Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 348 (2006) 206–211
BBRC
www.elsevier.com/locate/ybbrc

Hyphal formation of *Candida albicans* is controlled by electron transfer system

Toshihiko Watanabe *, Ayako Ogasawara, Takeshi Mikami, Tatsuji Matsumoto

Department of Microbiology, Tohoku Pharmaceutical University, 4-4-1, Komatsushima, Aobaku, Sendai 981-8558, Japan

Received 6 July 2006
Available online 24 July 2006

Abstract

Most *Candida albicans* cells cultured in RPMI1640 medium at 37 °C grow in hyphal form in aerobic conditions, but they grow in yeast form in anaerobic conditions. The hyphal growth of *C. albicans* was inhibited in glucose-deficient conditions. Malonic acid, an inhibitor of succinate dehydrogenase, enhanced the yeast proliferation of *C. albicans*, indicating that the hyphal-formation signal was derived from the glycolysis system and the signal was transmitted to the electron transfer system via the citric acid cycle. Thenoyl trifluoro acetone (TTFA), an inhibitor of the signal transmission between complex II and Co Q, significantly inhibited the hyphal growth of *C. albicans*. Antimycin, KCN, and oligomycin, inhibitors of complex III, IV, and V, respectively, did not inhibit the hyphal growth of *C. albicans*. The production of mRNAs for the hyphal formation signal was completely inhibited in anaerobic conditions. These results indicate that the electron transfer system functions upstream of the RAS1 signal pathway and activates the expression of the hyphal formation signal. Since the electron transfer system is inactivated in anaerobic conditions, *C. albicans* grew in yeast form in this condition.

Keywords: *Candida albicans*; Hyphae; Electron transfer system; RAS1; CPH1; EFG1

The opportunistic pathogen *Candida albicans* is an agent that causes infection in the immunocompromised host. *C. albicans* is a dimorphic fungus which transforms from a yeast to a hyphal form depending on the growth conditions [1]. Temperature, pH, and Ca^{2+} are known to be external factors affecting the transformation [2–4]. *C. albicans* has RAS1 protein, which activates factors involved in hyphal formation signaling, such as EFG1 and CPH1 [5–7]. However, the details of the RAS1 activation system have not been clarified. In this study, we demonstrated that the respiration system controlled the activation of RAS1 and other hyphal formation signals.

Materials and methods

Fungus. The *C. albicans* NIH A-207 strain was kindly donated by the Department of Microbiology, Meiji College of Pharmacy, Japan. *C. albicans* TIMM1768 and 2640 were kindly donated by the Institute of Medical Mycology, Teikyo University, Japan. The *C. albicans* JCM 1542, 1621, and 2076 strains were obtained from the Japan Collection of Microorganisms, Riken, Japan. The *C. albicans* IFO 0579 strain was provided by the Institute for Fermentation, Osaka, Japan. These strains were maintained in Sabouraud's liquid medium which was shaken at 27 °C for 24 h. For hyphal proliferation, these *Candida* cells were cultured in RPMI1640 medium (Nissui Pharmaceutical Co. Ltd., Japan) at 37 °C in a humidified atmosphere of 5% CO_2.

Reagents. Malonic acid disodium salt monohydrate (Nacalai Tesque, Inc., Japan), thenoyl-tri-fluoroacetone (TTFA, Sigma Chemical Co.), oligomycin (from *Streptomyces diastatochromogenes*, Nacalai Tesque, Inc.), and antimycin A (from *Streptomyces* sp., Sigma Chemical Co.) were used in this study.

Culture conditions. To produce anaerobic conditions, Na_2SO_3 (final 20 mg/ml) was added to RPMI1640 medium. Na_2SO_3 absorbs oxygen dissolved in RPMI1640 medium ($2Na_2SO_3 + O_2 = 2Na_2SO_4$). AnaeroPack (Mitsubishi Gas Chemical Co. Inc., Japan) was also used to produce anaerobic conditions. AnaeroPack is an absorbent for oxygen which makes the atomosphere in an airtight container anaerobic. *C. albicans* cells suspended in these anaerobic medium (final 1×10^5 cells/ml) were cultured at 37 °C in a humidified atmosphere of 5% CO_2. After the incubation, the growth form of *Candida* cells was examined by photographing the cells with an IX51 microscope (Olympus, Japan). The number of yeast cells in

* Corresponding author. Fax: +81 22275 2013.
E-mail address: twatanab@tohoku-pharm.ac.jp (T. Watanabe).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.07.066

suspension culture was counted using a hemocytometer and the total number of cells was calculated from the optical density (OD) at 620 nm. The absorbance was convert into the number of cells using calibration curves prepared with the yeast cells. The accuracy of this measurement was verified using NucleoCounter™ YC-100 (MS Techono Systems). NucleoCounter™ YC-100 can count the amount of nucleus and converted into the number of cells. When the number of hyphal cells of *C. albicans* was counted using this system, the resulted number was similar to the number calculated from the absorbance (data not shown).

Quantification of mRNAs in C. albicans. *C. albicans* (1×10^5 cells/ml in RPMI1640 medium) was incubated at 37 °C in 5% CO_2 for 1, 3, 5, and 7 h. After the incubation, the cells were harvested by centrifugation, and total RNA was extracted using a Total RNA Purification kit MagExtractor (Toyobo, Japan). To prepare cDNA, total RNA was mixed with dNTP mix (Toyobo) and random hexamers (Roche) at 70 °C for 3 min. M-MLV Reverse Transcriptase (Ambion), 10× RT buffer (Ambion), and RNase inhibitor (Promega) were added to this mixture and incubated at 42 °C for 1 h. The reaction was terminated by heating at 92 °C for 10 min and the resulting mixture was used as cDNA solution. The relative expression of the target gene in the cDNA library was analyzed using the 7500 Real Time PCR System (Applied Biosystems). The gene arrangement was checked using the Entrez System of the National Center for Biotechnology Information (USA). The PCR primers and TaqMan MGB probe were designed using Primer Express (Applied Biosystems). The results are shown as relative expression compared to 18 s rRNA.

Statistical analysis. Values are shown as means ± SE, and statistical analysis of these data was performed using Student's *t*-test. A value of $P < 0.05$ was considered significant.

Results

Growth form of C. albicans cultured in anaerobic conditions

To produce anaerobic conditions, Na_2SO_3 was added to RPMI1640 medium. Twenty milligrams of Na_2SO_3 per ml helped keep this medium in an aerobic condition for 24 h (data not shown). *C. albicans* NIH A207 strain cultured in RPMI1640 grew in hyphal form in aerobic conditions, but grew in the yeast form upon addition of sodium sulfite (Fig. 1). Sodium sulfite has mutagenic activity, and therefore we examined whether the yeast form of *C. albicans* was a variant or not. The yeast cells induced by sodium sulfite transformed to hyphal cells in RPMI1640 in the absence of sodium sulfite (data not shown), indicating that this yeast induction was not due to the mutagenic activity of sodium sulfite. To examine the fraction of yeast cells in the culture suspension, the number of yeast cells was measured using a hemocytometer and the total number of *C. albicans* cells was calculated from the absorbance at 620 nm. The results revealed that most cells in *C. albicans* NIH A207 strain grew in the hyphal form in aerobic conditions and in the yeast form in anaerobic conditions (Table 1). To confirm that this yeast induction was due to anaerobic

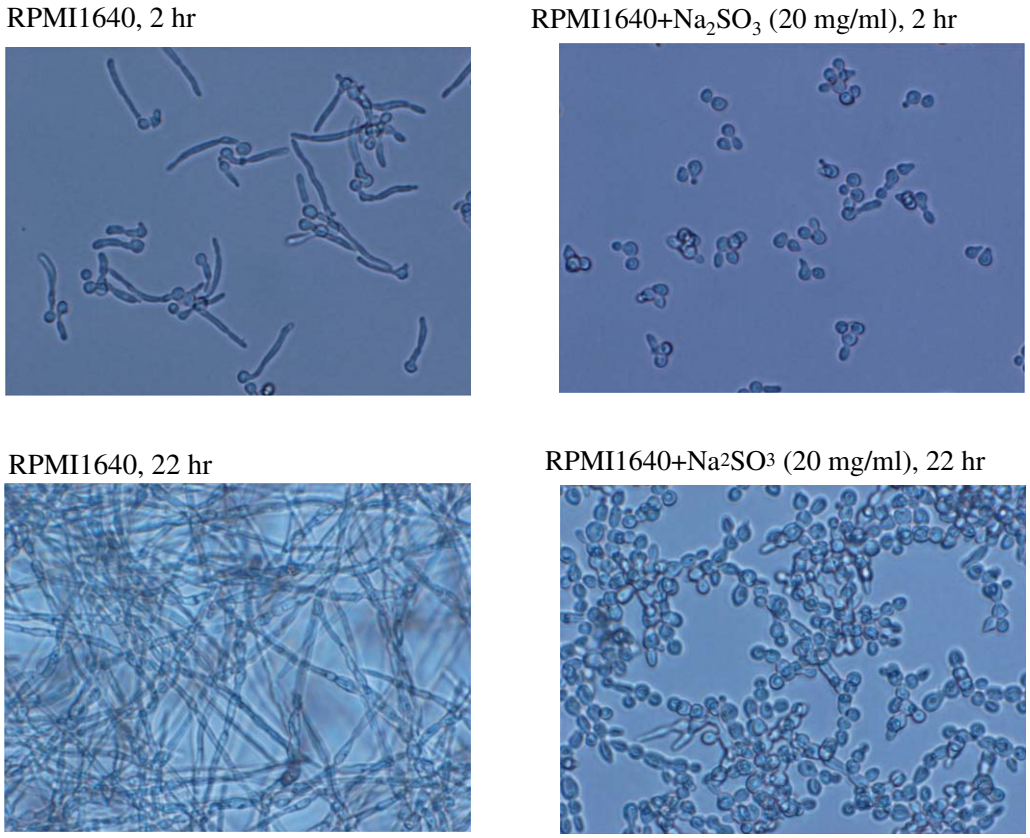

Fig. 1. Growth form of *C. albicans* NIH A-207 cultured in aerobic or anaerobic conditions. *C. albicans* (1×10^5 cells/ml in RPMI1640) was cultured at 37 °C for 2 or 22 h in anaerobic conditions induced by Na_2SO_3 (20 mg/ml). After the incubation, the growth form of *Candida* cells was examined by photographing the cultures with an IX51 microscope (400×).

Table 1
Effect of Na_2SO_3 on hyphal growth of *C. albicans* NIH A-207 in RPMI1640*

	Number of yeast cells ($\times 10^4$ cells/ml)	Number of total cells ($\times 10^4$ cells/ml)	(Yeast/total) × 100
Control	1 ± 1	583 ± 13	0
Na_2SO_3 (20 mg/ml)	384 ± 42	366 ± 30	105
AnaeroPac	187 ± 40	210 ± 25	97

* *C. albicans* (1×10^6 cells/ml in RPMI1640) was cultured at 37 °C for 24 h in anaerobic condition induced by Na_2SO_3 or AnaeroPack. After the incubation, the number of yeast cells and total cells were measured. AnaeroPack is an absorbent of oxygen, which make anaerobic condition in hermetic container.

Table 2
Effect of Na_2SO_3 on hyphal growth of *C. albicans* in RPMI1640*

Strain	Aerobic	Anaerobic
NIH A207	H	Y
TIMM1768	H	Y
JCM2076	H	Y
TIMM2640	H	Y
IFO0579	H	H,Y
JCM1542	H,Y	Y
JCM1621	H,Y	H,Y

H, Hyphae; Y, Yeast.
* *C. albicans* strains were cultured in RPMI1640 with/without Na_2SO_3 at 37 °C for 20 h. After the incubation, the growth form was examined.

cultivation, *C. albicans* NIH-A207 was cultured in an air-tight container which was made anaerobic using an oxygen absorbent. The yeast proliferation induction of *C. albicans* was also induced in the anaerobic conditions (Table 1), indicating that anaerobic cultivation was the cause of yeast induction. To examine whether the hyphal growth of other *C. albicans* strains was also controlled by oxygen, we examined the growth form of seven strains of dimorphic *C. albicans* in anaerobic conditions. These strains grew in the hyphal form in the aerobic condition and the hyphal transformation of them was inhibited in anaerobic conditions (Table 2), indicating that the hyphal formation signal of *C. albicans* was controlled by oxygen.

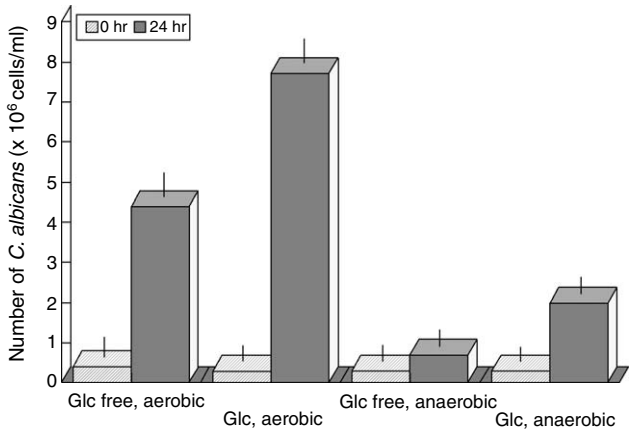

Fig. 2. Effect of glucose on growth of *C. albicans* NIH A-207 in aerobic or anaerobic conditions. *C. albicans* (1×10^5 cells/ml in glucose free-RPMI1640 or RPMI1640) was cultured at 37 °C for 24 h in aerobic conditions or anaerobic conditions induced by Na_2SO_3. After the incubation, the number of cells was measured as described in Materials and methods.

Effect of glucose on anaerobic growth of C. albicans

To study the growth system of *C. albicans* in anaerobic conditions, glucose-free RPMI1640 was used. *C. albicans* was cultured in glucose-free-RPMI1640 with/without glucose (final 2 mg/ml), and the growth rate was measured. In aerobic conditions, *C. albicans* was able to grow in glucose-free-RPMI1640 medium, suggesting that *C. albicans* could produce ATP using a glycolysis-independent system (Fig. 2). When *C. albicans* was cultured in anaerobic condition, it could grow slowly in anaerobic condition with glucose, but not in the glucose-free conditions. These results indicated that the electron transfer system and the oxidative phosphorylation of *C. albicans* were inactivated in the anaerobic conditions Fig. 3.

Restriction of the hyphal growth by glycolysis and citric acid cycle

The fact that the proliferation of *C. albicans* was dependent on glycolysis in anaerobic conditions suggested that the hyphal transformation was also controlled by glucose. To test this hypothesis, we examined the growth form of *C. albicans* cultured in glucose-free RPMI1640 medium with/without glucose. As shown in Table 3, the yeast cell proliferation was increased in the glucose-free conditions, indicating that the signal derived from glycolysis increased the hyphal transformation. Moreover, we examined the

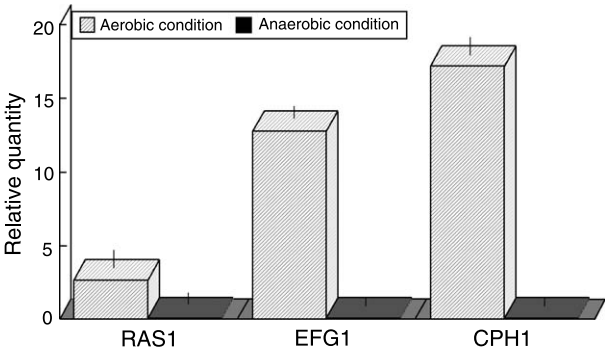

Fig. 3. Relative expression of mRNAs of *C. albicans* NIH A-207 cultured in aerobic or anaerobic condition. *C. albicans* (1×10^5 cells/ml in glucose free RPMI1640 or RPMI1640) was cultured at 37 °C for 3 h in aerobic conditions or anaerobic conditions induced by Na_2SO_3. After the incubation, the relative expression level of mRNAs was measured.

Table 3
Effect of malonic acid on hyphal growth of *C. albicans* NIH A-207 cultured in RPMI1640*

	Malonic acid (final, mg/ml)		
	0	1	10
Glucose-free RPMI1640			
Number of yeast cells ($\times 10^4$ cells/ml)	31 ± 4	63 ± 6	78 ± 7
Number of total cells ($\times 10^4$ cells/ml)	83 ± 21	145 ± 44	118 ± 19
(Yeast/total) × l00	37	43	66
RPMI640 (glucose, 2 mg/ml)			
Number of yeast cells ($\times 10^4$ cells/ml)	2 ± 1	29 ± 3	35 ± 4
Number of total cells ($\times 10^4$ cells/ml)	370 ± 82	570 ± 166	368 ± 121
(Yeast/total) × l00	1	5	10

* *C. albicans* (1×10^5 cells/ml in glucose-free RPMI 1640 or RPMI 1640) was cultured at 37 °C for 24 h in aerobic conditions with or without malonic acid. After the incubation, the numbers of yeast cells and total cells were measured.

relationship of the citric acid cycle to the hyphal transformation. Malonic acid is an inhibitor of succinate dehydrogenase in the citric acid cycle and inhibits the metabolism of succinic acid to fumaric acid [8]. The growth of yeast cells in glucose-free conditions was enhanced by the addition of malonic acid. These results suggest that the hyphal-formation signal from the glycolysis system is transmitted to the electron transfer system through the citric acid cycle.

Effect of inhibitors of electron transfer system on the growth form of C. albicans

We examined the effect of various inhibitors of the electron transfer system on the growth form of *C. albicans*. Rotenone, an inhibitor of complex I in the electron transfer

Table 4
Effect of mitochondrial respiration inhibitor on hyphal growth of *C. albicans* NIH A-207 in RPMI1640*

	Number of yeast cells ($\times 10^4$ cells/ml)	Number of total cells ($\times 10^4$ cells/ml)	(Yeast/total) × 100
Control	2 ± 1	300 ± 7	0
Rotenone (0.01 mg/ml)	7 ± 2	633 ± 132	1
TTFA (0.1 mg/ml)	763 ± 51	698 ± 41	109
Antimycin (0.1 mg/ml)	49 ± 5	370 ± 22	13
KCN (0.02 mg/ml)	6 ± 1	155 ± 41	4
Oligomycin (0.1 mg/ml)	38 ± 5	448 ± 70	8

* *C. albicans* (1×10^5 cells/ml in RPMI1640) was cultured at 37 °C for 24 h in aerobic conditions with various respiration inhibitors. After the incubation, the numbers of yeast cells and total cells were measured.

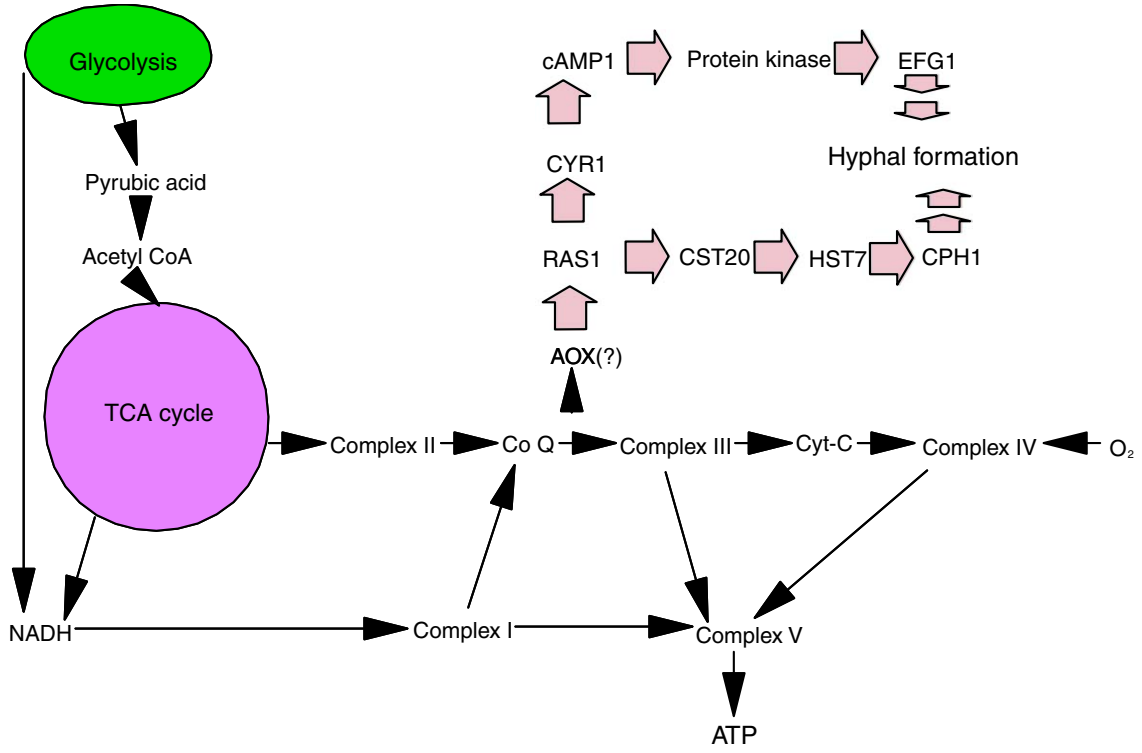

Fig. 4. Tentative signal transduction for hyphal formation of *C. albicans*.

system [9], did not inhibit the hyphal growth of *C. albicans* (Table 4). Thenoyl trifluoro acetone (TTFA), which inhibits the signal transmission between complex II and Co Q [10], significantly inhibited the hyphal growth of *C. albicans*. Antimycin, KCN, and oligomycin are complex III, complex IV, and complex V inhibitors, respectively [11–13]. These inhibitors also did not affect the hyphal growth of *C. albicans*.

These results indicate that the signal of hyphal transformation was derived at a signaling step between these involving Co Q and complex III.

Analysis of hyphal formation signal in C. albicans cultured in anaerobic conditions

The hyphal formation of *C. albicans* is regulated by the MAP kinase cascade and cAMP pathway, which are activated by RAS1 protein [14]. The expression of CPH1, a component of the MAP kinase cascade, and EFG1, a component of the cAMP pathway, are increased during the hyphal formation [15]. Therefore, we measured the expression of these mRNAs (CPH1, EFG1, and RAS1) as an indicator of the hyphal formation signal. *C. albicans* NIH A207 was cultured in aerobic or anaerobic conditions and the amounts of these mRNAs were measured using the 7500 Real-Time PCR System.

When the cells were cultured in anaerobic conditions, the expression of these mRNAs was completely inhibited (Fig. 4), indicating that the hyphal formation signals were activated in aerobic conditions, but not in anaerobic conditions.

Discussion

Candida albicans hyphal formation is known to be induced by RAS-signal transmission, but there is no reported evidence about what regulates RAS activation. In this study, we demonstrated that oxygen was essential to form hyphal cells of *C. albicans* and analyzed the relationship between respiration and RAS activation.

If the temperature, pH, and nutrition are appropriate, *C. albicans* NIH A-207 grows in the hyphal form in aerobic conditions. The hyphal formation was shown here to be prevented by a deficiency of oxygen (Fig. 1, Table 1). Other dimorphic *C. albicans* strains also showed the inhibition of the hyphal formation in anaerobic conditions (Table 2). These results indicate that the dimorphic system is regulated by respiration.

To examine the mode of respiration of *C. albicans*, the cells were cultured aerobically or anaerobically in glucose-free RPMI1640 medium. *C. albicans* could aerobically grow without glucose (Fig. 2), indicating that the system of ATP production was able to function without glycolysis in aerobic conditions. On the other hand, glucose was essential for anaerobic proliferation, which implies that the parts of the respiration system other than glycolysis, namely, the citric acid cycle, electron transfer system, and oxidative phosphorylation, were inactivated in anaerobic conditions. As hyphal formation was inhibited in anaerobic conditions, the signal of hyphal formation might be derived from the respiration system. The fact that the hyphal formation of *C. albicans* was inhibited in the absence of glucose supports this hypothesis (Table 3). The hyphal formation was also inhibited by malonic acid, a citric acid cycle inhibitor. These results indicate that the hyphal formation signal was derived at a step between the electron transfer system and oxidative phosphorylation.

The hyphal formation of *C. albicans* was inhibited by TTFA, but not other inhibitors of the electron transfer system or oxidative phosphorylation (Table 4). TTFA inhibits the signal transmission between Co Q and Complex III [10]. The electron transmission forks between Co Q and Complex III via an alternative oxidase (AOX1) [16], suggesting that AOX regulates the hyphal formation.

In conclusion, the present results indicated that the electron transfer system functioned upstream of the RAS1 signal pathway and activated the expression of the hyphal formation signal (Fig. 4). Since the electron transfer system was inactivated in anaerobic conditions, *C. albicans* grew in yeast form in such conditions.

References

[1] E. Blasi, L. Pitzurra, A. Bartoli, M. Puliti, F. Bistoni, Tumor necrosis factor as an autocrine and paracrine signal controlling the macrophage secretory response to*Candida albicans*, Infect. Immun. 62 (1994) 1199–1206.

[2] V. Maneu, P. Roig, D. Gozalbo, Complementation of *Saccharomyces cerevisiae* mutations in genes involved in translation and protein folding (EFB1 and SSB1) with *Candida albicans* cloned genes, Res. Microbiol. 151 (2000) 739–746.

[3] A. Porta, A.M. Ramon, W.A. Fonzi, PRR1, a homolog of *Aspergillus nidulans* palF, controls pH-dependent gene expression and filamentation in *Candida albicans*, J. Bacteriol. 181 (1999) 7516–7523.

[4] A.R. Holmes, R.D. Cannon, M.G. Shepherd, Effect of calcium ion uptake on *Candida albicans* morphology, FEMS Microbiol. Lett. 15 (1991) 187–193.

[5] Q. Feng, E. Summers, B. Guo, G. Fink, Ras signaling is required for serum-induced hyphal differentiation in *Candida albicans*, J. Bacteriol. 181 (1999) 6339–6346.

[6] P. Jain, I. Akula, T. Edlind, Cyclic AMP signaling pathway modulates susceptibility of *Candida* species and *Saccharomyces cerevisiae* to antifungal azoles and other sterol biosynthesis inhibitors, Antimicrob. Agents Chemother. 47 (2003) 3195–3201.

[7] E. Leberer, D. Harcus, D. Dignard, L. Johnson, S. Ushinsky, D.Y. Thomas, K. Schroppel, Ras links cellular morphogenesis to virulence by regulation of the MAP kinase and cAMP signalling pathways in the pathogenic fungus *Candida albicans*, Mol. Microbiol. 42 (2001) 673–687.

[8] A.A. Dukes, K.M. Korwek, T.G. Hastings, The effect of endogenous dopamine in rotenone-induced toxicity in PC12 cells, Antioxid. Redox Signal. 7 (2005) 630–638.

[9] J.G. Zhang, M.W. Fariss, Thenoyltrifluoroacetone, a potent inhibitor of carboxylesterase activity, Biochem. Pharmacol. 15 (2002) 751–754.

[10] K. Biswas, J. Morschhauser, The Mep2p ammonium permease controls nitrogen starvation-induced filamentous growth in *Candida albicans*, Mol. Microbiol. 56 (2005) 649–669.

[11] B. Lai, L. Zhang, L.Y. Dong, Y.H. Zhu, F.Y. Sun, P. Zheng, Inhibition of Qi site of mitochondrial complex III with antimycin A decreases persistent and transient sodium currents via reactive oxygen species and protein kinase C in rat hippocampal CA1 cells, Exp. Neurol. 194 (2005) 484–494.
[12] E.F. Bloch, M. Rahbar, A.K. Wright, A.M. Patterson, R.F. Souza, C.H. Hammer, T.A. Gaither, K.A. Joiner, Potassium cyanide protects *Escherichia coli* from complement killing by the inhibition of C3 convertase activity, Immunol. Invest. 22 (1993) 127–149.
[13] B.W. Kim, H.J. Choo, J.W. Lee, J.H. Kim, Y.G. Ko, Extracellular ATP is generated by ATP synthase complex in adipocyte lipid rafts, Exp. Mol. Med. 36 (2004) 476–485.
[14] T. Sato, T. Watanabe, T. Mikami, T. Matsumoto, Farnesol, a morphogenetic autoregulatory substance in the dimorphic fungus *Candida albicans*, inhibits hyphae growth through suppression of a mitogen-activated protein kinase cascade, Biol. Pharm. Bull. 27 (2004) 751–752.
[15] E.J. Helmerhorst, M. Stan, M.P. Murphy, F. Sherman, F.G. Oppenheim, The concomitant expression and availability of conventional and alternative, cyanide-insensitive, respiratory pathways in *Candida albicans*, Mitochondrion 5 (2005) 200–211.
[16] W.K. Huh, S.O. Kang, Molecular cloning and functional expression of alternative oxidase from *Candida albicans*, J. Bacteriol. 181 (1999) 4098–4102.