Med. Mycol. J.
Vol. 60, 29-37, 2019
ISSN 2185-6486

Original Article

Mild Heat Stress Affects on the Cell Wall Structure in *Candida albicans* Biofilm

Shojiro Ikezaki[1,2], Tamaki Cho[1], Jun-ichi Nagao[1], Sonoko Tasaki[3], Masashi Yamaguchi[4], Ken-ichi Arita-Morioka[1], Kanae Yasumatsu[1], Hiroji Chibana[4], Tetsuro Ikebe[2] and Yoshihiko Tanaka[1]

[1] *Section of Infection Biology, Department of Functional Bioscience, Fukuoka Dental College*
[2] *Section of Oral Surgery, Department of Oral and Maxillofacial Surgery, Fukuoka Dental College*
[3] *Section of Dentistry for the Disabled, Department of Oral Growth and Development, Fukuoka Dental College*
[4] *Medical Mycology Research Center, Chiba University*

ABSTRACT

We previously reported that *Candida albicans* responded to mild heat stress in a range of temperature elevations simulating fever, and concluded that mild heat stress increases susceptibility to antifungal drugs. In this study, we show that mild heat stress causes a morphological change in hyphae during the process of biofilm formation. We found that mild heat stress extended the period of hyphal stage maintenance in *C. albicans* biofilm. Although the rate of hyphal change from yeast form to hyphal form reached the maximum within 3 hr, later, almost every cell quickly reverted to the yeast growth phase within 6 hr at 37ºC but not at 39ºC, or under mild heat stress. Electron microscopy using a smart specimen preparation technique revealed that mild heat stress significantly increased the thickness of the inner cell wall accompanied by a decrease in density of the outer cell wall in the hyphae of *C. albicans* biofilm. To identify the gene responsible for the morphological changes associated with mild heat stress, we performed microarray gene expression analysis. Eleven genes were upregulated and 17 genes were downregulated under mild heat stress in biofilm cells. The increased *PHR1* gene expression in response to mild heat stress was confirmed in quantitative RT-PCR analysis. The mutant upregulated *PHR1* expression showed the same sensitivity against antifungal drug micafungin as dependent on mild heat stress. Our findings point to possible therapeutic effects of hyperthermia as well as to the effect of fever during infections.

Key words : biofilm, *Candida albicans*, cell wall, mild heat stress, *PHR1*

Introduction

Candida albicans is a commensal of human mucosal surfaces, but it causes serious clinical diseases in immunosuppressed hosts[1)]. The organism forms biofilms that make antifungal agents on medical implants and intravascular catheters ineffective. On the other hand, treatments against infectious diseases are administered to reduce risks on the host. We have been interested in exogenous mild heat stress (fever-range heat stress) that has been used in physical therapies to treat muscle injuries and malignant tumors[2-6)]. Faster pathogen clearance by immunological mechanisms was reported as a beneficial effect of fever in the infected host[7)]. Cowen and Lindquist have focused on fungal Hsp90 as a new therapeutic target based on a clinical benefit of fever[8)]. We reported the effect of mild heat stress on the antifungal susceptibility of biofilm formation in *C. albicans*[9)]. In particular, mild heat stress at two or four degrees higher than 37ºC led to increased susceptibility of *C. albicans* biofilm to antifungal agents such as micafungin, fluconazole, and amphotericin B. The difference in component ratio of yeast and hyphae at an early stage of biofilm formation may be the reason for the increased susceptibility of *C. albicans* biofilm to antifungal agents under conditions of mild heat stress, although the difference was observed only microscopically[9)]. Moreover, biofilm at over 39ºC was observed to somewhat repress further biofilm growth. Pumeesat et al. reported that

Address for correspondence: Yoshihiko Tanaka, MD, PhD
Section of Infection Biology, Department of Functional Bioscience, Fukuoka Dental College, 2-15-1 Tamura, Sawara-ku, Fukuoka, 814-0193, Japan
Received: 7, January 2019, Accepted: 28, January 2019
E-mail: tanakayo@college.fdcnet.ac.jp

the activity of biofilm formation of *C. albicans* at 42ºC was lower than at 37ºC[10]. Effectiveness of antifungal agents under mild heat stress was also demonstrated at a late stage of biofilm formation in *Aspergillus fumigatus*[11].

In this study, we investigated the alteration of the cell morphology, the ultrastructure of the cell wall, and the gene expression of *C. albicans* exposed to an increase in temperature by two degrees higher than 37ºC, as effects of mild heat stress. Using the rapid-freezing method for transmission electron microscopy of living samples revealed that the ultrastructure of the cell wall of *C. albicans* during biofilm formation was altered in response to mild heat stress. Moreover, the analysis of gene expression indicated an upregulation of *PHR1* due to mild heat stress. The mutant upregulated *PHR1* expression exhibited high sensitivity against micafungin as observed in conditions of mild heat stress. Our findings point to possible therapeutic effects of hyperthermia as well as to the effect of fever during infections.

Material and methods

Fungal strains

C. albicans SC5314[12] and *C. albicans* THE1[13] were maintained as frozen glycerol stocks at −80ºC. *C. albicans* SC5314 was used for biofilm assay, electron microscopy experiment, microarray analysis, and quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis. *C. albicans* THE1 was used for construction of mutants of *PHR1* gene.

Biofilm formation

Biofilms of the organism were formed using a partially modified method of Chandra et al.[14]. Briefly, cultures of the organism were grown on yeast extract-peptone-dextrose (YPD) agar (1% yeast extract, 2% Bacto peptone, 2% glucose, 1.5% agar) at 37ºC for 24 hr. A cell suspension of approximately 1×10^7 cells/ml was prepared in phosphate-buffered saline (PBS). Polystyrene, 96-well, flat bottom tissue culture plates were covered with fetal bovine serum (FBS) for 24 hr at 37ºC on a rocking platform. The pretreated plates were washed with PBS three times to remove residual FBS. One hundred µl of the cell suspension was inoculated into the FBS-coated plates. The cells were allowed to adhere to the plates for 90 min at 37ºC. Wells were gently washed with PBS three times to remove non-adherent cells. Subsequently, yeast nitrogen base (YNB; Difco, Detroit, MI, USA) medium supplemented with 50 mM glucose (YNB-Glc) and with or without antifungal agents was added to the wells and incubated at 37ºC and 39ºC. The temperatures were controlled exactly.

Plasmid construction

Plasmid pSFS2-PHR1 was constructed by cloning the 5′ and 3′ flanking regions of *PHR1* into the *Kpn*I/*Xho*I site (for the 5′ region) and the *Sac*II/*Sac*I site (for the 3′ region) in plasmid pSFS2. The 5′ region was amplified by PCR with the primers PHR1-KpnI and PHR1-XhoI, while the 3′ region was amplified by PCR with the primers PHR1-SacII and PHR1-SacI.

To construct the plasmid p99PHR1, the 5′ flanking region (nucleotides −460 to −148) and 5′ portion of the coding sequence region (nucleotides −6 to 308) of *PHR1* were amplified by PCR using the primers PHR1-AF and PHR1-AR or PHR1-BF and PHR1-BR, respectively. The amplified fragments were cloned into the *Kpn*I/*Sal*I site (for the 5′ flanking region) and the *Spe*I/*Sac*I site (for the 5′ portion of coding sequence) in plasmid p99CAU1[13].

Plasmid p99RP10 was constructed by cloning the 5′ and 3′ flanking regions of *RPS10* into the *Kpn*I/*Sal*I site (for the 5′ region) and the *Spe*I/*Not*I site (for the 3′ region) in plasmid p99CAU. The 5′ region was amplified by PCR with the primers RP10tet-AF and RP10tet-AR, while the 3′ region was amplified by PCR with the primers RP10tet-BF and RP10tet-BR. Primers used in this study are listed in Table 1.

Construction of strains

Yeast cells were transformed by a modified version of the lithium acetate method of Umeyama et al.[15]. The SAT1 flipper was used to disrupt one of the *PHR1* alleles to generate the heterozygous strain[16]. The *PHR1* deletion cassette (the DNA fragments between the *Kpn*I and *Sac*I sites) of the plasmid pSFS2-PHR1 was amplified with the primers PHR1-KpnI and PHR1-SacI and the amplified fragments were used to transform into *C. albicans* THE1[13]. Nourseothricin-resistant transformants were selected on YPD agar plates containing 200 µg/ml of nourseothricin to obtain the *PHR1* heterozygous mutants. For excision of the SAT1 flipper from nourseothricin-resistant transformants by the site-specific recombinase FLP-mediated recombination, the strains were cultivated overnight in YPD medium without selective pressure. In order to replace the endogenous promoters with the tet-regulatable promoter in the remaining *PHR1* allele, the DNA fragment between the *Kpn*I and *Sac*I sites in plasmid p99PHR1 was amplified with the primers PHR1-AF and PHR1-BR and used to transform the *PHR1* heterozygous mutants. After selection on synthetic dextrose medium lacking uracil, the resulting $URA3^+$ transformants were plated on 5-fluoroorotic acid-containing medium to isolate the ura segregants. The resulting strain was designated tetPHR1.

We also constructed a control strain, contPHR1 strain, in which the tet-promoter element was integrated at the *RP10* locus of *C. albicans* genome. The DNA fragment between the *Kpn*I and *Not*I sites in plasmid p99RP10 was amplified with the primers RP10tet-AF and RP10tet-BR and used to transform into *C. albica*ns THE1 strain. Construction of the strains is described in Fig. S1(a).

Table 1. Primers used in this study

Primer name	Purpose	A Nucleotide sequence (5′–3′)[a]
PHR1-KpnI	Construction of plasmid pSF2-PHR1	GCCGGGTACCGTTAGATACCTTCGTTTC
PHR1-XhoI		GCCGCTCGAGAATGAAAAGAGTGTGGCA
PHR1-SacII		GCCGCCGCGGTTATTACTATTGTTACTG
PHR1-SacI		GCCGGAGCTCGCTACTCCGCCCCCAATT
PHR1-AF	Construction of plasmid p99PHR1	TTTTGGTACCAAATGAAGTGTTATTTCTAACCA
PHR1-AR		TTTTGTCGACTGTTAATTATATTGCCAATGGAC
PHR1-BF		TTTTACTAGTAAAAAAATGTATTCATTAATCAA
PHR1-BR		TTTTGAGCTCTCAATAGCATAAACTCTCAAAGT
RP10tet-AF	Construction of plasmid p99RP10	TTTTGGTACCAACAAGAGATTGTCCAAAG
RP10tet-AR		TTTTGTCGACTTCATCAACTCTCAATTTGA
RP10tet-BF		GGAAACTAGTTACGCTCAATCCTCTAAATT
RP10tet-BR		AAAAGCGGCCGCTTGAAACCAGAAGAAACTTTCT
P1(PHR1-AF)	Confirmation of the constructed strain shown in Fig. S1(a)	TTTTGGTACCAAATGAAGTGTTATTTCTAACCA
P2(PHR1-BR)		TTTTGAGCTCTCAATAGCATAAACTCTCAAAGT
P3		CTGACTTTCTGTATCTAAAAATT
P4		GGAATTCCTTAGTGGTATCAACGTC

[a] Restriction enzyme sites are underlined.

Total RNA extraction

Total RNA was extracted from biofilm-forming cells either at 37ºC or 39ºC for 3 hr in YNB-Glc medium using 24-well plates. All cultures collected from the plates with scrapers were quickly filtrated on cellulose acetate membranes *in vacuo*. They were then quick-frozen and stored at −80ºC. Extraction of total RNA was performed using RNeasy Mini (QIAGEN GmbH, Germany) according to the kit instructions. Cells on the frozen membrane filters were transferred to microtubes containing 0.3 g of zirconia/silica disruption beads and the first solution of the kit, and then shaken vigorously in a Disruptor Genie (Scientific Industries, Inc., NY, USA) for 5 × 45 sec with periodic chilling on ice-water.

Microarray design and gene expression microarray

For the custom microarray slides of *C. albicans*, we used the *C. albicans* SC5314 ORF sequence data (https: //fungi. ensembl.org/Candida_albicans_sc5314/info/index). Probes were generated from 6251 ORF sequences utilizing the e-array portal hosted by Agilent for array design (https://earray.chem.agilent.com/earray/). Finally, 43718 probes were selected for a 4 × 44K array format. The cRNA was amplified, labeled using a Low Input Quick Amp Labeling Kit (Agilent Technologies), and hybridized to a 4 × 44k Agilent custom array of *C. albicans*, according to the manufacturer' s instructions. All hybridized microarray slides were scanned by an Agilent scanner. Relative hybridization intensities and background hybridization values were calculated using Agilent Feature Extraction Software (9.5.1.1).

Microarray data analysis and filter criteria

The raw signal intensities and flags for each probe were calculated from hybridization intensities and spot information according to the procedures recommended by Agilent Technologies using the Flag criteria in the Gene Spring Software. The raw signal intensities of two samples were $\log_2$-transformed and normalized by quantile algorithm with 'preprocessCore' library package on Bioconductor software[17, 18]. To identify up- or downregulated genes, we calculated Z-scores and ratios (non-log scaled fold-change) from the normalized signal intensities of each probe for comparison between control and experimental samples[19]. Then, we established criteria for regulated genes: (upregulated genes) Z-score ≥ 2.0 and ratio ≥ 1.0 - fold, (downregulated genes) Z-score ≤ −2.0 and ratio ≤ 1.0.

Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

To synthesize cDNA, the Advantage RT-for-PCR kit (Clontech, San Jose, CA, USA) was used according to the manufacturer's protocol. Quantitative PCR was performed and analyzed using the CFX Manager (ver. 3.1) (BIO-RAD). The SSO advanced universal SYBR Green Supermix kit (BIO-RAD) was used for quantitative PCR according to the manufacturer' s protocol. The reference gene used for this experiment was 18S rRNA and *ACT1* as described previously[20]. Results were analyzed using the $2^{-\Delta\Delta Ct}$ relative expressions methods[21, 22]. The value given for each gene at 37ºC and 39ºC for 3 hr relative to the expression of each gene

in biofilm-starting cells that were adhered to wells for 90 min at 37ºC was determined by evaluating the expression: $2^{-\Delta\Delta Ct}$.

Microscopy

Biofilm development in wells was monitored with a Keyence BZ-9000 (KEYENCE, Osaka, Japan).

Electron microscopy

Living cells scraped from biofilm culture wells were collected by centrifugation in a microcentrifuge for 10 sec. A small amount of the cell pellet was sandwiched between two copper disks and rapidly frozen by plunging them into melting propane cooled in liquid nitrogen[23]. They were freeze-substituted in acetone containing 2% osmium tetroxide at −80ºC for 2 days, embedded in epoxy resin (Quetol 812, Nisshin EM Co. Ltd., Tokyo), and polymerized at 60ºC. Ultrathin sections were cut to a thickness of 80 nm with a diamond knife (Diatome Co. Ltd., Switzerland) on an Ultracut S ultramicrotome (Leica Microsystems, Vienna) and picked up on 300-mesh grids. They were stained with uranyl acetate and lead citrate in a staining tube, covered with Super Support Film (Nisshin EM Co. Ltd., Tokyo), and observed in a JEM-1400 electron microscope (JEOL, Tokyo) at magnifications of 2,500 to 40,000 at 100 kV[24].

Spot assay

C. albicans cells were grown overnight on YPD agar medium at 37ºC. The colonies were suspended on YPD liquid medium and adjusted to cell concentration of 1×10^7 cells/ml. Five µl aliquots of 10-fold serial dilutions (from 1×10^7 cells/ml) were spotted onto YPD agar plates supplemented with 0.05 µg/ml micafungin in the presence or absence of 10 µg/ml doxycycline (DOX). Minimum inhibitory concentration (MIC) of micafungin for the contPHR1 strain at 37ºC based on E-test was 0.032 µg/ml. Concentrations of micafungin were used based on the MIC value and our previous report[25]. The plates were incubated at 37ºC or 39ºC for 36 hr.

Statistical analysis

Statistical significance was determined by a two-tailed Student's *t*-test.

Results

Mild heat stress extends the period of hyphal maintenance in *C. albicans* biofilm

Mild heat stress here means stress by increasing only two degrees from 37ºC. This condition simulates fever symptoms in infectious diseases. In our previous report, mild heat stress induced the antifungal susceptibility of *C. albicans* SC5314 biofilm[9]. However, what occurred in the cells due to mild heat stress remained to be solved. We monitored the process of biofilm formation at 37ºC and 39ºC as shown in Fig. 1(a) using *C. albicans* SC5314. Zero time represent the cells that adhered at 37ºC for 90 min on the culture wells. The non-adherent cells were removed by washing with PBS three times as mentioned in Materials and methods. Although most cells showed the yeast form at 0 hr, both of the temperatures completely induced a morphological change to the hyphal form within 3 hr. Later, almost all cells reverted back into the yeast form from the hyphal form at 37ºC after 6 hr. However, it seemed that 39ºC was insufficient to cause reversion to the yeast form within 6 hr. Therefore, we assessed the hyphal rate in the process of biofilm formation every hour at 37ºC and 39ºC as shown in Fig. 1(b). Both of the hyphal rates similarly increased in a time-dependent manner until 3 hr. Then, at 4 hr, the culture at 37ºC was suddenly induced to switch from hyphal growth phase into yeast growth phase. However, hyphal growth phase at 39ºC continued significantly until 6 hr. These results indicated that mild heat stress at 39ºC caused a morphological change in the process of biofilm formation.

Mild heat stress affects the structure of *C. albicans* cell wall

Fig. 1 shows that the significant difference of the hyphal rate in the process of biofilm formation at 37ºC and 39ºC appeared between 3 hr and 4 hr, showing dependency on mild heat stress. To understand what occurred in the cells under mild heat stress, we focused on cell surface architecture of hyphae at 3.5 hr following exposure to mild heat stress and observed them in detail through electron microscopy using a smart specimen preparation[23]. The yeast cells that adhered to culture wells at 37ºC for 90 min with PBS were set as the 0 hr of biofilm formation. The cells were grown in a hyphal form for 3.5 hr culture at both temperatures. The cells cultured for 3.5 hr in biofilm medium at 37ºC and 39ºC were scraped from the culture wells, and then were immediately flushed by a microcentrifuge. The living cells were rapidly treated for TEM as mentioned in Materials and methods. Fig. 2(a) showed TEM images of the cell wall from *C. albicans* SC5314 cells. An apparent difference in images of hyphal wall between 37ºC and 39ºC was observed in the outer cell wall. Electron density of the outer cell wall at 39ºC seemed lower compared to 37ºC. The thickness of cell wall was measured based on TEM pictures in Fig. 2(b). The total cell wall thickness at 39ºC was significantly higher than at 37ºC ($P < 0.01$). Although the outer cell wall thickness did not change between cells grown at 37ºC and 39ºC, the inner cell wall at 39ºC was significantly thicker compared to 37ºC ($P < 0.01$). According to a previous paper, the outer cell wall is the mannan fibrillar layer and the inner cell wall is the chitin and β-glucans layer[25]. Therefore, mild heat stress leads to decrease in electron density of the mannan fibrillar layer and to increase in thickness of the chitin and β-glucans layer.

***PHR1* gene expression in cells that responded to mild heat stress is increased**

Results shown in Fig. 1 and Fig. 2 revealed that the changes in cell wall protein expression in growth cell morphology occurred between 3 hr and 4 hr, and is dependent on the temperature. We first explored whether mild heat stress leads

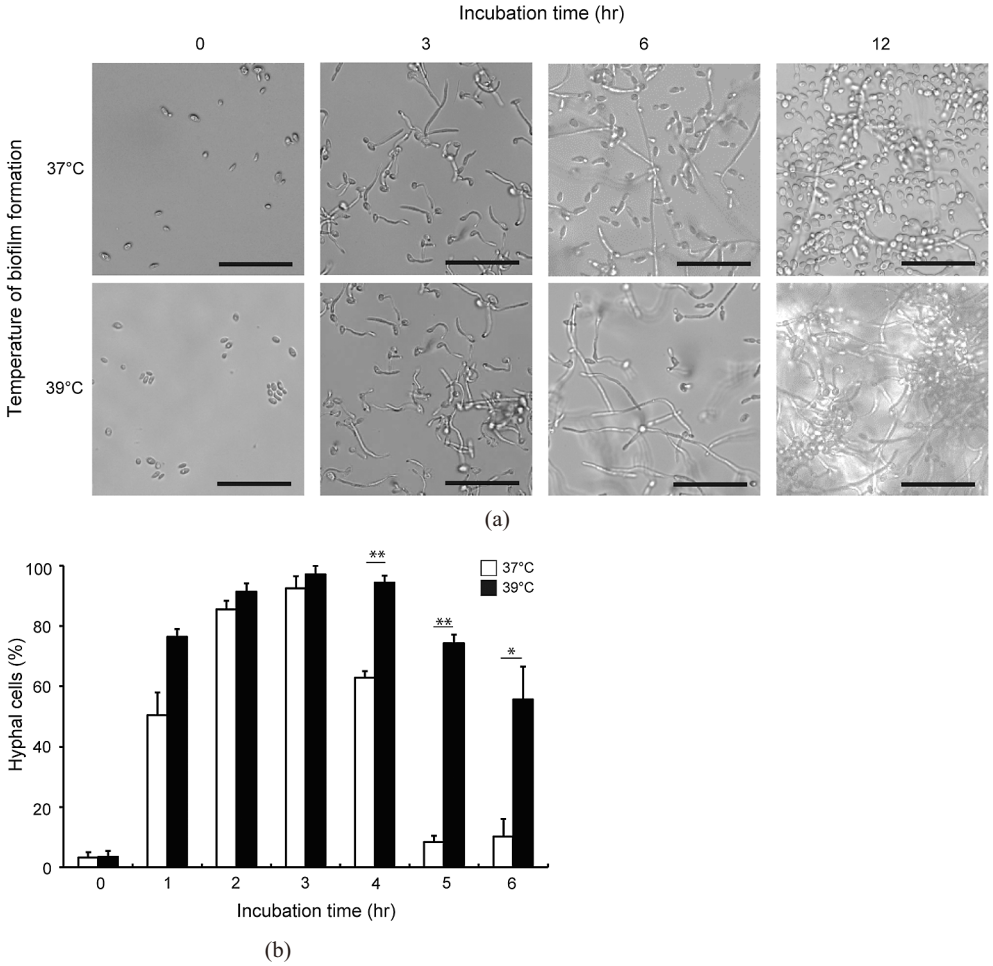


Fig. 1. Mild heat stress extends the period of hyphal growth phase in *C. albicans* biofilm.

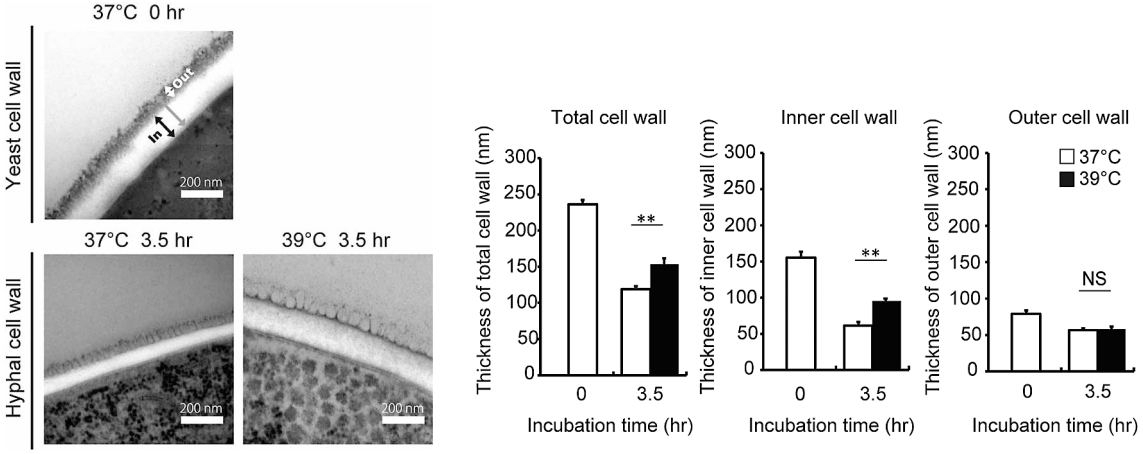
(a) Images of biofilm development of *C. albicans* SC5314 at 37ºC and 39ºC obtained with an optical microscope. Scale bar = 40 μm.

(b) Biofilm development of *C. albicans* SC5314 at 37ºC and 39ºC as analyzed based on hyphal rate. The thickness of biofilm increased over 6 hr under both temperatures. The hyphal rate was visually unmeasurable after 6 hr. Mean of hyphal rate from at least 300 cells per sample was calculated. Data are representative means and SD from five independent experiments. Error bars represent standard deviations. Statistical significance was assessed using a two-tailed Student's *t*-test (*P < 0.05, **P < 0.01).

to changes in expression of genes that affect the cell surface properties by using microarray gene expression analysis. In Fig. 3(a), genome-wide gene expression in biofilm cells of *C. albicans* SC5314 at 37ºC and 39ºC for 3-hr cultures was evaluated at the mRNA level. Eleven genes were upregulated in biofilm cells cultured at 39ºC for 3 hr compared to 37ºC. On the other hand, 17 genes were downregulated at 39ºC in biofilm cells. Since the apparent difference was observed in cell wall structure between at 37ºC and at 39ºC in electron microscopy, we focused on cell surface molecules in further experiments. The upregulated genes related to cell surface molecules were 3 genes; namely, *WOR1*, *TRY6*, and *PHR1*, and the downregulated genes were 5 genes; namely, *MNN22*, *PGA22*, *YWP1*, *PGA26*, and *PGA45*. The expression of these eight upregulated or downregulated genes was confirmed in detail using quantitative RT-PCR, as shown in Fig. 3(b and c). In quantitative RT-PCR analysis, the gene expression level in cells that adhered to culture wells at 37ºC for 90 min prior to biofilm culture was used as the basal expression level. The quantitative RT-PCR analysis revealed that *PHR1* gene expression was significantly upregulated at 39ºC compared to 37ºC. These results suggest that *PHR1* might have some function in the cell surface architecture of hyphae at 39ºC in biofilm cells.

The mutant upregulated *PHR1* expression shows the same sensitivity against micafungin as dependent on mild heat stress

To confirm the relationship with *PHR1* gene expression and mild heat stress, we constructed a tetPHR1 in THE1 strain where *PHR1* gene expression was regulated by a tet-repressive promoter, which is repressed in the presence of the tetracycline analog DOX and is overexpressed in the absence

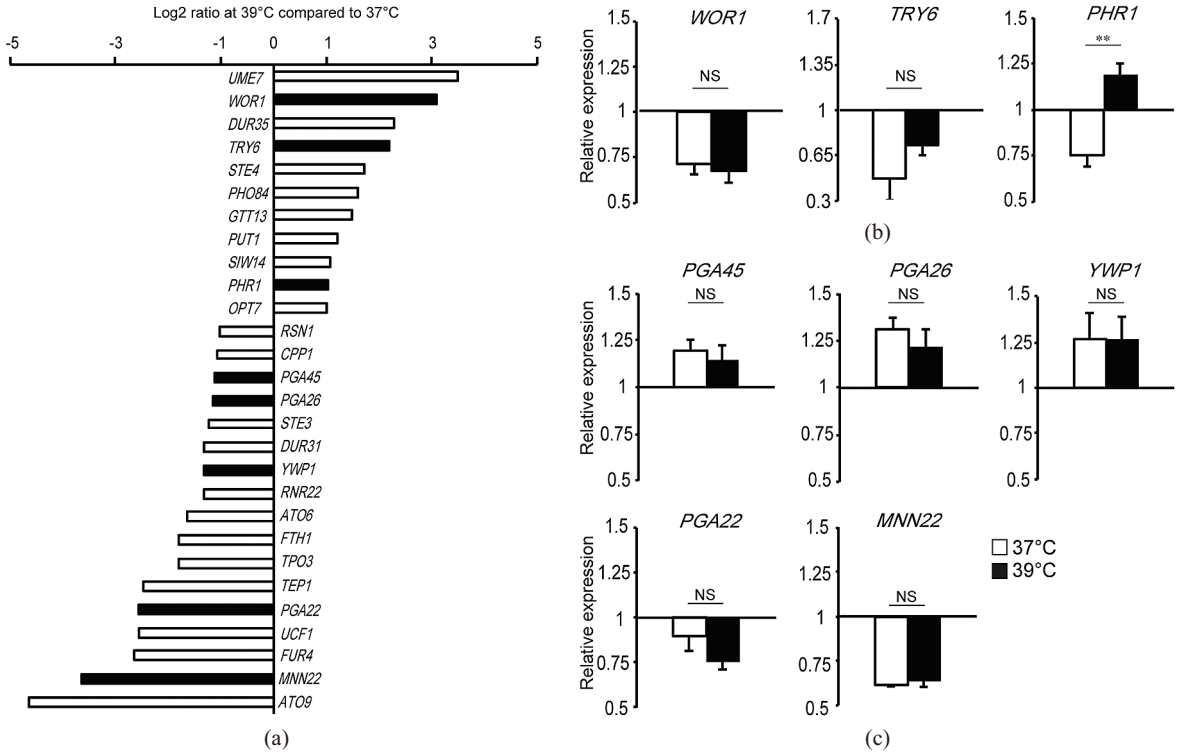


(a) (b)

Fig. 2. Mild heat stress affects the structure of the *C. albicans* cell wall.

(a) TEM images of cell walls from yeast form *C. albicans* SC5314 that adhered to culture well and from hyphal form *C. albicans* grown in biofilm medium at 37ºC and 39ºC for 3.5 hr (representative images from > 15 cells imaged). Out, outer wall; In, inner wall. Scale bars = 200 nm.

(b) Quantification of the thickness of the inner and outer layers of the *C. albicans* SC5314 cell wall based on TEM pictures (n > 15 cells). Total wall, outer, and inner wall. Error bars represent standard deviations. Statistical significance was assessed using a two-tailed Student's *t*-test (**P < 0.01; NS, not significant)



(a) (b) (c)

Fig. 3. *PHR1* gene expression in cells increased in response to mild heat stress.

(a) Relative quantification of gene expression using microarray. The graph represents $\log_2$ ratios of gene expression at 39ºC versus 37ºC, for both 3-hr cultures for biofilm formation of *C. albicans* SC5314. Positive values represent an increase and negative values represent a decrease of gene expression at 39ºC compared to 37ºC. Black bars represent genes related to cell surface structures.

(b) and (c) Relative quantification of gene expression by quantitative RT-PCR using the comparative C_T analysis. Quantification RT-PCR of eight genes selected by microarray in Fig. 3(a) was performed. Gene expression level in cells that adhered to culture wells at 37ºC for 90 minutes prior to biofilm culture was used as the basal expression level. (b) Upregulated genes. (c) Downregulated genes. Statistical significance was assessed using a two-tailed Student's *t*-test (**P < 0.01; NS, not significant)

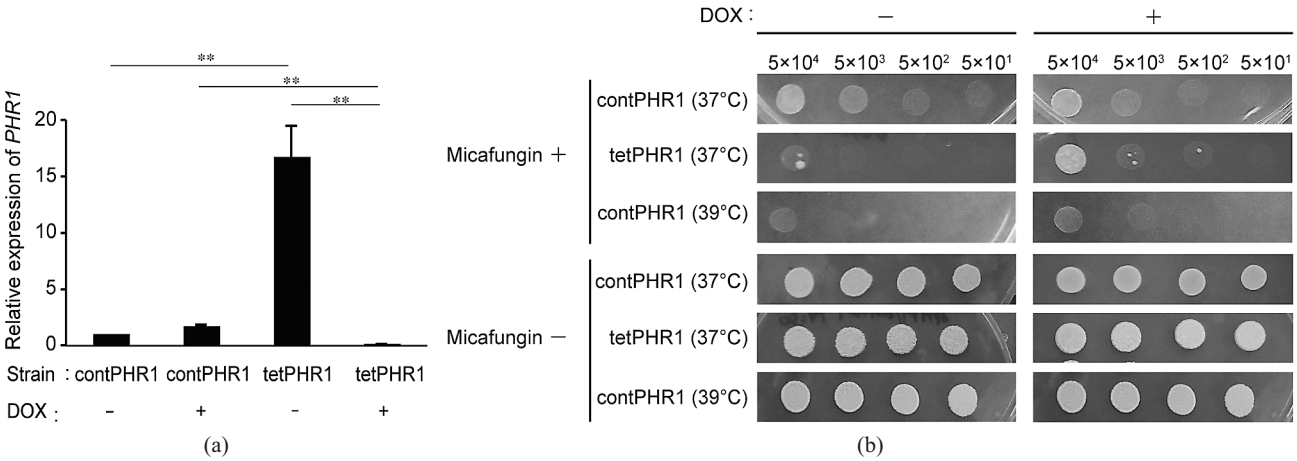


Fig. 4. The mutant upregulated *PHR1* expression shows the same sensitivity against micafungin as dependent on mild heat stress.

(a) The contPHR1 and tetPHR1 strains were cultured in YPD liquid medium at 37° C until the mid-log phase (1×10^6 cells/ml). DOX was added to the cell culture at 10 μg/ml, and the cell culture was then incubated at 37ºC for 30 min. Total RNA was extracted as described in Materials and methods. The *PHR1* expression level was measured by quantitative RT-PCR and normalized against that of the *ACT1* control. Statistical significance was assessed using a two-tailed Student's *t*-test (**P < 0.01)

(b) Aliquots of 10-fold serial dilutions of each strain (1×10^7 cells/ml) were spotted on YPD agar plates containing 0.05 μg/ml micafungin in the presence or absence of 10 μg/ml DOX. The plates were spotted with 5 μl of each dilution and then incubated at 37ºC for 36 hr. Data are representative of two independent experiments.

of DOX (Fig. S1(a)). We also constructed the contPHR1 strain in which the tet-promoter element was integrated at the *RP10* locus. The integration of the tet-promoter component was confirmed by PCR using appropriate primers as shown in Fig. S1(b). A quantitative RT-PCR analysis revealed that *PHR1* gene expression level in the tetPHR1 strain was 17-fold higher compared to the contPHR1 strain in the absence of DOX. Meanwhile, *PHR1* gene expression level in the tetPHR1 strain was completely repressed more than 10-fold compared to the contPHR1 strain in the presence of DOX at 37ºC as shown in Fig. 4(a). We found that mild heat stress increased *PHR1* gene expression level (Fig. 3(b)). Therefore, since the tetPHR1 strain expressed *PHR1* highly without DOX as shown in Fig. 4(a), we assumed that the effect of mild heat stress would be observed more significantly at 37ºC in the tetPHR1 strain compared to the contPHR1 strain.

First, we performed the biofilm formation assay using the contPHR1 strain. However, it was impossible to assess morphological changes because the biofilm of contPHR1 strain showed yeast-dominant formation unlike the SC5314 strain, even though the contPHR1 strain possessed the ability for morphogenesis at 37ºC (data not shown). We next performed spot assay to confirm the effectiveness of antifungal drugs at 37ºC in the tetPHR1 strain with or without DOX. The sensitivity of micafungin under mild heat stress in *C. albicans* SC5314 strain was higher than amphotericin B or fluconazole in our previous report[9]. When the tetPHR1 strain was grown at 37ºC without DOX, wherein *PHR1* was overexpressed, it showed higher sensitivity to micafungin compared to the contPHR1 strain at 37ºC (Fig. 4(b)). This phenomenon was similar to the sensitivity of micafungin in the contPHR1 strain at 39ºC. The sensitivity to micafungin of the tetPHR1 strain recovered to the control level when grown with DOX at 37ºC. Meanwhile, the high sensitivity of the tetPHR1 strain to amphotericin B or fluconazole at 37ºC was not observed through this assay (Fig. S2). These results suggest that the mild-heat-stress-dependent expression of *PHR1* induced sensitivity to micafungin.

Discussion

Multiple environmental stresses, including osmotic stress and thermal stress, lead to remodeling of cell wall structure[26, 27]. We reported that *C. albicans* biofilm responded to mild heat stress (elevation in the temperature from 37ºC to 39ºC and 41ºC) by showing increased susceptibility against antifungal drugs[9]. It was reported that the mass, thickness, and metabolic activity of biofilm produced at 42ºC were lower than those at 37ºC[10]. There have been few reports, however, on the analysis of mild heat stress of about two-degree-increase simulating human fever during infection. Mild heat stress (37ºC to 39ºC) was reported as a useful physical therapy in orthopedic surgery[5]. In this study, we focused on the cell wall structure and gene expression to examine changes in the cell surface in response to mild heat stress by a two-degree temperature elevation.

As shown in Fig. 1, the main form of *C. albicans* cell morphology occupying biofilm beyond 3 hr at 37ºC changed from hyphae to yeast. On the other hand, maintenance of hyphae continued until 6 hr at 39ºC. It appeared that the

elevation in the temperature by two degrees induces the maintenance of hyphal growth of the cell in biofilm. We examined the structural changes of the cell surface layer of hyphae at 3.5-hr culture under mild heat stress (Fig. 2). The two distinct layers of the cell wall, the inner and outer layers, were clearly visible. The outer wall is composed of the mannan fibrillar layer, and the inner wall is composed of β-glucan and chitin[28, 29]. Mild heat stress did not affect the thickness of the outer wall, but decreased the electron density of the mannan fibrillar layer. Furthermore, the thickness of the inner wall increased under mild heat stress for 3.5 hr. The increased thickness of the inner wall in response to mild heat stress may be to compensate for the fragility of the outer wall. The fungal cell wall is affected by environmental changes including osmotic shock, carbon source, and oxygen concentration[25, 30]. In this study, the vulnerability of the mannan layer appeared in response to a two-degree temperature elevation from 37ºC in the early stage of biofilm formation in *C. albicans* as shown in Fig. 2(a). Zeng et al. reported that the sensitivity of antifungal drugs to mild heat stress in *Aspergillus fumigatus* biofilm was more evident at the late stage of biofilm formation[11]. *A. fumigatus* differs from *C. albicans* in structure and composition of cell walls, and these phenomena in response to mild heat stress may occur in other kinds of fungi as well.

Based on the data in Fig. 1, RNAs were extracted from cells cultured at 37ºC and 39ºC for 3 hr, and gene expression levels were compared at 37ºC and 39ºC using microarray analyses as shown in Fig. 3 (a). The gene expression levels were confirmed using quantitative PCR method. The gene expression in cells that adhered at 37ºC for 90 min in the wells was used as the basal expression level. Therefore, this difference in comparison criteria for gene expression between microarray analysis and quantitative PCR method may yield differences in results. The β-glucan transglycosylase gene *PHR1*[31, 32] was selected as a gene whose expression is suppressed at 37ºC and is promoted at 39ºC (Fig. 3(b)). This result was similar to the result reported by Heilmann et al. showing that *PHR1* gene expression was higher at 42ºC compared to 37ºC[27]. In addition, they also reported an increase in other cell surface proteins at 42ºC; namely, chitin-related proteins Crh11 and Utr2 and cell wall maintenance protein Ecm33. Therefore, it is also conceivable from their data that mild heat stress affects genes related to cell surface structure. *PHR1* is believed to be involved in the phenomenon resulting to the increase in thickness of the mycelial inner cell wall at 39ºC than that at 37ºC as shown in Fig. 2. It will be necessary to analyze the cell wall thickness under the electron microscope using the tetPHR1 strain.

Plaine et al. reported that *PHR1*-deficient strains are resistant to caspofungin[33]. This result is consistent with our data that *PHR1* overexpression in the tetPHR1 strain without DOX at 37ºC led to increased sensitivity to micafungin as shown in Fig. 4(b). Our results suggest that the sensitivity phenotypes against micafungin under mild heat stress as we previously reported could be attributed to *PHR1* functions. In particular, we believe that the upregulation of *PHR1* expression in response to mild heat stress leads to remodeling of cell wall structure via the transglycosylase function of PHR1, which in turn confers the susceptibility to micafungin. The new findings obtained in this study point to a possible application of hyperthermia as a therapeutic method. Further studies including *in vivo* experiments will be required to substantiate the therapeutic effect of hyperthermia as well as the effect of fever during infections.

Acknowledgements

This work was supported in part by grants from Japan Society for the Promotion of Science KAKENHI Grant Number JP18K09547 (to C. T.), JP18H06301 (to S. T.), JP17H04371, JP17H05804, and JP18K19657 (to Y. T.); Science Research Promotion Fund (to T.C. and Y.T.); Astellas Research Support (to Y. T.); Terumo Foundation for Life Sciences and Arts (to Y.T.); and Private University Research Branding Project (to J.N.). This work was partly performed as part of the Cooperative Research Project Program of the Medical Institute of Bioregulation, Kyushu University. The research was also partly supported by the Cooperative Research Program of the Medical Mycology Research Center, Chiba University.

Conflicts of interest

None.

References

1) Calderone RA, Clancy CJ (eds): *Candida* and candidiasis. ASM Press, Washington, 2011.
2) Hanson DF: Fever, temperature, and the immune response. Ann N Y Acad Sci **813**: 453-464, 1997.
3) Shui C, Scutt A: Mild heat shock induces proliferation, alkaline phosphatase activity, and mineralization in human bone marrow stromal cells and Mg-63 cells *in vitro*. J Bone Miner Res **16**: 731-741, 2001.
4) Park HG, Han SI, Oh SY, Kang HS: Cellular responses to mild heat stress. Cell Mol Life Sci **62**: 10-23, 2005.
5) Yamaguchi T, Suzuki T, Arai H, Tanabe S, Atomi Y: Continuous mild heat stress induces differentiation of mammalian myoblasts, shifting fiber type from fast to slow. Am J Physiol Cell Physiol **298**: C140-C148, 2010.
6) Griffin RJ, Dings RP, Jamshidi-Parsian A, Song CW: Mild temperature hyperthermia and radiation therapy: role of tumor vascular thermotolerance and relevant physiological factors. Int J Hyperthermia **26**: 256-263, 2010.

7) Hasday JD, Fairchild KD, Shanholtz C: The role of fever in the infected host. Microbes Infect **2**: 1891-1904, 2000.
8) Cowen LE, Lindquist S: Hsp90 potentiates the rapid evolution of new traits: drug resistance in diverse fungi. Science **309**: 2185-2189, 2005.
9) Cho T, Nagao J, Imayoshi R, Kodama J, Morotomi T, Kaminishi H: *In vitro* efficacy of continuous mild heat stress on the antifungal susceptibility of *Candida albicans* biofilm formation. Biol Pharm Bull **35**: 1371-1373, 2012.
10) Pumeesat P, Muangkaew W, Ampawong S, Luplertlop N: *Candida albicans* biofilm development under increased temperature. New Microbiol **40**: 279-283, 2017.
11) Zeng R, Li M, Chen Q, Wang L, Zhan P, Wang C, Lv G, Shen Y, Liu W: *In Vitro* analyses of mild heat stress in combination with antifungal agents against *Aspergillus fumigatus* biofilm. Antimicrob Agents Chemother **58**: 1443-1450, 2014.
12) Gillum AM, Tsay EY, Kirsch DR: Isolation of the *Candida albicans* gene for orotidine-5' -phosphate decarboxylase by complementation of S. cerevisiae ura3 and *E. coli* pyrF mutations. Mol Gen Genet **198**: 179-182, 1984.
13) Nakayama H, Mio T, Nagahashi S, Kokado M, Arisawa M, Aoki Y: Tetracycline-regulatable system to tightly control gene expression in the pathogenic fungus *Candida albicans*. Infect Immun **68**: 6712-6719, 2000.
14) Chandra J, Mukherjee PK, Leidich SD, Faddoul FF, Hoyer LL, Douglas LJ, Ghannoum MA: Antifungal resistance of candidal biofilms formed on denture acrylic *in vitro*. J Dent Res **80**: 903-908, 2001.
15) Umeyama T, Kaneko A, Nagai Y, Hanaoka N, Tanabe K, Takano Y, Niimi M, Uehara Y: *Candida albicans* protein kinase CaHsl1p regulates cell elongation and virulence. Mol Microbiol **55**: 381-395, 2005.
16) Reuss O, Vik A, Kolter R, Morschhäuser J: The SAT1 flipper, an optimized tool for gene disruption in *Candida albicans*. Gene **341**: 119-127, 2004.
17) Bolstad BM, Irizarry RA, Astrand M, Speed TP: A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics **19**: 185-193, 2003.
18) Gentleman RC, Carey VJ, Bates DM, et al: Bioconductor: open software development for computational biology and bioinformatics. Genome Biol **5**: R80, 2004.
19) Quackenbush J: Microarray data normalization and transformation. Nat Genet **32 Suppl**: 496-501, 2002.
20) Toyoda M, Cho T, Kaminishi H, Sudoh M, Chibana H: Transcriptional profiling of the early stages of germination in *Candida albicans* by real-time RT-PCR. FEMS Yeast Res **5**: 287-296, 2004.
21) Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) Method. Methods **25**: 402-408, 2001.
22) Lan CY, Rodarte G, Murillo LA, Jones T, Davis RW, Dungan J, Newport G, Agabian N: Regulatory networks affected by iron availability in *Candida albicans*. Mol Microbiol **53**: 1451-1469, 2004.
23) Yamaguchi M, Okada H, Namiki Y: Smart specimen preparation for freeze substitution and serial ultrathin sectioning of yeast cells. J Electron Microsc (Tokyo) **58**: 261-266, 2009.
24) Yamaguchi M, Chibana H: A method for obtaining serial ultrathin sections of microorganisms in transmission electron microscopy. J Vis Exp **131**: e56235, 2018.
25) Nagao J, Cho T, Uno J, Ueno K, Imayoshi R, Nakayama H, Chibana H, Kaminishi H: *Candida albicans* Msi3p, a homolog of the *Saccharomyces cerevisiae* Sse1p of the Hsp70 family, is involved in cell growth and fluconazole tolerance. FEMS Yeast Res **12**: 728-737, 2012.
26) Ene IV, Walker LA, Schiavone M, Lee KK, Martin-Yken H, Dague E, Gow NA, Munro CA, Brown AJ: Cell wall remodeling enzymes modulate fungal cell wall elasticity and osmotic stress resistance. MBio **6**: e00986-15, 2015.
27) Heilmann CJ, Sorgo AG, Mohammadi S, Sosinska GJ, de Koster CG, Brul S, de Koning LJ, Klis FM: Surface stress induces a conserved cell wall stress response in the pathogenic fungus *Candida albicans*. Eukaryot Cell **12**: 254-264, 2013.
28) Cappellaro C, Baldermann C, Rachel R, Tanner W: Mating type-specific cell-cell recognition of *Saccharomyces cerevisiae*: cell wall attachment and active sites of a- and α-agglutinin. EMBO J **13**: 4737-4744, 1994.
29) Levin DE: Regulation of cell wall biogenesis in *Saccharomyces cerevisiae*: the cell wall integrity signaling pathway. Genetics **189**: 1145-1175, 2011.
30) Pradhan A, Avelar GM, Bain JM, Childers DS, Larcombe DE, Netea MG, Shekhova E, Munro CA, Brown GD, Erwig LP, Gow NAR, Brown AJP: Hypoxia promotes immune evasion by triggering β-glucan masking on the *Candida albicans* cell surface via mitochondrial and cAMP-protein kinase A signaling. MBio **9**: e01318-18, 2018.
31) Calderon J, Zavrel M, Ragni E, Fonzi WA, Rupp S, Popolo L: *PHR1*, a pH-regulated gene of *Candida albicans* encoding a glucan-remodelling enzyme, is required for adhesion and invasion. Microbiology **156**: 2484-2494, 2010.
32) Popolo L, Degani G, Camilloni C, Fonzi WA: The PHR family: the role of extracellular transglycosylases in shaping *Candida albicans* cells. J Fungi (Basel) **3**: 59, 2017.
33) Plaine A, Walker L, Da Costa G, Mora-Montes HM, Mckinnon A, Gow NA, Gaillardin C, Munro CA, Richard ML: Functional analysis of *Candida albicans* GPI-anchored protein: role in cell wall integrity and caspofungin sensitivity. Fungal Genet Biol **45**: 1404-1414, 2008.