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Effect of Environmental Stress on Morphological Change of an Extremely Cadmium-Tolerant Yeast, *Hansenula anomala* B-7

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Abstract An extremely cadmium-tolerant budding yeast, *Hansenula anomala* B-7 underwent a morphological switch in response to either heat shock treatment or cadmium stress, respectively. It exhibited a morphological transition from a unicellular yeast form to a pseudohyphae-like coagulation when subjected to prolonged heat shock treatment. In contrast, the yeast cells showed an irregularity in surface morphology when given thermal stress for a short time. Patterns of proteins expressed in the pseudohyphae-like cells demonstrated that several proteins were overexpressed while others were underexpressed in comparison with those prepared from the cells in the yeast form. It was a striking feature, however, that nearly 40% of the proteins extracted from the cells in the pseudohyphae form appeared to be composed of a single polypeptide. This polypeptide was apparently overexpressed during the pseudohyphae phase and its molecular weight was estimated to be 58 kDa according to SDS-PAGE analysis. However, a significant level of the protein was not observed in the cells before transition to pseudohyphae. The architecture of the cell shape was also damaged when incubated in a medium containing more than 1,000 ppm (8.9 mM) of cadmium ions, although able to proliferate at a slow rate. However, the irregularity in the cell morphology exerted either by the brief heat shock treatment or by the cadmium stress with the high concentrations of the metal ions was not repaired, even though the damaged cells were allowed to grow for sufficient time in fresh, cadmium-free medium.

Key words: Environmental stress, cadmium-tolerant yeast, morphological transition, yeast form, pseudohyphae form, pseudohyphae-specific protein

From microorganisms to plants and mammals, a group of proteins called stress-responsible proteins have been synthesized in response to environmental stress such as heat shock stress, heavy metals, and carbon starvation. The synthesis of such stress-responsible proteins are induced very rapidly to protect the cells against toxicity caused by the stresses, thereby enabling the organisms to survive in harmful environmental conditions [2, 3, 10, 12, 21]. The response is one of the most highly conserved genetic control systems, particularly the synthesis of a metal binding protein known as metallothionein (MT), and heat shock proteins (hsp) in response to heavy metals and elevated temperature, respectively.

Dimorphism has been used to define several fungi that can grow vegetatively in either a unicellular yeast form or a multicellular filamentous form called pseudohyphae [8, 14]. A pseudohyphae is defined as a fragile chain of cells (usually yeasts, which have arisen by budding and have elongated without detaching from adjacent cells), with morphological characteristics intermediate between a chain of yeast cells and hyphae [8]. Dimorphism is a key feature of several pathogenic fungi [22] and is believed to be associated with pathogenicity in *Candida albicans* [5]. The worldwide resurgence of *Candidiasis* gives an emphasis on the importance of a non-pathogenic model with genetically more tractable organism such as *Saccharomyces cerevisiae* [26].

In *S. cerevisiae*, starvation for nitrogen was found to induce the dimorphic transition via the pheromone-response mitogen activated protein (MAP) kinase cascade [17]. The signal for the switch from yeast to pseudohyphae seems complex because several reports indicate that filamentous growth was not nitrogen dependent. For example, when *KIR*, one of the Ras-family G protein genes, is expressed, the MAP kinase pathway is activated and pseudohyphae form, but this pathway is not nitrogen dependent [6].

Recent work on the budding yeast, *S. cerevisiae*, has revealed that activation of Ras results in elevated intracelluar cAMP levels that in turn activate the A kinase,

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which is composed of an inhibitory subunit Bcy1 and a catalytic subunit encoded by three redundant genes. Activation of the A kinase, either by a dominant $Ras2^{Val19}$ mutation or *bcy1* null mutation confers sensitivity to heat shock and nutrient starvation, loss of carbohydrate reserves, and a block to sporulation [4, 20]. In addition, activated $Ras2^{Val19}$ stimulates filamentous growth in response to starvation for nitrogen [8], suggesting that there may be some relation between heat shock and filamentous growth in *S. cerevisiae.*

There has been considerable discrepancy, however, in defining the role of dimorphism. Ginemo *et al.* [8] suggested that the role of pseudohyphae in *S. cerevisiae* was to accelerate foraging for nutrients and substrates at a distance from their initial colonization site. In contrast, on the basis of observation that pseudohyphae only form in continuous culture under oxygen limitation but not under nitrogen limitation, some insist that dimorphism should be regarded as a response to environmental conditions rather than foraging for nutrients in *S. cerevisiae* [26]. However, there has been no report yet demonstrating that high concentrations of cadmium can produce morphological irregularity in unicellular yeast *S. cerevisiae,* although the metal cadmium is of no known biological utility but highly toxic at relatively low concentrations [13, 14, 18].

Little is known about morphological transition in extremely cadmium-resistant yeast, *Hansenula anomala* B-7, induced by environmental stress such as high concentrations of cadmium or thermal stress. *H. anomala* B-7 is the same budding yeast as *S. cerevisiae* but this yeast not only can accomodate growth in a medium supplemented with 10 mM of highly toxic cadmium ions but also absorb upto 30 mg/g dry cell of the metal ions [11, 25]. *Hansenula anomala* B-7 though the same budding yeast as *S. cerevisiae* not only can accomodate growth in a medium supplemented with 10 mM of highly toxic cadmium ions but also absorb upto 30 mg/g dry cell of the metal ions [11, 25]. However, little is known about morphological transition induced by environmental stress such as high concentrations of cadmium or thermal stress in this extremely cadmium-resistant yeast. To explore the role of the environmental stresses in morphological transition, *H. anomala* B-7 cells were subjected either to heat shock at 43°C or to various concentrations of cadmium ions. The treatment revealed that in *H. anomala* B-7, more than 10 mM of cadmium in the medium induced severe irregularity in cell morphology and the damage in cellular architecture seemed to be permanent. The cells grown in the presence of 500 ppm (4.5 mM) of cadmium ions, however, did not show the irregularity in cell shape. The cells given heat shock stress for more than 4 h also showed the abnormality in cell morphology, which appeared to be non-repairable. Prolonged heat shock treatment, however, conferred a morphological transition from a unicellular yeast form to a filamentous pseudohyphae-like coagulation on the cells which appeared to more resemble the hyphae observed in *Candida albicans* rather than the pseudohyphae form known to be induced by nitrogen starvation in the same budding yeast S. cerevisiae. Protein expression patterns in the hyphae-like coagulation form demonstrated that some proteins were overexpressed while others underexpressed. Interestingly, a polypeptide with molecular weight of 58 kDa was remarkably overexpressed and comprised approximately 40% of the total proteins prepared from the cells undergoing morphological transition from the yeast to the pseudohyphae-like form. Taken together, these findings lead us to suggest that a novel mechanism playing a crucial role in the induction of morphological transition in response to environmental stress exists in extremely cadmium-resistant yeast *H. anomala* B-7 which has not yet been revealed in the same budding yeast *S. cerevisiae.*

MATERIALS AND METHODS

Cells, Growth Conditions, and Heat Shock Protocols

A strain of extremely cadmium-resistant and budding yeast, *Hansenula anomala* B-7, was obtained from Dr. Tae-Shick Yu in the Department of Microbiology, Keimyung University, Taegu, Korea for this work. The morphological and cultural properties of cells have been characterized by Yu *et al.* [25]. The cells were grown in the in the YPD medium (1% glucose, 1% polypeptone, 0.5% yeast extract, 0.1% NaCl, 0.03% $MgSO_4 \cdot 7H_2O$, 0.01% KH_2PO_4, pH 6.0) as previously described by Huh *et al.* [11] at 28°C unless otherwise specified. The cultured cells were harvested by centrifugation at 1,500×g for 10 min at 4°C, and washed three times with ice cold water as described [24]. The cell growth was determined by measuring either optical density at 600 nm or the weight of cell mass after drying overnight at 105°C under vacuum. To give cells heat shock stress, the cells grown at 28°C to an optical density of 1.0 at 600 nm were transferred to a glass flask prewarmed to 43°C in a shaking water bath, and cultivation was continued for the time indicated in the text at the same temperature.

Scanning Electron Microscopy (SEM) Methods

Yeast cells proliferating in liquid media were transferred to small squares of wet filter paper. The cells on the paper were then fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2) at 24°C for 60 min and dehydrated in a graded ethanol series at the same temperature. SEM was performed with a scanning electron microscope

(Hitachi 600) and images were photographed on Polaroid 55 film as described [8].

Preparation of Yeast Protein

Extraction of proteins from the yeast was carried out as described previously with the following modifications [24]. The cell pellets washed with ice-cold deionized water were suspended in three volumes of cell disruption buffer (200 mM Tris HCl, pH 8.0, 10% glycerol, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1 mM PMSF) [7]. Then, 4 volumes of chilled acid-washed glass beads (0.45 to 0.55 mm, Sigma) were added. The cells were disrupted by vortexing with maximum speed at 4°C until 30% of the cells were broken. The cell debris were removed by centrifugation at 12,000×g for 60 min [1]. Protein concentrations were determined by the Bradford assay (Bio-Rad).

SDS-PAGE Analysis

SDS-polyacrylamide gel electrophoresis (PAGE) was performed by a modification of the procedure of Laemmli [16] as follows: Proteins were boiled in the presence of 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (w/v) glycerol for 4 min, and were applied onto a 12% SDS-PAGE slab gel prepared according to a standard protocol [10]. Protein bands were visualized by the Coomassie Brilliant blue (R250) staining method. Low molecular weight polypeptide standards for SDS-PAGE (molecular weight range; 10,000~100,000, Bio-Rad) were resolved together with protein preparations to estimate the molecular weight of the target bands detected in gels.

RESULTS

Effect of Heat Shock Treatment on Cell Surface Morphology

Effect of heat shock treatment on the change of cell surface morphology was investigated with the *H. anomala* B-7 cells grown under various conditions but in the cadmium-free standard medium (Fig. 1). Characterization of morphological change induced by thermal stress in the cadmium-resistant yeast cell was performed by scanning electron microscopic analysis. For this experiment, the cells were grown under normal condition at 28°C to an optical density of 1.0 at 600 nm (Fig. 1A) and then subjected to heat shock treatment by incubation at 43°C for various times upto 200 min. A part of the cells grown at 43°C for 200 min for prolonged heat shock treatment was shifted to 28°C, and cultivation was continued for another 500 min to allow for recovering from the heat shock treatment (Fig. 1C). Then, the heat shock-treated cells were harvested for SEM analysis. The morphology of the cells given the heat shock as shortly as 30 min was not changed significantly in comparison with the cells grown at normal growth temperature (data not shown). However, the cells, which received the heat shock treatment for 200 min, revealed severe damage on

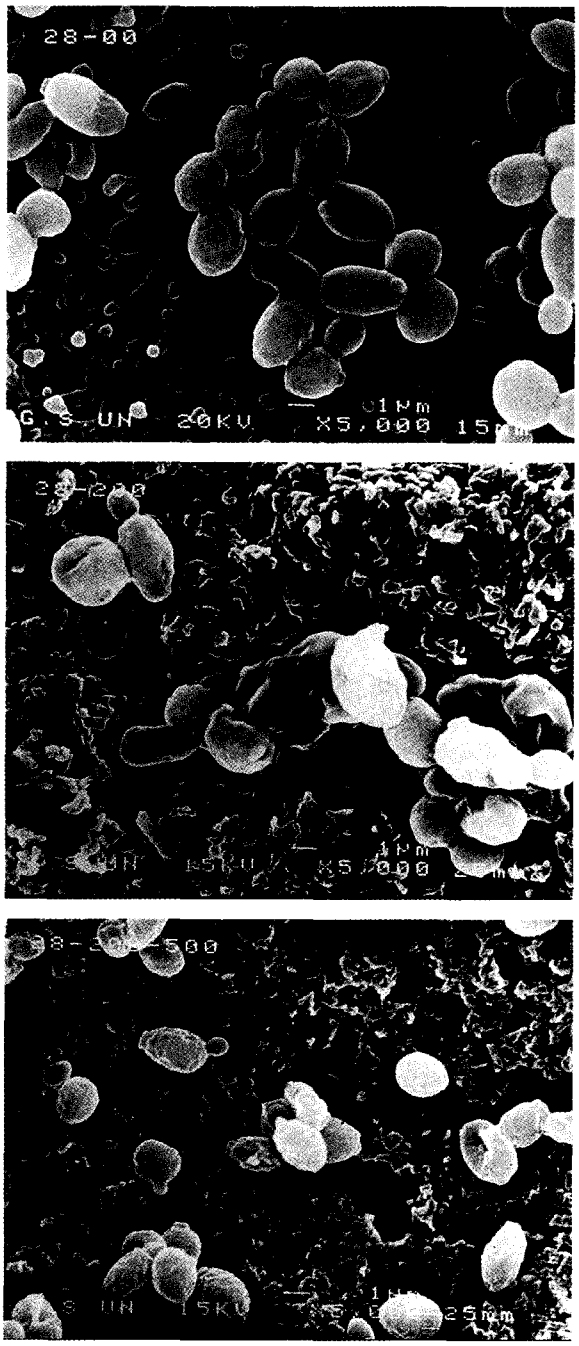

Fig. 1. Scanning electron microscopy (SEM) of a cadmium-resistant yeast *Hansenula anomala* B-7 treated with heat shock.

The cells grown at 28°C upto 1.0 by OD at 600 nm were transferred to 43°C and further incubated for either 0 min (panel A), or 200 min (panel B), respectively. A part of the cells treated with the heat shock at 43°C for 200 min was moved to 28°C and cultivation was continued for 500 min (panel C). Morphological change was investigated by SEM analysis.

cell surface architecture (Fig. 1B). Furthermore, the damaged cells were not able to return to a healthy morphology completely, although they were allowed to grow at normal growth temperature (28°C) as long as for 500 min after being subjected to heat shock treatment (Fig. 1C).

Effect of Cadmium Shock on Cell Morphology Change

To investigate the effect of Cd^{2+} ions on cell morphology change, cells were grown in the cadmium-free medium to mid-log phase, and subsequently spun down to remove the medium. Then, the pelleted cells were resuspended in the fresh medium containing 1,000 ppm of cadmium ions and were incubated further for another 2 days (Fig. 2A), or to mid-log phase, for which it took another 4 or 5 days (Fig. 2B), respectively, at 28°C. The cells were then harvested to be fixed for SEM analysis as described in Materials and Methods. On the other hand, a part of the cells grown to mid-log phase in the presence of 1,000 ppm cadmium was harvested and subsequently transferred to the fresh, cadmium-free medium for further cultivation for another 1 day (Fig. 3A), or 4 days (Fig. 3B), respectively, under the normal growth condition. The morphology of the cells was also characterized by SEM analysis as described.

The SEM analysis revealed that when the healthy cells cultivated to mid-log phase in the cadmium-free medium were shocked with 1,000 ppm of cadmium ions for upto 2 days, they did not show a significant irregularity in cell phenotype (Fig. 2A), although proliferated at 1/3 to 1/4 of the growth rate in the cadmium-free medium [11]. The cells, however, appeared to receive a mild damage in morphology when being subjected to the cadmium shock

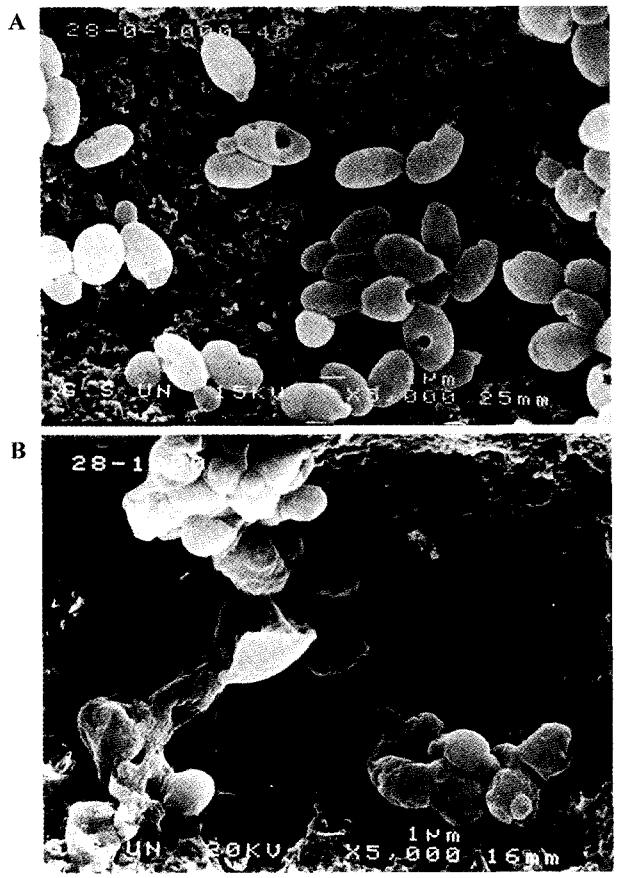

Fig. 2. Effect of cadmium treatment on morphological change in the cadmium-resistant yeast, *H. anomala* B-7.

The yeast cells grown in a cadmium-free medium to mid-log phase were spun down to replace the medium with the fresh ones but supplemented with 1,000 ppm (8.9 mM) of Cd^{2+} ions. The concentration of the cells in the fresh media was adjusted to an optical density of 1.0 at 600 nm and subsequently subjected to cadmium treatment by incubation for 2 days (panel A) or to mid-log phase (panel B), respectively. The cells were harvested as described in Materials and Methods SEM analysis.

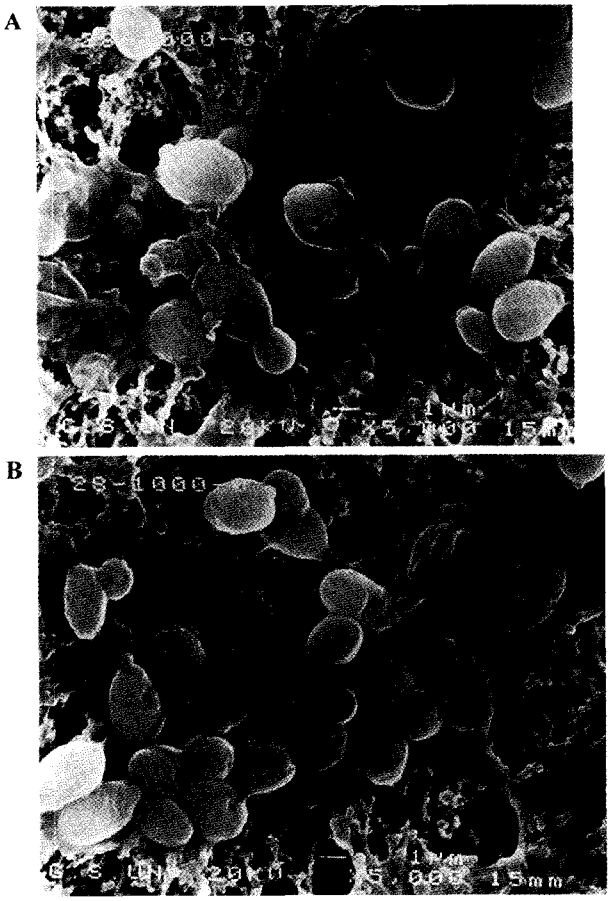

Fig. 3. Effect of incubation of the cells in a fresh medium on repair of the cellular architecture destructed by high concentrations of cadmium ions.

The yeast cells of *H. anomala* B-7 treated with 1,000 ppm (8.9 mM) of cadmium ions to mid-log phase as described at the legend of Fig. 2 were resuspended in the fresh cadmium-free medium and further incubated for 1 day (panel A) or 4 days (panel B), respectively. Cellular architecture was characterized by SEM as described in Materials and Methods.

for 4 days at the same concentration of the metal ions (data not shown), and a severe damage if given the cadmium shock until they were grown to mid-log phase (Fig. 2B). Furthermore, the damage derived from the cadmium shock given to the mid-log phase seemed to be permanent because they did not restore the normal cellular architecture, although they were allowed to grow in the cadmium-free medium even for 4 days further (Fig. 3B). Interestingly, destruction of cell wall and leakage of intracellular substances which could lead to formation of ghost-like cells or cell lysis was observed in the cells treated with the high concentrations of cadmium ions (Fig. 2B). However, we can not rule out the possibility that the structure revealed in the Figs. 2B and 3, respectively, is a phenomenon resulted from the cell fusion through cell-cell interaction which may be an intermediate phenotype produced during the transition from the unicellular yeast to the multicellular pseudohyphae.

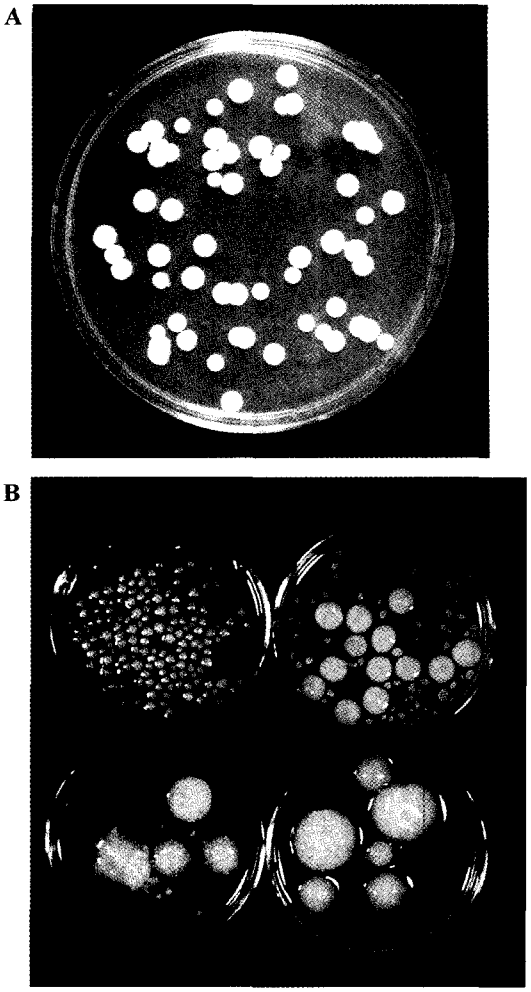

Fig. 4. Morphological phenotype of the yeast cells produced by heat shock treatment.

The yeast cells grown at 28°C to an optical density of 1.0 at 600 nm were either transferred to the YPD-agar medium for formation of colonies at the same temperature (panel A), or subjected to heat shock treatment by incubation at 43°C for 4 days resulting in formation of pseudohyphae-like coagulation from unicellular yeast cells. Various forms of the coagulated cells were photographed after brief sonication (panel B).

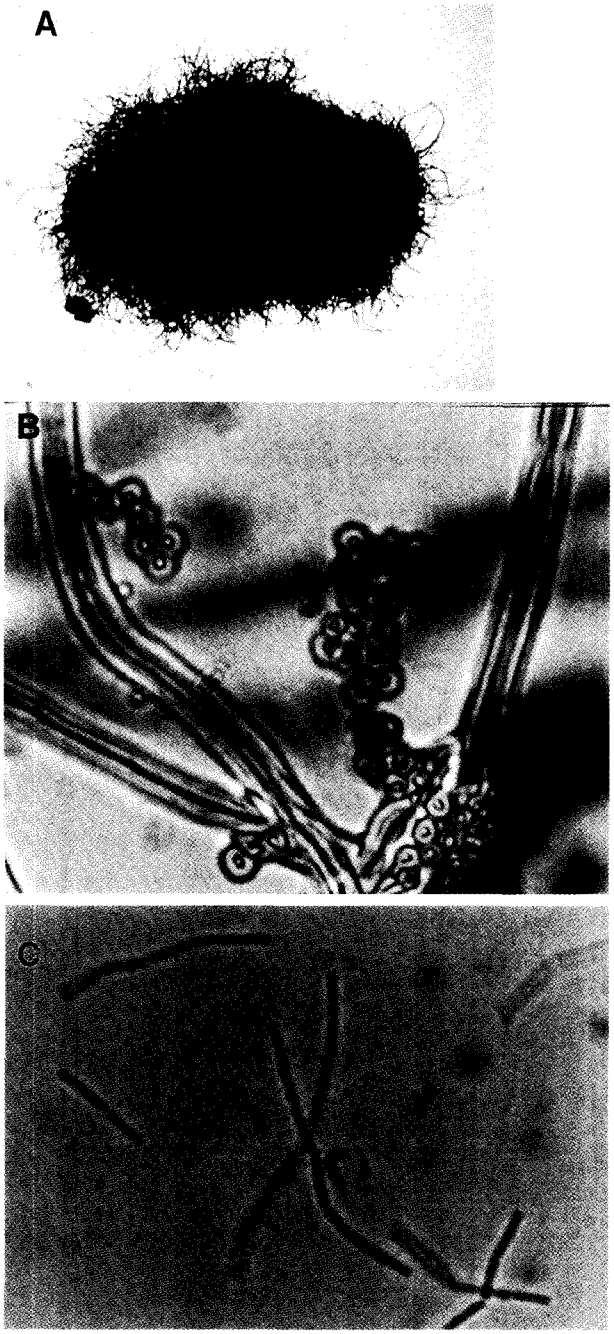

Fig. 5. Photo-microscopic analysis of the *H. anomala* B-7 cells in pseudohyphal coagulation form.

An enlargement view of the pseudohyphal growth was photographed with magnification of ×40 (panel A) and a part of the morphology revealed at the top of the panel A was examined by brightfield micrography with magnification of ×2,000 (panel B). The cells in the course of dimorphic transition from unicellular yeast cells to filamentous pseudohyphal growth were also micrographed with magnification of ×500 (panel C).

Transition of Morphology from Yeast to Pseudohyphae by Heat Shock

On the basis of the findings demonstrated above by SEM analysis, the extremely cadmium-resistant yeast *H. anomala* B-7 was believed to receive non-repairable damage in cellular architecture (Fig. 1C) as well as in cell growth [11] when given heat shock treatment for more than 200 min at 43°C. Furthermore, the cells were not able to return to the healthy morphology, even though they were allowed to grow at normal growth temperature (28°C) for more than 500 min after being subjected to the heat shock treatment. Therefore, it will be of interest to examine whether prolonged heat shock treatment induces morphological transition or not, because *S. cerevisiae*, the same budding yeast as *H. anomala* B-7, has been revealed to be a dimorphic fungus capable of interconverting between a unicellular yeast form and a multicellular filamentous form called a pseudohyphae [8].

When *H. anomala* B-7 cells in liquid culture were subjected to heat shock for more than 4 days, the cells started showing morphological transition from unicellular yeast resulting in formation of big, coagulated substances (Fig. 4), which revealed to be a pseudohyphal growth by photo-microscopic analysis (Fig. 5). This morphological transition from yeast to pseudohyphae was coincident with the previous observation in *S. cerevisiae* [8].

Pattern of Proteins Expressed in the Pseudohyphae Form of the Cells

In order to distinguish expression patterns of proteins in the pseudohyphal coagulation phase from those in the yeast phase, proteins were extracted from the cells harvested immediately before completely switching to pseudohyphal coagulation and were resolved on a 12% polyacrylamide gel together with the proteins prepared from the cells in the yeast phase (Fig. 6). A striking feature was that more than 40% of the total proteins extracted from the pseudohyphae-like cells appeared to be composed of a single polypeptide. In order to characterize the size of the polypeptide accurately, the protein preparations obtained from the pseudohyphal cells were sequentially diluted and subsequently analyzed by SDS-PAGE (data not shown). The molecular weight of the highly overexpressed polypeptide was estimated to be 58 kilodalton (kDa). The protein patterns resolved on the gel demonstrated that a group of proteins including the 58 kDa polypeptide (p58) was overexpressed while several others underexpressed

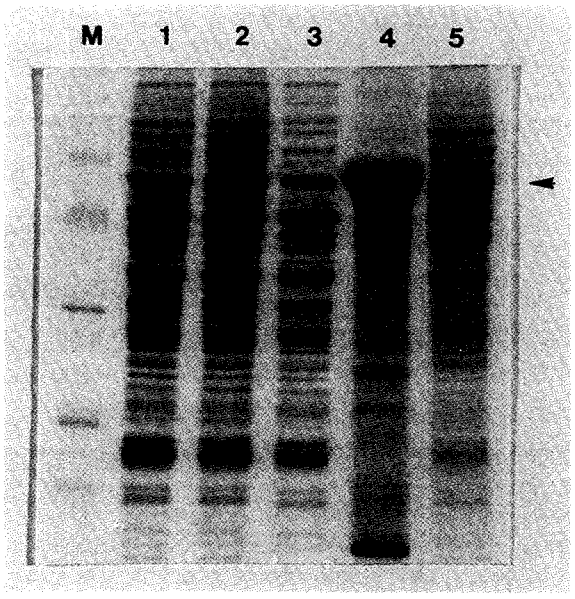

Fig. 6. SDS-PAGE electrophoresis patterns of proteins prepared from the heat shocked *H. anomala* B-7.

The cells were grown at 28°C in the standard medium to early-log (lane 1), mid-log (lane 2), and late-log (lane 3) phase, respectively. The cells grown to the early-log phase were also subjected to heat shock treatment at 43°C to induce a morphological transition to pseudohyphae-like coagulation (lane 4). The cells were harvested to prepare proteins as described in Materials and Methods. The proteins resolved in the lane 5 were prepared from the cells grown to mid-log phase in the presence of 1,000 ppm of Cd^{2+} ions. Size markers (lane M) included rabbit muscle phosphorylase B (97.4 kD), bovine serum albumin (66.2 kD), egg white ovalbumin (45 kD), bovine carbonic anhydrase (31.0 kD), and egg white lysozyme (14.4 kD), respectively. Indicated is a signal for the protein highly overexpressed in the cells switched to the pseudohyphae form by the heat shock treatment.

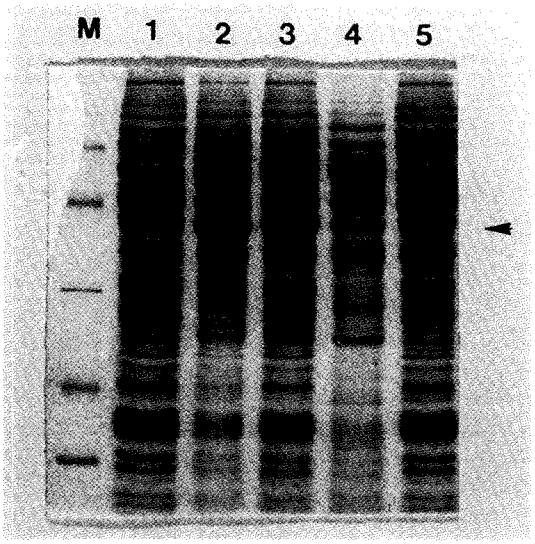

Fig. 7. SDS-PAGE electrophoresis of proteins extracted from the heat or cadmium shocked *H. anomala* B-7 cells.

The proteins were extracted from the cells cultivated at the various conditions as followings: lane1, at 23°C; lane 2, at 28°C; lane 3, at 28°C to early-log phase followed by shifting to 43°C for 30 min; lane 4, at 28°C in a medium containing 1,000 ppm of Cd; lane 5, at 23°C to early-log phase followed by incubation at 37°C for 30 min further. All cells except the ones used for the lanes 3 and 5 were harvested at mid-log phase to prepare proteins and they were resolved by the methods described in Fig. 6. Indicated is the signal expected to be the polypeptide of molecular weight 58 kDa.

in comparison with those from the yeast phase cells, which were cultivated without subjecting to the thermal treatment at 43°C (Fig. 6). The rest of them appeared to be correspondent from each other. However, overexpression of the 58 kDa polypeptide was not observed in the cells treated either with heat shock for 30 min or with 1,000 ppm of Cd^{2+} ions upto mid-log phase (Fig. 7), although these growth conditions are not sufficient for the production of pseudohyphae. When the cell mass in the form of pseudohyphae-like coagulation was transferred to the fresh standard medium and was incubated further at 28°C, it was split into small, but unproliferative substances. The size of these was estimated to be 1/10 of the normal yeast cells (data not shown).

DISCUSSION

Dimorphism is a key feature of several pathogenic fungi and is believed to be associated with pathogenicity in *Candida albicans* [5]. In yeast *S. cerevisiae*, starvation for nitrogen has been found to induce the dimorphic transition from a unicellular yeast form to a multicelluar filamentous pseudohyphae form for accelerating foraging for nutrient at a distance [8]. In contrast, when the polyploid strain *S. cerevisiae* IFO 0203 was propagated by continuous cultivation, they showed extensive pseudohyphae formation under oxygen limitation, but not under nitrogen limitation [26]. On the basis of these data, a controversial suggestion has been made that aeration conditions play an important role in cell morphology of *S. cerevisiae*. Those experiments also showed that dimorphism in *S. cerevisiae* was associated with the expression of specific proteins suggesting that the yeast phase-specific proteins either maintain the yeast form or repress development of the pseudohyphae form [26]. Several lines of evidence, however, indicate that signal for the switch seems complex, because not only various protein factors but also transcription elements seem to work for mediating the pseudohyphal development by cooperative interaction from each other [19, 26].

In this report, we describe that a putative dimorphic transition to filamentous pseudohyphal coagulation from unicellular form can be induced in a highly cadmium-tolerant yeast *H. anomala* B-7, which can accumulate 30 mg of cadmium per gram dry cell though the same budding yeast as *S. cerevisiae*, by subjecting to prolonged heat shock treatment. Furthermore, the switch can be accomplished with the cells grown in the liquid medium made with YPD under batch growth manner for creating a condition of neither oxygen limitation nor starvation for nitrogen. When the cells in unicellular yeast form were subjected to thermal stress at 43° for 200 min, they showed irregularity in cell morphology instead of forming the filamentous pseudohyphal coagulation (Fig. 1), suggesting that sufficient thermal treatment is required for inducing morphological transition to the multicellular coagulation form. Furthermore, the cells showing the morphological irregularity did not return to a healthy morphology completely, even though they were further cultivated at normal growth temperature in the cadmium-free fresh medium (Fig. 1C). However, we cannot rule out the possibility that this abnormality is a morphological characteristic intermediate between the unicellular yeast form and the filamentous pseudohyphal coagulation. The reason is that the structure revealed in the Fig. 1B seems to be a fragile chain of the yeast cells, which has been addressed to be a typical intermediate phenotype observed in cells undergoing the morphological transition [8]. Protein patterns expressed in the pseudohyphae form revealed that several proteins were overexpressed while others underexpressed. It is of interest to report that 40% of the proteins prepared from the cells in the form of the pseudohyphal coagulation are composed of a single polypeptide with molecular weight estimated to be 58 kDa. This polypeptide appears to be expressed only in the pseudohyphae phase, (Figs. 6 and 7). Therefore, these observations lead us to suggest that the polypeptide may be expressed in a phase-specific as well as in a thermal stress-dependent manner, although it is yet to be elucidated whether this polypeptide is exclusively associated with heat shock treatment, or with transition of morphogenecity, or both. In addition, it is of value to construct an expression vector from the gene encoding the 58 kDa polypeptide because the promoter of the gene is believed to be extremely strong in the transcription.

To-date, it has not been investigated whether cadmium stress rendered to the *H. anomala* B-7 in yeast form induces an abnormality in morphology; the present work by electron microscopic analysis has been undertaken to answer this question. The *H. anomala* B-7 cells subjected to cadmium stress by incubation at 8.9 mM (1,000 ppm) of the metal ions induce severe abnormality in cell surface morphology (Fig. 2) which is not eliminated by further cultivation for 4 days in a fresh, cadmium-free medium (Fig. 3). In contrast, the cells grown in the presence of 4.5 mM (500 ppm) of the metal ions show the abnormality, but restore the healthy morphology in 24 h under normal growth conditions (unpublished data). It remains to be elucidated, however, why the induction of the unrepairable damage on the cell morphology is dependent upon the concentration of cadmium ions. However, on the basis of the previous work showing that the effect of heat shock treatment on cell growth was different from that of cadmium treatment [11], it is speculated that the mechanism for the formation of the irregular morphology in the cells by the cadmium stress is different from that for the induction of the morphological transition due to the thermal stress.

However, the morphological transition due to the thermal stress seems to be produced by genetic change, because the pseudohyphae were split into small but unproliferative particles, instead of returning to unicellular yeasts when further cultivated at normal growth temperature in a fresh medium (unpublished data).

Acknowledgments

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