

Full Length Research Paper

***Candida albicans* TRR1 heterozygotes show increased sensitivity to oxidative stress and decreased pathogenicity**

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The thioredoxin reductase gene *TRR1* has been reported to be overexpressed when *Candida albicans* is exposed to oxidative stress. To elucidate the role of *TRR1* in the response to oxidative stress and in the pathogenicity of *C. albicans*, we attempted to disrupt both alleles of this gene using gene-disruption cassettes containing *LEU2* and *HIS1* as selection markers. The disruption cassettes were transformed into *C. albicans* SN87 (*leu2*, *his1*) to knockout both *TRR1* alleles. Despite the successful creation of heterozygous *TRR1* mutants, several attempts to create homozygous mutants were unsuccessful, indicating that this gene is required for survival. Exposure to different concentrations of hydrogen peroxide indicated that the heterozygotic mutants, NZ1 (*TRR1/trr1*, *LEU2/leu2*) and NZ2 (*TRR1/trr1*, *HIS1/his1*), were more sensitive to oxidative stress, compared with wild-type. Virulence studies carried out in mice revealed that the average survival time of mice infected with wild-type *C. albicans* was 4 days, whereas the average survival time of mice infected with the NZ1 and NZ2 mutants was 17.2 and 16.8 days, respectively. These results indicate that *TRR1* is crucial for *C. albicans* survival and pathogenicity.

Key word: *Candida albicans*, oxidative stress, thioredoxin reductase, gene disruption and pathogenicity.

INTRODUCTION

Candida albicans is an opportunistic fungal pathogen of humans that causes life-threatening infections, especially in individuals with compromised immune systems. It causes diverse symptoms, ranging from irritating superficial infections (especially of the epithelial tissues of the oral and urogenital tracts) to life-threatening disseminated bloodstream infections. The number of reported infections is constantly on the rise, mainly because of increases in the number of immunocompromised individuals due to organ transplants, chemotherapy and the prevalence of AIDS (Pfaller and

Diekema, 2007). Although there are a number of antifungal drugs available for the treatment of *C. albicans* infections (for example, azole drugs such as ketoconazole, fluconazole and itraconazole), the emergence of clinical strains resistant to these drugs has been reported worldwide (Akortha et al., 2009; Abaci and Haliki-Uztan, 2011; Amran et al., 2011; Pfaller et al., 2011). Therefore, the identification of potential new drug targets, as well as the continuous development of new drugs, will be crucial for combating this pathogen in the future.

One of the most important means used by the human body to combat invading microorganisms is phagocytosis by neutrophils and macrophages, which destroy pathogens by exposing them to high doses of reactive oxygen species (ROS) (Missall and Lodge, 2005) that

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can cause fatal oxidative stress. ROS can modify the lipid membrane and proteins and cause mutations in nucleic acids. Hence, to successfully invade their hosts, microbes must be able to overcome the threat of ROS. Fungal pathogens have developed several defensive strategies to overcome this threat, including the expression of detoxifying enzymes, such as superoxide dismutases (SODs), catalase and thiol peroxidase, as well as the production of other non-enzymatic scavenging substances, such as melanin, mannitol and trehalose (Misall et al., 2004). Genome-wide transcriptome analysis has revealed that a total of 184 *C. albicans* genes were overexpressed after exposure to oxidative stress (Enjalbert et al., 2006). Among the genes whose functions are known are enzymes used for the detoxification of H₂O₂, such as superoxide dismutase (*SOD2*) and catalase (*CTA1*), as well as enzymes involved in the glutaredoxin (*GPX1* and *GSH1*) and thioredoxin (*TSA1*, *TRX1* and *TRR1*) systems.

The *C. albicans* *TRR1* gene encodes thioredoxin reductase and is responsible for catalyzing the reduction of thioredoxin to its oxidized form. *TRR1* has been studied in several microorganisms, including prokaryotes and eukaryotes. Elevated transcription of thioredoxin reductase in *Staphylococcus aureus* is observed after diamide, menadione, and t-butyl hydroperoxide treatment (Uziel et al., 2004). In *Saccharomyces cerevisiae*, *TRR1* is induced in response to hydrogen peroxide stress (Godon et al., 1998), while its homologs in *Cryptococcus neoformans* were seen to be induced in response to oxidative and nitrosative stress (Missal and Lodge, 2005). Additionally, by replacing the endogenous *TRR1* promoter with an inducible copper-transporter promoter, Missal and Lodge (2005) showed that *TRR1* is essential for viability in *C. neoformans*. In *C. albicans*, in addition to being induced during exposure to hydrogen peroxide, *TRR1* expression is also induced when cells are exposed to whole blood and antibiotics (Enjalbert et al., 2003; Fradin et al., 2003). In addition, *C. albicans* *TRR1* has no human homologs (Abadio et al., 2011) and could therefore be a promising target for new antifungal drugs, if it proves to be important for viability and pathogenicity. Therefore, the aim of this study was to determine the importance of the *TRR1* gene in *C. albicans* pathogenicity by creating and analyzing *trr1* mutant strains.

MATERIALS AND METHODS

Strains, media and growth condition

A clinical isolate of *C. albicans*, strain C288, was used as the DNA source for the *TRR1* gene isolation (Ibrahim et al., 2006). This strain was obtained from the Institute of Medical Research (IMR) in Kuala Lumpur, Malaysia. For use in the *TRR1* gene disruption scheme, *C. albicans* strain SN87 (*leu2*, *his1*) was provided by Suzanne Noble from the University of California, San Francisco, USA (Noble and Johnson, 2005). Yeast cultures were grown on

either yeast potato dextrose (YPD) medium (1% yeast extract, 2% peptone and 2% glucose) or SD medium (2% glucose, 6.78 g/l yeast nitrogen base without amino acids) supplemented with the appropriate amino acids required by the auxotrophic mutants (Noble and Johnson, 2005). The cultures were incubated at 30°C for 24 or 48 h.

Genomic DNA extraction and isolation of the *TRR1* gene

C. albicans wild-type strain C288 was grown in YPD at 30°C in an orbital incubator at 200 rpm for 24 h. Genomic DNA extraction was carried out as described by Hoffman and Winston (1987). The *TRR1* gene was amplified using PCR with the primers *Trr1_nz_F* and *Trr1_nz_R* (Table 1). The primers were designed to amplify the *TRR1* open reading frame as well as approximately 600 bp of sequence upstream of the *TRR1* start codon and the approximately 370 bp of sequence downstream of the stop codon. The amplified product was cloned into the pGEM-T Easy Vector (Promega, USA) and sequenced using the BigDye[®] Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA). The nucleotide sequence obtained was analyzed using BLAST (NCBI) (<http://www.ncbi.nlm.nih.gov/>) and was compared to the *TRR1* amino acid sequences from other fungi using ClustalW (<http://www.ebi.ac.uk/clustalw/>; Larkin et al., 2007) and BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

Deletion of the *TRR1* gene

The fusion Polymerase chain reaction (PCR) strategy used in this study was carried out according to the protocols developed by Noble and Johnson (2005). A list of all of the primers used in this experiment is given in Table 1. An initial round of PCRs was carried out to immediately amplify the upstream (536 bp) and downstream (603 bp) regions of the *TRR1* open reading using primer pairs 1_*trr1* and 3_*trr1* (to generate the 5' flanking region fragment), and 4_*trr1* and 6_*trr1* (to generate the 3' flanking region fragment). The selection markers (*HIS1* and *LEU2*) were amplified from plasmids pSN52 and pSN40, respectively, using the primer pair 2_*leu_his* and 5_*leu_his* (Noble and Johnson, 2005), which contain tails complementary to the primers used to amplify the flanking regions. The flanking sequence and marker fragments were gel purified and subjected to a second fusion-PCR round using the primer pair 1_*trr1* and 6_*trr1*. The fusion PCR product was gel purified prior to transformation. Linear fragments of the gene disruption cassettes were transformed into *C. albicans* SN87 using the lithium acetate transformation method as described by Sanglard et al. (1996).

Verification of positive transformants

Histidine and leucine prototrophs were isolated after 4 days of growth at 30°C and then replica plated on SD agar to detect knockouts. Correct integration of the construct was confirmed by PCR analysis with primers specific to sites inside the gene disruption cassette and to flanking genomic regions (Table 1). Transformants were further analyzed with Southern blotting according to Sambrook and Russel (2006). A total of 20 µg of genomic DNA was digested with 2 µl of *Bgl*II, and the probe used for the hybridization was specific to the *TRR1* coding region.

Sensitivity towards oxidative stress

Wild-type and mutant cells were subjected to different concentrations of hydrogen peroxide to analyze the sensitivity of *C. albicans* towards oxidative stress. To determine the generation

Table 1. PCR primers used in this study.

Sequence # 5'-3'	Name of primers	Function
ATGAACAAGGTAGCAGGAA 3	Trr1_nz_F	Amplification of <i>TRR1</i>
ATAGCCAACCACCGTCTCT	Trr1_nz_R	Amplification of <i>TRR1</i>
GTATGTGTGTGACAAATAAACGAGG	1_trr1	Amplification of <i>TRR1</i> 5' flanking region
cacggcgcgctagcagcggACGAAGGTATGTTGGCTA	3_trr1	Amplification of <i>TRR1</i> 5' flanking region
gtcagcggccgcatccctgcCAATGGGTTATTCTACGC	4_trr1	Amplification of <i>TRR1</i> 3' flanking region
ATAGCCAACCACCGTCTC	6_trr1	Amplification of <i>TRR1</i> 3' flanking region
ccgctgctagcgcgcccgtgACCAGTGTGTGATGGATATCTGC	2_leu_his	Amplification of the <i>LEU2</i> marker from pSN40 and the <i>HIS1</i> marker from pSN52
gcagggatcgggccgctgacAGCTCGGATCCACTAGTAACG	5_leu_his	Amplification of the <i>LEU2</i> marker from pSN40 and the <i>HIS1</i> marker from pSN52
CATCGGCACCCAAAGC	trr1_G	To detect the presence of the <i>LEU2</i> gene cassette in mutants
CTTCATTGCCAGATACCA	trr1_H	To detect the presence of the <i>LEU2</i> gene cassette in mutants
CGTCGCTTCGTCTACTACTG	trr1_I	To detect the presence of the <i>HIS1</i> gene cassette in mutants
GTTGAGGCTTCTTGC	trr1_J	To detect the presence of the <i>HIS1</i> gene cassette in mutants

* Sequences in lowercase correspond to exogenous, complementary sequences introduced into primers 2, 3, 4, and 5 for mutually primed synthesis in the second round of fusion PCR.

time, *C. albicans* SN87, NZ1 and NZ2 cells (at a concentration of 1×10^6 cells/ml) were subcultured in one the following media: YPD medium without H_2O_2 ; YPD supplemented with 0.4 mM H_2O_2 ; and YPD supplemented with 4.0 mM H_2O_2 (Ibrahim et al., 2006). The cells were incubated at 30°C with agitation at 200 rpm. OD_{600} readings were taken every hour to determine the cell growth rates.

To analyze the resistance of cells towards oxidative stress, cells at a density of 1×10^6 cells/ml were subcultured and grown to a mid-log phase in YPD medium. A total of 9 ml of these cultures was aliquoted into Falcon tubes, and H_2O_2 was added to one of the following final concentrations: 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 mM. The cells were exposed to H_2O_2 for 60 min at 30°C, and then 0.1 ml of cells were plated on YPD agar. Colonies were counted after 48 h of incubation at 30°C.

Murine model virulence testing of *C. albicans*

A total of 60 healthy BALB/c mice (female, aged 6 to 8 weeks, weighing 12 to 18g) were infected by tail vein injection with approximately 1×10^5 cells of SN87, NZ1 or NZ2 (each group consisted of 20 mice). Survival was monitored daily over a period of 20 days. The average

survival time of the mice was determined using the Kaplan-Meier method and log-rank tests were carried out using GraphPad Prism 5 software. The handling of the animals was carried out according to the guidelines established by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) (Hara et al., 2009). To quantify the infection of host organs, kidneys and livers were aseptically removed from the mice on the third day after infection, weighed and homogenized in sterile saline, and inoculated on YPD agar. Colonies were counted after incubation at 30°C for 48 h.

Statistical analyses

Statistical analyses were performed using the SAS version 9.2 (SAS Institute Inc., USA) applying the one-way ANOVA test. Means were compared using the Duncan's Multiple Range Test; when the p-value was less than 0.05, the difference was regarded as statistically significant.

RESULTS AND DISCUSSION

Several genes have been identified and they are

important for the survival of *Candida albicans* upon exposure to oxidative stress (Enjalbert et al., 2007). We focused our attention on the *TRR1* gene because it shows an elevated level of expression in response to oxidative stress. Moreover, *TRR1* is specific to fungi (no homolog has been identified in humans) (Abadio et al., 2011); therefore, it could be an ideal candidate target for new antifungal drugs.

In this study, the *TRR1* gene was amplified from the clinical isolate *C. albicans* C288. The *TRR1* gene is 963 bp in length and encodes a protein of 320 amino acids. When comparing the *TRR1* sequence from *C. albicans* C288 to the reference sequence from *C. albicans* SC5314, no differences were found between the two nucleotide sequences. BLAST analyses showed that the deduced amino acid sequence exhibited 96% identity with thioredoxin reductase from *S. cerevisiae* and 87% identity with thioredoxin reductase from *Pichia stipitis*. In addition, the sequence shows a high level of homology

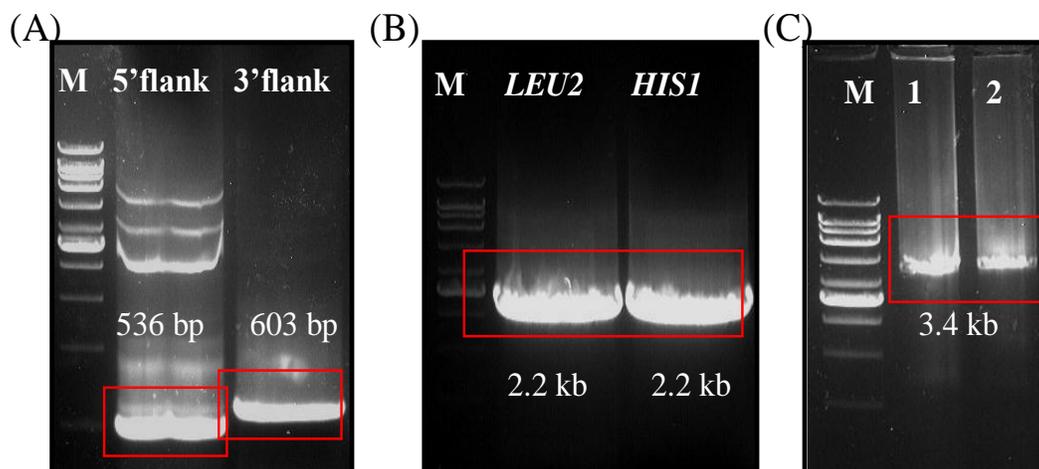


Figure 1. Agarose gel electrophoresis of various PCR products used in the construction of *TRR1* gene disruption cassettes. (A) PCR products of both *TRR1* 5' (536 bp) and 3' flanking (603 bp) regions. (B) Amplification of the *LEU2* (approximately 2.2 kb) and *HIS1* (approximately 2.2 kb) gene cassettes. (C) Lane 1: Fusion PCR products of the gene disruption cassette containing *LEU2* (3.4 kb); Lane 2: fusion PCR product of the gene disruption cassette containing *HIS1* (3.4 kb). Lane M: 1 kb DNA marker (Promega, USA).

to the thioredoxin reductase proteins from other fungal pathogens, ranging from 99% identity with Trr1 from *C. dubliniensis*, to 67% with Trr1 from *Paracoccidioides brasiliensis*, demonstrating a high degree of sequence conservation in fungi. However, when the sequence was BLASTed against the human, mouse and *Caenorhabditis elegans* genomes, no orthologs were identified. Several important domains, including thioredoxin reductase, pyridine nucleotide-disulphide oxidoreductase and FAD-dependent pyridine nucleotide-disulphide oxidoreductase, were detected in *C. albicans* Trr1 when the protein was analyzed using InterProScan.

To disrupt the *TRR1* gene, a fusion PCR strategy developed by Noble and Johnson (2005) was used. Briefly, the regions upstream and downstream of *TRR1* were amplified in an initial round of PCR, along with amplification of the selection markers leucine and histidine (from the pSN40 and pSN52 plasmids, respectively). These fragments were then combined in a second round of fusion-PCR amplification (using primers with complementary linkers) to generate disruption cassettes containing either *LEU2* or *HIS1* (as the selection markers) flanked by the *TRR1* sequences. Using this approach, Noble and Johnson (2005) managed to disrupt a total of three *C. albicans* genes. Since the method was published, this strategy has been adapted by several other groups to disrupt numerous genes in *C. albicans* (Al-Rawi et al., 2010; Lettner et al., 2010).

Using the fusion-PCR technique, disruption cassettes containing either the leucine or histidine selection markers flanked by the *TRR1* upstream and downstream sequences (Figure 1) were created. To accomplish

disruption of the first allele of the *TRR1* gene, the disruption cassette containing the *LEU2* selection marker was transformed into *C. albicans* SN87. Mutants with a disrupted *TRR1* allele were confirmed by PCR and Southern blot analyses. A schematic representation of the wild-type and disrupted alleles of *TRR1* is shown in Figure 2. The disruption of one copy of the *C. albicans* *TRR1* gene was confirmed using PCR with two different sets of primers. As a negative control, PCR amplification using wild-type SN87 genomic deoxyribonucleic acid (DNA) as the template and the primer pairs Trr1_nz_F and trr1_H, and trr1_G and Trr1_nz_R was carried out. These reactions did not yield any products, as the binding site for one of the primers in each pair was located within the disruption cassette (which was absent in the non-transformed strain). However, PCR amplification using heterozygotic mutant DNA as the template and the primers Trr1_nz_F and trr1_H yielded a product of 1113 bp, while the amplification using primers trr1_G and Trr1_nz_R yielded a product of 1147 bp (Figure 2A). These results indicate that the *trr1/LEU2* gene disruption cassette was successfully integrated into one of the *TRR1* alleles, thus generating a *TRR1* heterozygotic mutant (NZ1; *TRR1/trr1: LEU2/leu2*).

Next, we attempted to disrupt the second allele of *TRR1* using the gene disruption cassette with *HIS1* as the selection marker. Although the transformation generated 12 transformants that could survive on SD plates without histidine, none of the transformants produced the expected-sized product when their DNA was subjected to PCR analysis. The transformation was repeated twice, but both procedures failed to knock out the second *TRR1* allele.

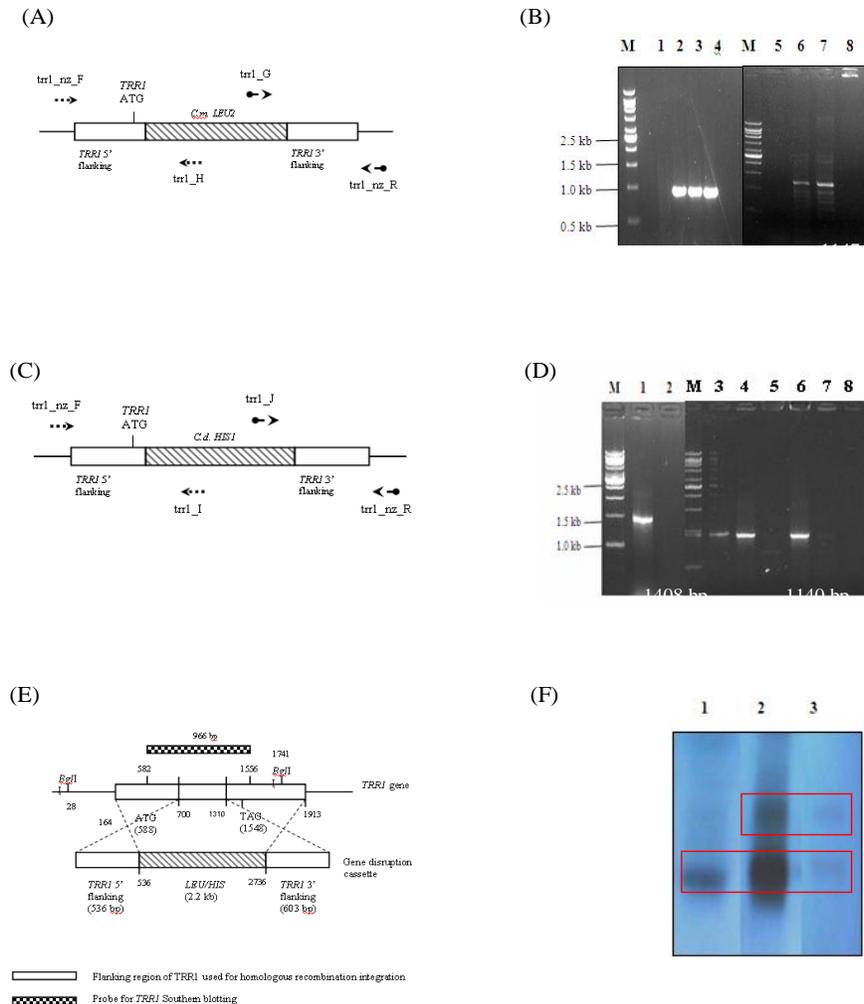


Figure 2. Validation of the *TRR1* gene disruption using PCR and Southern blot analyses. (A) The locations of the primers *Trr1_nz_F*, *trr1_G*, *trr1_H* and *Trr1_nz_R* used for PCR analysis of the integrated *LEU2* disruption cassette transformants. (B) PCR confirmation of the disruption of one copy of the *C. albicans TRR1* by the *LEU2* disruption cassette. Lane M: 1 kb DNA marker (Promega, USA); Lane 1: PCR amplification using wild-type SN87 genomic DNA with the primers *Trr1_nz_F* and *trr1_H* yielded no product; Lanes 2-4: PCR amplifications using genomic DNA from three *TRR1/trr1* mutants using genomic DNA with primers *Trr1_nz_F* and *trr1_H* yielded 1113 bp PCR products; Lane 5: PCR amplification using wild-type SN87 genomic DNA with the primers *trr1_G* and *Trr1_nz_R* yielded no product; Lanes 6 and 7: PCR amplifications using genomic DNA from two *TRR1/trr1* mutants with primers *trr1_G* and *Trr1_nz_R* yielded 1147 bp PCR products; Lane 8: negative control. (C) The locations of the primers *Trr1_nz_F*, *trr1_I*, *trr1_J* and *Trr1_nz_R* used for PCR analysis of the transformants created with the *HIS1* disruption cassette. (D) PCR confirmation of the disruption of one copy of the *C. albicans TRR1* gene with the *HIS1* disruption cassette. Lane M: 1 kb DNA marker; Lane 1: PCR amplification of *TRR1/trr1* using genomic DNA with primers *Trr1_nz_F* and *trr1_I* yielded a 1408 bp PCR product; Lane 2: PCR amplification of the wild-type SN87 genomic DNA using the primers *Trr1_nz_F* and *trr1_I* yielded no product; Lanes 3-7: PCR amplification using genomic DNA from several *TRR1/trr1* mutants with primer pairs *Trr1_nz_F* and *trr1_J* yielded 1140 bp PCR products; Lane 8: PCR amplification using wild-type SN87 genomic DNA with primers *Trr1_nz_R* and *trr1_J* yielded no PCR product. (E) The genomic arrangement of the *TRR1* locus in wild-type strains and in the heterozygous mutant. The location of the probe used in the Southern blot analysis is indicated on the map. (F) Confirmation of the disruption of one copy of the *C. albicans TRR1* gene by Southern blot analysis. Lane 1: SN87 genomic DNA; Lane 2: genomic DNA from NZ1 (*TRR1/trr1*, *LEU2/leu2*); Lane 3: genomic DNA from NZ2 (*TRR1/trr1: HIS1/his1*).

Due to the initial failure in generating a homozygotic *TRR1* mutant, the gene knockout process was repeated again, this time swapping the order of the cassettes used. In this approach, *TRR1* in *C. albicans* SN87 was first disrupted using the disruption cassette containing the *HIS1* selection marker. This was then followed by transformation using the disruption cassette containing *LEU2* as the selection marker. Similarly to the results of the initial attempt, disruption of the first *TRR1* allele using the disruption cassette containing *HIS1* selection marker was successful. PCR with the primers *Trr1_nz_F* and *trr1_I* yielded a product of 1408 bp using the template from transformants harboring the gene disruption cassette, but not from wild-type template (Figure 2D). Similarly, PCR with the primer pair *trr1_J* and *trr1_nz_R* yielded a product of 1140 bp using a heterozygotic *TRR1* DNA template but not from wild-type template (Figure 2D). These results indicate that the gene disruption cassette containing *HIS1* was integrated into one of the *TRR1* alleles, giving rise to the heterozygotic mutant NZ2 (*TRR1/trr1: HIS1/his1*). Again, several attempts were made to disrupt the second *TRR1* allele using gene disruption cassette containing the *LEU2* selection marker. However, none of the generated transformants gave the desired products when their DNA was subjected to PCR analysis with the primer pairs *Trr1_nz_F* and *trr1_H* or *trr1_G* and *Trr1_nz_R* to validate the gene disruption process.

The correct integration of the disruption cassettes into the *TRR1* locus was also confirmed by Southern blot analysis (Figure 2F). When genomic DNA of wild-type *C. albicans* SN87 was hybridized with a probe specific to the *TRR1* ORF, a single band was obtained; on the other hand, when genomic DNA from the mutants NZ1 or NZ2 were hybridized with the same probe, two bands (corresponding to the disrupted and non-disrupted alleles) were obtained. These results demonstrated that the gene disruption cassettes were integrated into the correct location and that only one allele of the *TRR1* gene had been disrupted in each heterozygous strain.

The failure to generate homozygous *TRR1* mutants, even after several attempts, strongly suggests that this gene is essential for the growth and viability of *C. albicans*. Missal and Lodge (2005) have reported that *TRR1* double mutants could not be generated in *C. neoformans* and concluded that this gene is likely to be essential in fungi. The *S. cerevisiae* genome encodes a full mitochondrial thioredoxin system in addition to its cytoplasmic system (Pedrajas et al., 1999), which could explain why *TRR1* is not essential in baker's yeast (*S. cerevisiae* is also non-pathogenic). In addition to its extreme growth defects, the *S. cerevisiae trr1* mutant is very sensitive to H_2O_2 and high temperatures, and it is auxotrophic for methionine (Machado et al., 1997; Pearson and Merrill, 1998). In a study performed by Dib et al. (2008) on *C. albicans DDR48* (a stress responsive gene), they showed that homozygous *DDR48* mutants

could not be generated. The heterozygous mutants, however, showed defects in filamentation on all kinds of hyphal-formation media, indicating that even the loss of one copy of the *DDR48* gene had an impact on physiology. Therefore, we carried out our studies on the *TRR1* heterozygous mutants.

To further investigate the effects of oxidative stress on the growth of wild-type *C. albicans* and *trr1* heterozygous mutants, growth curves for each strain were plotted. Figure 3 shows that the growth rates of the wild-type and the heterozygous mutants were similar when cells were exposed to 0.4 mM H_2O_2 compared with untreated cells. This indicates that both wild-type and mutant *C. albicans* can adapt well to the presence of 0.4 mM H_2O_2 . The generation times for wild-type SN87, NZ1 and NZ2 cells exposed to 0.4 mM H_2O_2 were 55 ± 0.3 , 59.3 ± 0.41 and 60.2 ± 0.11 min, respectively. However, when the cells were exposed to higher doses of H_2O_2 (4.0 mM), the times taken for the cells to enter the log phase was longer, compared with non-treated cells (Figure 3). The generation times of SN87, NZ1 and NZ2 were 60.5 ± 0.32 , 61.2 ± 0.28 and 64.4 ± 0.14 min, respectively in the presence of 4.0 mM H_2O_2 . It is likely that these hydrogen-peroxide-treated cells remain in the G1 phase of the cell cycle for longer periods of time in order for the yeast to express certain proteins necessary for adaptation towards oxidative stress. It was also observed that the heterozygous mutants had a slower growth rate compared with wild-type.

To compare the abilities of the *TRR1* heterozygous mutants and wild-type strain to withstand higher concentrations of H_2O_2 , cells were exposed to different concentration of H_2O_2 for 1 h, and the numbers of viable cells were quantified by growing the cells on yeast potato dextrose (YPD) agar. Figure 4 shows that both NZ1 and NZ2 yielded decreasing cell counts on YPD agar as the concentration of H_2O_2 was increased compared with wild-type. At 1.0 mM H_2O_2 , 83% of the SN87 cells were still viable. In contrast, 81 and 78.8% of the NZ1 and NZ2 cells were viable, respectively (Figure 4). When cells were exposed to a higher concentration of H_2O_2 (10.0 mM), 40% of the wild-type SN87 cells remained viable, while only 35.2% of NZ1 and 34% of NZ2 cells were viable (Figure 4). These results indicate that the ability of *C. albicans* to adapt towards oxidative stress is decreased as the concentration of H_2O_2 is increased. Additionally, heterozygous *trr1* mutants appeared to be more sensitive to the oxidative stress compared with the wild-type.

To investigate the importance of *TRR1* in pathogenicity, wild-type *C. albicans* and the heterozygous mutant strains were subjected to *in vivo* virulence assays. Figure 5 shows that mice infected with SN87 have an average survival time of 4 days, whereas mice infected with NZ1 or NZ2 had an average survival time of 17.2 or 16.8 days, respectively ($p < 0.0001$). These data indicate that loss of one copy of *TRR1* makes *C. albicans* significantly less

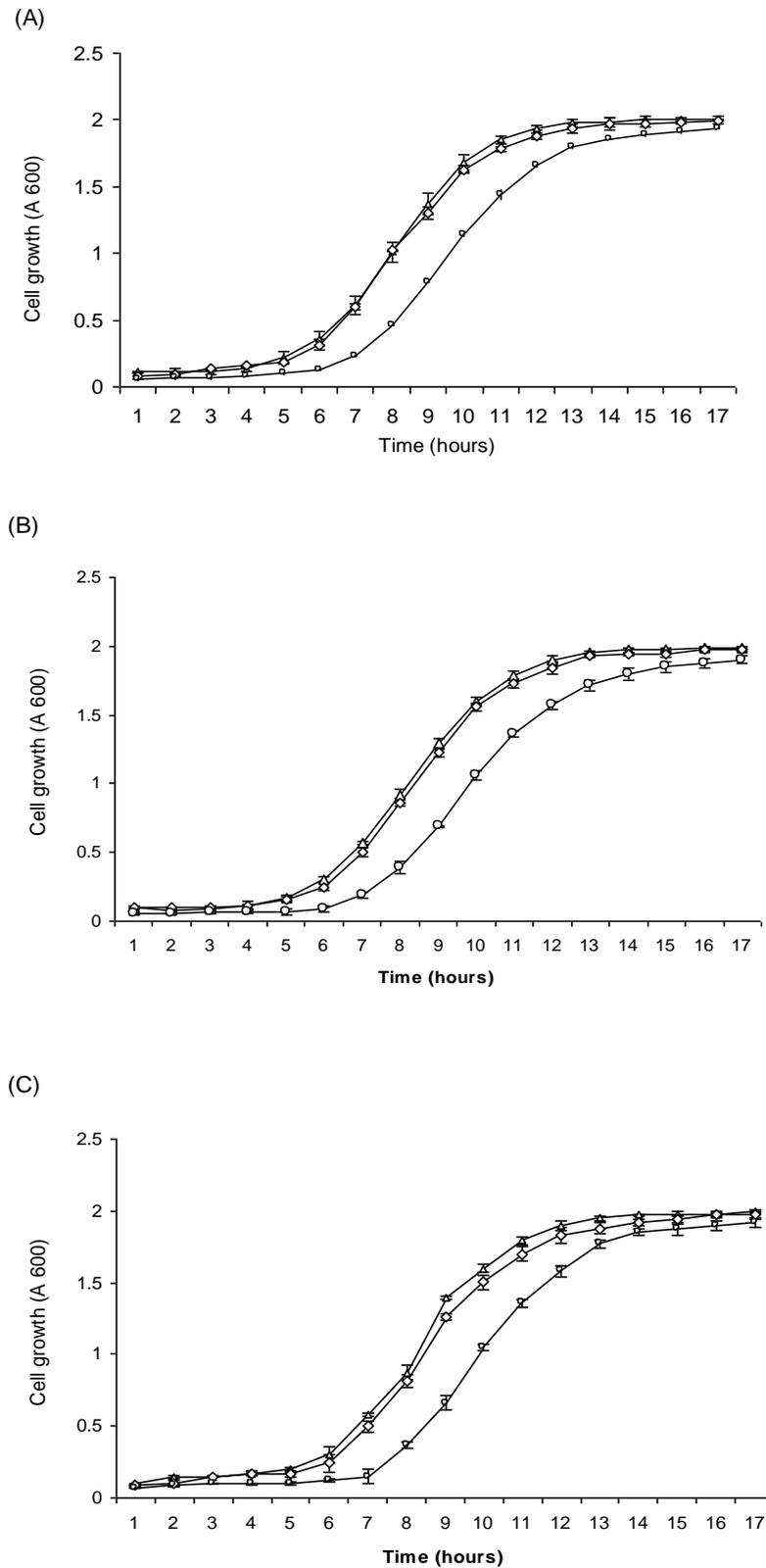


Figure 3. A comparison of the growth curves of *C. albicans* on different concentrations of H₂O₂. (A) SN87, (B) NZ1 and (C) NZ2. The following symbols denote the different H₂O₂ exposure: YPD (control) (Δ), YPD supplemented with 0.4 mM H₂O₂ (◊), YPD supplemented with 4.0 mM H₂O₂ (○). All p-values are less than 0.001.

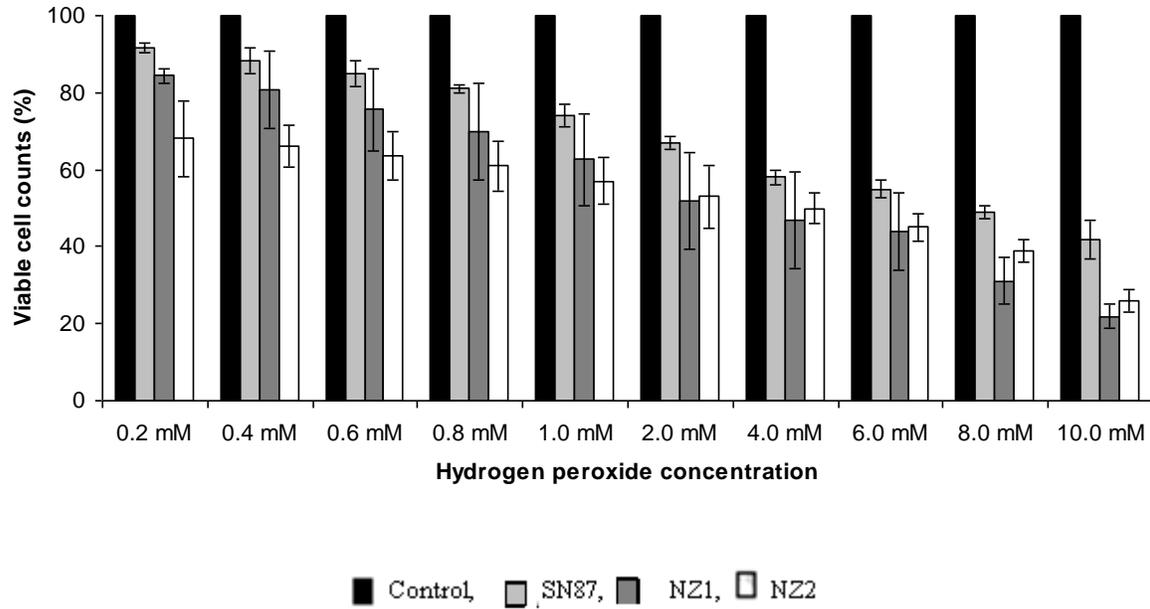


Figure 4. Determination of the number of viable cells when wild-type SN87 and the NZ1 and NZ2 mutants were exposed to different concentration of H₂O₂. As a control, cells were exposed to dH₂O instead of H₂O₂.

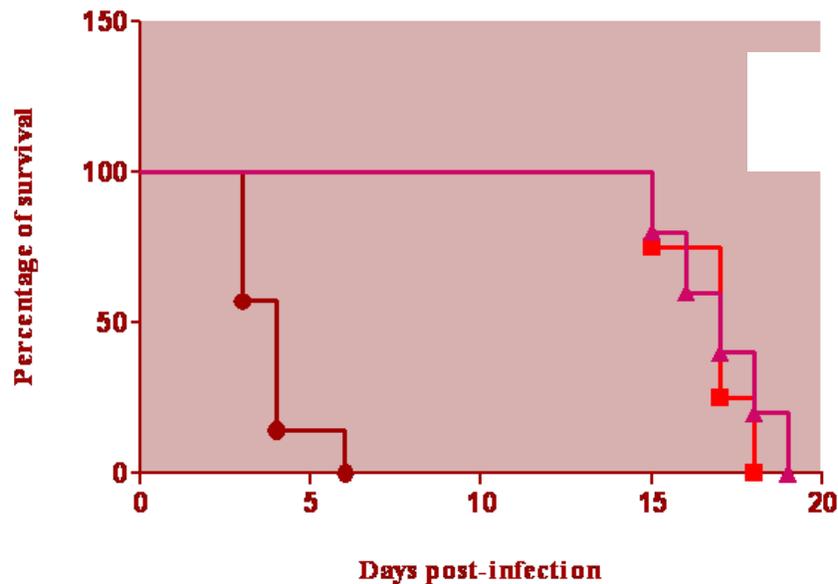


Figure 5. Survival analyses performed on mice infected with the *C. albicans* SN87, NZ1 or NZ2 strains analyzed using the Kaplan-Meier method over a period of 20 days. The following symbols denote the different *C. albicans* strains: SN87 (●), NZ1 (■), NZ2 (▲).

virulent in a mouse model. We also found that mice infected with SN87 have higher colony forming unit (CFU) counts in the kidney and liver compared with NZ1 and NZ2 (p-value < 0.05), also indicating a higher degree of infection in SN87-treated animals (Table 2).

These results correlate with the average survival times of the mice (that is, higher colony numbers in mouse organs correlate with a shorter average survival time in mice infected with the wild-type *C. albicans* strain). Furthermore, CFU counts from kidneys were greater in

Table 2. Number of *C. albicans* colonies isolated from kidney and liver samples 72 h post-infection.

Time of post-infection (h)	Strains	Log ₁₀ CFU (mean ± SD)	
		Kidney	Liver
72	SN87	5.25 ± 0.48	3.87 ± 0.14
	NZ1	4.77 ± 0.49	3.26 ± 0.06
	NZ2	4.19 ± 0.19	3.69 ± 0.26

SD: Standard deviation.

magnitude compared with the CFU counts obtained from the liver, suggesting that the kidney is a target organ for systemic *C. albicans* infection. These data are also in agreement with the findings of Calera et al. (1999) and Murad et al. (2001).

It appears that *C. albicans* requires more time to adapt to stressful host conditions when *TRR1* levels are low. The inactivation of one copy of *TRR1* had a significant effect on *C. albicans* pathogenicity, as the mutants took much longer to kill their hosts compared with wild-type strains. It has been postulated that some cells cannot survive the oxidative stress exerted by the defense mechanisms of their hosts and are destroyed. The surviving cells require additional time to adapt and proliferate to eventually reach the population numbers required to cause serious illness. Tsuchimori et al. (2000) found that mice infected by *C. albicans* mutants for *HWP1*, which encodes a surface protein involved in hyphal formation, have a 10 days average survival time, compared with a 3.5 days average survival time for mice infected by wild-type yeast. Calera et al. (1999) also demonstrated that mice infected with a heterozygous mutant of *CHK1* live up to 17 days, as opposed to mice infected with wild-type *C. albicans*, which only lived for 3 days. These findings suggest that the loss of one allele of a virulence-related gene is often sufficient to reduce *C. albicans* virulence in mice.

Conclusion

In this study, we characterized the effects of the loss of one copy of *TRR1* in *C. albicans*, a gene implicated in the oxidative stress response. We demonstrate that a loss-of-function deletion of only one *TRR1* allele is sufficient to reduce the virulence of *C. albicans* in mice. Additionally, the loss of one allele decreases the ability of *C. albicans* to survive oxidative stress. Our failure to generate homozygous *trr1* mutants suggests that the product of the *TRR1* gene is essential for cell viability. Taken together, results from this study indicate that *TRR1* is required for the survival and pathogenicity of *C. albicans*. Because thioredoxin reductase is highly conserved across fungal species (but not in humans) and *TRR1* has been shown to be essential for the survival of *C. albicans*, this gene may be a promising new target for antifungal therapy.

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