *C. glabrata* *ace2* null mutant was confirmed as hypervirulent: it led to early terminal illness and kidney, brain, and lung fungal burdens substantially and significantly larger than those in controls. The *C. glabrata* *swi5* null mutant did not lead to terminal illness but generated significantly larger brain and lung burdens than those in controls. The *C. albicans* *ace2* null mutant was very slightly attenuated and the *S. cerevisiae* *ace2* and *swi5* null mutants were substantially attenuated relative to their parental control strains. The phenotype of aggressive hypervirulence, unique to disruption of the *C. glabrata* *ACE2* gene among the strains tested, was not seen when the *C. glabrata* *ace2* strain was tested in immunologically intact mice. The different effects seen with these mutants rule out the clumping phenotype as the explanation for hypervirulence in the *C. glabrata* *ace2* mutant. The absence of *C. glabrata* *ace2* hypervirulence in healthy mice may be a tool for definitive future study of host-parasite cross talk in microbial opportunism.
TABLE 1. Oligonucleotide primers used for C. albicans and C. glabrata deletions in *S. cerevisiae*.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMDACE2-F</td>
<td>ATGAGACGTAGCTCATGTC</td>
</tr>
<tr>
<td>BMDACE2-R</td>
<td>TCGGAGATCTGCTGTTC</td>
</tr>
<tr>
<td>ACE2-TEST-F</td>
<td>TCCAGTACGACATGCTCATCTTC</td>
</tr>
<tr>
<td>ACE2-TEST-R</td>
<td>GCACCCCTCTCTGCTCCTCA</td>
</tr>
<tr>
<td>KAN-1</td>
<td>GCGGTGGAGCGAGACGA</td>
</tr>
<tr>
<td>KOS-1</td>
<td>ACAATATAGTACACCATATACCTGTCTT</td>
</tr>
<tr>
<td>KOS-2</td>
<td>GGGAGTACGCTGAGCTGCCTGAC</td>
</tr>
<tr>
<td>ACE2-TEST-F</td>
<td>AAATGGTCTACTAATATAATTCTACATAGTA</td>
</tr>
<tr>
<td>ACE2-TEST-R</td>
<td>TACATAAGCCATAGGCACTAGTCGATCTG</td>
</tr>
<tr>
<td>ACE2-TEST-F</td>
<td>AGAGGCAATCAAATATACGAG</td>
</tr>
<tr>
<td>ACE2-TEST-R</td>
<td>CATAACCTGGTCGTTACAGTCC</td>
</tr>
</tbody>
</table>

* Underlined sequences were derived from the pF66-kanMX2 multiple cloning site or from the *C. glabrata* HIS3 gene.

To produce challenge yeast inocula free of cell clumps for animal experiments, two alternative approaches were investigated. For the first approach, the test strains were grown for 18 h in NGY at 30°C with constant rotation at 20 rpm. Yeast cells were centrifuged at 2,500 × g for 5 min, resuspended in sterile saline, and then treated with chitinase (Sigma, Poole, Dorset, United Kingdom) (9). For the second approach, chitinase was included at 2 U/ml in the NGY medium for the duration of growth.

**Construction of ACE2 and SWI5 null mutants of C. cerevisiae.** A 1.3-kb fragment from the MannX gene cassette was amplified from pF66-kanMX2 (21), using primers BMDACE2-F and BMDACE2-R for ACE2 and KOS-1 and KOS-2 for SWI5 (Table 1). Both primer pairs were designed to remove the entire open reading frame of ACE2 or SWI5. The deletion cassettes were transformed into *S. cerevisiae* strain YJM145 (13) by the TRAFO method (7).

Disruption of *ACE2* or *SWI5* in confirmed G418-resistant transformants was achieved with diagnostic PCR, using the primers ACE2-TEST-F, ACE2-TEST-R, and KAN-2 and KOS-2 for ACE2 (Table 1), and ACE2-TEST-F for SWI5, KOS-1 for SWI5 (Table 2), and KOS-2 and KOS-3 for SWI5 (2), respectively. To disrupt both alleles of *ACE2* or *SWI5* in the diploid *S. cerevisiae* isolate YJM145, suitable heterozygous deletion transformants were induced to sporulate in SPO1 buffer at 25°C for 4 to 6 days. Tetrads were dissected with the aid of a Singer MSM microscope. Since YJM145 is a homothallic isolate, haploid spores spontaneously undergo mating-type switching and immediately form diploids. Heterozygous deletions (derived from two of four tetrads) were verified by PCR as described above. Single representative *ACE2* (BMEY-5) and *SWI5* (YCK2) knockout mutants were chosen for subsequent analysis.

**Construction of SWI5 null mutant of C. glabrata.** The *C. glabrata* HIS3 gene was amplified using primers SWI5-HIS-F and SWI5-HIS-R. These primers include 60 bp of *SWI5* flanking sequences and were designed to delete the entire open reading frame. The purified deletion cassette was used to transform *C. glabrata* ΔH1 (22). Thirty histidine prototrophs were selected and analyzed by diagnostic PCR. Primers SWI5-F and SWI5-R were used to distinguish *SWI5* wild-type cells (generating a PCR product of 2.4 kb) from *swi5A* mutant cells (generating a PCR product of 1.5 kb). The primer combination of SWI5-F and HIS-T (generating a product of 1.3 kb) confirmed that the *SWI5* locus was replaced with HIS3. Three colonies were detected that had undergone the desired recombination event. One was selected and named MK1-1.

**Mouse model.** All animal experimentation was done in accordance with UK Home Office regulations and was approved by both the Home Office and an institutional ethical review committee. Female BALB/c mice (Harlan, United Kingdom) with a weight range from 17 to 23 g were maintained in groups of up to 12 animals per cage. The mice were supplied with food and water ad libitum. To induce temporary neutropenia, mice were injected intraperitoneally with cyclophosphamide at 150 mg/kg of body weight 3 days before challenge and on the day of challenge. The mice were injected intraperitoneally with cyclophosphamide at 150 mg/kg of body weight 3 days before challenge and on the day of challenge.

**RESULTS**

**Influence of chitinase treatment on infection outcomes.** No significant differences were found in survival curves or mean kidney fungal burdens determined for groups of six cyclophosphamide-treated mice challenged with *C. glabrata* suspensions treated with chitinase after growth or grown with chitinase added to the medium (Table 2). Microscopic examination of challenge inocula of both *Candida* species showed no difference in appearance of the cells: both methods of chitinase treatment effectively reduced cell clumps to pairs or occasional tetrad. For all subsequent experiments, inocula were grown in the presence of chitinase.

**Virulence for mice of ace2 and swi5 mutants of C. albicans, C. glabrata, and S. cerevisiae.** Temporarily neutropenic mice infected with *C. albicans* CAI-4 containing C1p10 survived with a similar curve to that for mice infected with the *ace2* null strain MK106. The small difference was statistically significant by log rank analysis (*P < 0.001*) (Fig. 1a) but was the consequence of prolonged survival of a single animal. The difference in mean survival times (Table 3) was only 4 days. Tissue burdens of viable *C. albicans* (Table 3) for mice challenged with the two strains indicated that mean kidney and lung burdens were slightly but significantly (*P < 0.05*) smaller in animals infected with the *ace2* null mutant than in mice infected with the CAI-4 control. In terms of both survival and two of three sets of tissue burden data, the *ace2* null mutant, given at the same level of challenge as its parental strain, showed a very minor though statistically significant level of virulence attenuation. The significant differences remained even when data for the MK106-infected mouse were excluded from analysis.
control strain (HLS121) survived until 28 days after challenge, unlike mice infected with the ace2 null strain (HLS122), which all died by day 4 (Fig. 1b) \((P < 0.001\) versus control), even though the challenge dose was 10 times lower than that of the control strain, thus confirming the hypervirulence of this mutant. All mice challenged with the C. glabrata swi5 null strain (CK1-1) at the same dose as that of HLS122 survived for 28 days after challenge, essentially the same result as that for the control strain (Fig. 1b). Data for tissue burdens of C. glabrata (Table 3) confirmed the hypervirulence of the ace2 null mutant, which was already evident from the survival rates (Fig. 1b). For HLS122, the mean fungal burdens in kidneys \((P = 0.002)\), brains \((P < 0.001)\), and lungs \((P < 0.001)\) were all significantly larger than those of the control strain HLS121, even though the challenge dose was approximately 10 times lower. These results paralleled the short survival times for mice challenged with this strain. For the swi5 mutant, also given as a challenge at a 10-fold lower dose than that of the control strain, the mean burdens in brains and lungs were significantly larger than those of controls \((P < 0.001)\), while the mean kidney burden was not significantly different from that of controls. The C. glabrata swi5 null mutant’s mean brain and lung burdens were also significantly lower than the corresponding means for the ace2 null mutant.

Groups of mice challenged intravenously with \(1.0 \times 10^5\) to \(1.6 \times 10^5\) yeast cells of wild-type S. cerevisiae (pathogenic isolate YJM145) or the ace2 (BMEY5) or swi5 (YCK2) null mutants all survived to day 28 and had negative cultures for kidney, brain, and lung burdens at postmortem (details not shown). With a higher challenge dose of \(4.2 \times 10^5\) yeast cells/g, S. cerevisiae YJM145 led to fatal disease in 50% of a group of six mice (Fig. 1c), while five of six mice survived the same dose of the ace2 and swi5 null mutants (Fig. 1c). These survival differences were significant by the log rank test \((P = 0.039)\). None of the tissue burden differences between S. cerevisiae control and mutant strains reached statistical significance, even though mean kidney and brain burdens were >10-fold smaller in animals challenged with the swi5 null mutant than in controls.

**Effects of C. albicans and C. glabrata ace2 null mutants in immunologically normal BALB/c mice.** Because our previous work with the C. albicans ace2 null mutant was done with DBA/2 mice, not the BALB/c mouse strain used in the present study, we challenged six unmodified BALB/c mice with the MK106 (ace2 null) mutant grown in the presence of chitinase at a dose of \(1.9 \times 10^5\) CFU/g. All mice survived to 28 days

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**TABLE 3. Summary of virulence parameters for temporarily neutropenic mice challenged intravenously with three fungal species and their ace2 or swi5 null mutants**

<table>
<thead>
<tr>
<th>Fungal strain</th>
<th>Challenge dose (CFU/g body wt)</th>
<th>No. of animals tested</th>
<th>Mean survival time (days) ± SD</th>
<th>Mean burden (log CFU/g) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Kidneys</td>
</tr>
<tr>
<td>C. albicans CAI-4 + Ctp10</td>
<td>(1.5 \times 10^4)</td>
<td>12</td>
<td>2.1 ± 0.7</td>
<td>6.7 ± 0.3</td>
</tr>
<tr>
<td>C. albicans MK106 (ace2 null)</td>
<td>(1.6 \times 10^4)</td>
<td>12</td>
<td>6.0 ± 0.7</td>
<td>6.0 ± 0.1</td>
</tr>
<tr>
<td>C. glabrata HLS121 (control)</td>
<td>(1.2 \times 10^6)</td>
<td>12</td>
<td>27.9 ± 0.3</td>
<td>3.4 ± 1.1</td>
</tr>
<tr>
<td>C. glabrata HS122 (ace2 null)</td>
<td>(1.6 \times 10^5)</td>
<td>12</td>
<td>3.0 ± 0.6</td>
<td>4.8 ± 0.4</td>
</tr>
<tr>
<td>C. glabrata CK1-1 (swi5 null)</td>
<td>(1.3 \times 10^5)</td>
<td>12</td>
<td>28.0 ± 0.0</td>
<td>4.0 ± 0.7</td>
</tr>
<tr>
<td>S. cerevisiae YJM145 (parent)</td>
<td>(4.2 \times 10^5)</td>
<td>6</td>
<td>9.7 ± 9.5</td>
<td>5.5 ± 1.9</td>
</tr>
<tr>
<td>S. cerevisiae BMEY-5 (ace2 null)</td>
<td>(4.2 \times 10^5)</td>
<td>6</td>
<td>23.8 ± 10.2</td>
<td>5.0 ± 1.8</td>
</tr>
<tr>
<td>S. cerevisiae YCK2 (swi5 null)</td>
<td>(4.2 \times 10^5)</td>
<td>6</td>
<td>23.8 ± 10.2</td>
<td>3.4 ± 1.2</td>
</tr>
</tbody>
</table>
postchallenge. Five of six kidney samples and all six brain samples were negative for *C. albicans* at postmortem examination. These data confirm the attenuation of the *ace2* mutant seen in DBA/2 mice.

We challenged three pairs of healthy BALB/c mice with the *C. glabrata* *ace2* and *swi5* null mutants at doses of $3 \times 10^5$, $3 \times 10^6$, and $3 \times 10^7$ CFU/g. At the two lowest doses of the *ace2* null mutant, all mice survived to day 28. At $3 \times 10^7$ CFU/g, one of the mice was terminated 19 days after challenge, and the other survived to day 28. Kidneys were negative for *C. glabrata* for both animals challenged with $3 \times 10^5$ CFU/g; one animal challenged with $3 \times 10^7$ CFU/g had a positive kidney result, with $4.7 \log$ CFU/g, and the mean kidney burden ($\pm$ standard deviation) for the two mice challenged with $3 \times 10^6$ CFU/g was $4.5 \pm 0.1 \log$ CFU/g. All animals survived to day 28 with all three doses of the *swi5* null mutant. The mean kidney burdens for the pairs of mice were $3.5 \pm 0.1$, $3.9 \pm 0.1$, and $4.0 \pm 0.4 \log$ CFU/g for doses of $3 \times 10^5$, $3 \times 10^6$, and $3 \times 10^7$ CFU/g, respectively. No evidence suggestive of hypervirulence was obtained for either mutant in these experiments with immunologically intact mice.

**DISCUSSION**

We undertook this study to determine if deletion of *ACE2* or *SWI5* from *S. cerevisiae* leads to hypervirulence in the immunocompromised mouse model, in a manner analogous to that after deletion of *ACE2* from *C. glabrata* (9), since these two fungal species are quite closely related and *C. glabrata* may have lost its mating ability relatively recently (2). However, the results of our experiments show that the consequence of *ACE2* and *SWI5* deletion in *S. cerevisiae* is a reduction in mouse virulence from the already low virulence of the parental strain. We used a segregant from a clinical isolate of *S. cerevisiae* (YJM145) that was previously shown to colonize the organs of infected mice (8). The *ACE2* and *SWI5* genes were replaced with dominant drug resistance markers that have no effect on virulence (8). Our *S. cerevisiae* challenge dose was more than twice as high as that used with the hypervirulent *ace2* null mutant of *C. glabrata* and approximately 30 times higher than the challenge dose used for *C. albicans* strains, so it is unlikely that any change in the direction of hypervirulence in the *S. cerevisiae* *ace2* and *swi5* null mutants would have been missed in our experiments.

The results for the *swi5* null strain of *C. glabrata* make an interesting comparison with those for the *C. glabrata* *ace2* null strain. Only the latter demonstrated hypervirulence by causing severe illness in infected mice that appeared as reduced survival. The mean brain and lung burdens of the *C. glabrata* *swi5* mutant at postmortem were substantially (1.5 log) and significantly ($P < 0.001$) larger than those in mice challenged with a 10 times higher dose of the *C. glabrata* control strain, but kidney burdens did not differ (Table 3). These mean burdens were also smaller, at similar orders of magnitude and the same statistical significance, than the brain and lung burdens in mice challenged at the same level with the *ace2* null mutant. By these criteria, the *swi5* null mutant has only moderate virulence effects in temporarily neutropenic mice. The terminal illness resulting from *C. albicans* infection is due to the development of sepsis, with kidney pathology being a primary contributor to terminal events and the kidney burden being directly correlated with renal failure (18). From our data, it seems reasonable to conclude that the aggressive hypervirulence of the *C. glabrata* *ace2* null mutant results from the development of large kidney burdens combined with unusually large burdens in the brain. The contribution of lung burdens to gross symptoms may be less important or negligible, since the *S. cerevisiae* strains tested also achieved large postmortem lung burdens without leading to overt symptomatology. Compared with the control strain, the *C. glabrata* *swi5* null mutant generates high brain burdens but does not achieve renal dysfunction sufficient to lead to terminal sepsis. Since the *ace2* and *swi5* null mutants of all three fungal species have a clumping phenotype, yet obvious differences were found in the pathological consequences of their infections, our data support the interpretation that the *C. glabrata* *ace2* mutant’s increased virulence is not due to the clumpy growth phenotype but rather is a specific result of some other attribute associated with *ACE2* inactivation.

Our aim was to compare the virulence of *ace2* and *swi5* null mutants of three fungal species under conditions that were as identical as possible. This included preparing all challenge inocula identically, with chitinase included in the growth medium to minimize the clumping phenotype of the mutants, and conducting all tests in temporarily neutropenic mice. We adjusted challenge doses species by species: the wild-type strains of *C. glabrata* and *S. cerevisiae* are inherently less virulent than the *C. albicans* wild type, so identical challenge inocula were not possible in the experimental design if we wished to detect possible attenuation in the mutants of the less virulent species. The results of the comparison show that in the immunosuppressed mouse host, the *C. albicans* *ace2* null mutant is slightly attenuated in virulence, the *C. glabrata* *ace2* null mutant is notably hypervirulent, the *C. glabrata* *swi5* null mutant gives larger lung and brain burdens than those of the control, but without consequences for survival, and the *S. cerevisiae* *ace2* and *swi5* null mutants are attenuated.

It is perhaps worth emphasizing that challenges with all three strains of *S. cerevisiae* in the immunosuppressed animals led to lung burdens that were of the same order of magnitude as those achieved by the hypervirulent *C. glabrata* *ace2* null mutant (Table 2) and larger than the burdens seen with either of the *C. albicans* strains tested. It is unclear why cyclophosphamide-treated mice should be less effective at removing *C. glabrata* and *S. cerevisiae* from the lungs than they are with *C. albicans*. The effect might be a technical artifact—a consequence of *C. albicans* forming hyphae in the lungs which artificially reduce viable counts or of CFU counts being affected by the clumping phenotype—and quantitative PCR may be needed to obtain a more accurate estimate of pulmonary fungal burdens.

The *C. albicans* *ace2* null mutant was previously shown to be highly attenuated in mouse virulence compared with its parent (10). In the previous study, immunologically unaltered DBA/2 mice were used as hosts. This mouse strain is inherently more susceptible to *C. albicans* challenge than are the BALB/c mice used in the present experiments (12), so we retested the mutant at an appropriate intravenous challenge dose in immunologically normal BALB/c mice. The data replicated the nearly total attenuation of the null mutant in an intact host. However, in our main experiments, where the parent and null mutant
strains of three fungal species were compared in mice rendered neutropenic at the time of challenge, only a low degree of attenuation was found with the C. albicans ace2 null construct (Fig. 1a; Table 3), emphasizing the importance of host status in susceptibility to C. albicans challenge. Our tests with the C. glabrata ace2 and swi5 mutants in a small number of immunologically unaltered mice gave no evidence of the hypervirulence phenomenon. We concluded that the positive and negative virulence differences seen in challenge experiments of the type we have described are very highly influenced by the host immune status. The differences we have found in this study provide a strong basis for detailed investigation of the differences in immune recognition of and response to C. glabrata strains.

The observation that hypervirulence was seen only in temporarily neutropenic hosts is particularly notable in the context of studies already done with the C. glabrata ace2 mutant. A search for gene products that might account for hypervirulence revealed more than 60 proteins which are altered significantly in quantity in response to the presence or absence of Ace2p (19). This result confirms the central regulatory role played by Ace2p in the growth and development of C. glabrata, but it also suggests that the hypervirulence noted for the mutant in the immunosuppressed mouse model may result from changes in expression of more than one gene. If transcript profiling in vivo can be achieved with current models, it may become possible to pinpoint the most significant genes that lead to hypervirulence in immunosuppression.

We undertook these experiments in collaboration to establish the comparative virulence of the mutants in a single experimental program. Our individual laboratories work separately with the different species involved and are currently testing individual hypotheses for the virulence differences seen. The very different consequences of ACE2 disruption between C. albicans and C. glabrata show that these species are handled differently by murine immune defenses. The absence of C. glabrata ace2 mutant hypervirulence in normal mice and the relative attenuation of the swi5 mutant in normal and immunosuppressed mice may be tools for definitive future studies of microbial opportunism.

ACKNOWLEDGMENTS

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REFERENCES


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