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Article

Thermoresistant properties of bacterioferritin comigratory protein against high temperature stress in *Schizosaccharomyces pombe*

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*Schizosaccharomyces pombe*에 존재하는 bacterioferritin comigratory protein의 고온 스트레스에 대한 열저항적 성질

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ABSTRACT: The *Schizosaccharomyces pombe* structural gene encoding bacterioferritin comigratory protein (BCP) was previously cloned using the shuttle vector pRS316 to generate the BCP-overexpressing plasmid pBCP10. The present work aimed to evaluate the thermoresistant properties of BCP against high temperature stress using the plasmid pBCP10. When the *S. pombe* cells were grown to the early exponential phase and shifted from 30°C to 37°C or 42°C, the *S. pombe* cells harboring pBCP10 grew significantly more at both 37°C and 42°C than the vector control cells. After 6 h of the shifting to higher incubation temperatures, they contained the lower reactive oxygen species (ROS) and nitrite content, an index of nitric oxide (NO), than the vector control cells. After the temperature shifts, total glutathione (GSH) content and total superoxide dismutase (SOD) activities were much higher in the *S. pombe* cells harboring pBCP10 than in the corresponding vector control cells. Taken together, the *S. pombe* BCP plays a thermoresistant role which might be based upon its ability both to down-regulate ROS and NO levels and to up-regulate antioxidant components, such as total GSH and SOD, and subsequently to maintain thermal stability.

Key words: *Schizosaccharomyces pombe*, bacterioferritin comigratory protein, glutathione, reactive oxygen species, thermal stress

Peroxiredoxins (Prxs), also known as thioredoxin (Trx)- or glutaredoxin (Grx)-dependent peroxidases, belong to a family of antioxidant proteins which are ubiquitously conserved in a wide variety of living organisms. They catalyze the reduction of hydrogen peroxide and organic hydroperoxides and don't share any sequence homology with other antioxidant enzymes, such as catalase, superoxide dismutase (SOD) and glutathione (GSH) peroxidase (Bast *et al*., 2002). They participate in the cellular protection against oxidative and nitrosative stress via their peroxidase and peroxynitrite reductase activities supported by Trx, cyclophilin, and Grx (Lee *et al*., 2001; Wong *et al*., 2002). Although Prxs, produced at high levels in living cells, are located primarily in cytosol, they are also identified within mitochondria, chloroplasts, membranes, peroxisomes, and nuclei (Hofmann *et al*., 2002).

The bacterioferritin comigratory protein (BCP) family of Prxs,

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first identified both as a monomeric protein in *Escherichia coli* and as a member of the thiol-specific antioxidant (TSA)/alkyl hydroperoxide peroxidase C (AhpC) family, is a bacterial Trx-dependent thiol peroxidase that reduces a variety of peroxide substrates. The BCP family of bacterial Prxs is subdivided into two classes based on the basis of catalytic activity. For example, *E. coli* BCP is an atypical 2-Cys Prx that functions through the formation of an intermediate disulfide bond between the active and resolving cysteines, whereas the *Burkholderia cenocepacia* BCP functions through 1-Cys catalytic pathway (Clarke *et al*., 2010). BCP-deficient *B. cenocepacia* displays a growth-phase-dependent hypersensitivity to oxidative killing (Clarke *et al*., 2010). An *E. coli bcp* mutant grows more slowly than its wild type in aerobic culture and displays a hypersensitivity toward various oxidants such as hydrogen peroxide, *t*-butyl hydroperoxide, and linoleic acid peroxide (Jeong *et al*., 2000). Expression of the *E. coli bcp* gene elevates during exponential growth until mid-log phase growth, beyond which the expression level is diminished, and is induced by the oxidative stress given by changing the growth conditions from the anaerobic to aerobic cultures (Jeong *et al*., 2000).

In the previous work, the structural gene encoding BCP, based upon the bioinformatics information, was cloned into the shuttle vector pRS316, which resulted in the recombinant plasmid pBCP10, and its genuine expression was conformed using RT-PCR (Kang *et al*., 2009). The *S. pombe bcp* gene was found to participate in the cellular protection against hydrogen peroxide, *t*-butyl hydroperoxide (*t*-BOOH), cadmium, nitrogen starvation and high concentrations of glucose and sucrose as a sole carbon source (Kang *et al*., 2009). In the continuing work, we demonstrate that the *S. pombe* BCP is involved in the cellular protection against high temperature stress possibly via up-regulating some antioxidant components, such as GSH and SOD, and maintaining thermal stability.

Materials and Methods

Chemicals

Bradford reagent, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), Griess reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), glutathione reductase (GR), reduced glutathione (GSH), NADPH, cytochrome c, catalase, xanthine, and xanthine oxidase were obtained from Sigma-Aldrich Chemical Co. Yeast extract, peptone and agar were obtained from Amersham Life Science. All other chemicals used were of the highest grade commercially available.

Strain and growth condition

S. pombe KP1 (h^+ *leu1-32 ura4-294*), a derivative of *S. pombe* heterothallic haploid strain 975h^+, was used in the present work. The BCP-overexpressing recombinant plasmid pBCP10 was previously constructed using a yeast-*E. coli* shuttle plasmid vector pRS316 (Kang *et al*., 2009). The yeast cells were grown in YEPD medium (pH 6.5) which contained 1% yeast extract, 2% peptone, and 1% glucose. The yeast cells were incubated with shaking at 30°C prior to transferring to higher incubation temperatures and their growth was monitored by measuring the absorbance at 600 nm. Yeast cells used in the experiments were obtained preferentially from the early exponential growth phase.

Preparation of cellular extracts

The desired number of the yeast cells was obtained by centrifugation. They were re-suspended in 20 mM Tris buffer (pH 8.0) with 2 mM EDTA and disrupted using glass beads and ultrasonication. The cellular extracts, taken after centrifugation, were used for total GSH and SOD activity, and protein determinations detailed below. Protein content in cellular extracts was determined by the Bradford assay (Bradford, 1976) with bovine serum albumin as the reference protein.

Quantitation of intracellular ROS

To determine intracellular ROS levels, the redox-sensitive fluorescent probe DCFH-DA was used as described previously (Royall and Ischiropoulos, 1993). When DCFH-DA enters the cells, its diacetate group is cleaved by nonspecific esterases, leaving a non-fluorescent molecule, which is amenable to oxidization to fluorescent dichlorofluorescein (DCF) in the presence of ROS (Kiani-Esfahani *et al*., 2012). Yeast cells were incubated with 5 μM DCFH-DA for 30 min at 30°C. The treated cells were analyzed immediately using a microplate fluorometer.

Determination of nitrite concentration

Accumulated nitrite (NO_2^-), as an index of nitric oxide (NO), in conditioned medium was determined using a colorimetric assay based on the Griess reaction (Sherman *et al*., 1993). Conditioned medium (100 μl) were reacted with 100 μl Griess reagent (6 mg/ml) at room temperature for 10 min, and NO_2^- concentration was determined by the absorbance at 540 nm. The calibration curve was constructed using the known concentrations (0–160 μM) of sodium nitrite.

Quantitation of total GSH

As previously described (Nakagawa *et al*., 1990), the total GSH content in cellular extracts was determined using an enzymatic recycling assay based on GR. The reaction mixture (200 μl) contained 175 mM KH_2PO_4, 6.3 mM EDTA, 0.21 mM NADPH, 0.6 mM DTNB, 0.5 units/ml GR, and cellular extract at 25°C. The absorbance at 412 nm was monitored using a microplate reader. The total GSH content was reported as μg/mg protein.

Determination of total SOD activity

As previously described (Lee *et al*., 2002), the total SOD activity in cellular extracts was spectrophotometrically determined as reduced cytochrome c with the xanthine/xanthine oxidase system. The reaction mixture (200 μl) contained 50 mM phosphate buffer (pH 7.4), 0.01 units/ml xanthine oxidase, 0.1 mM EDTA, 1 μM catalase, 0.05 mM xanthine, 20 μM cytochrome c, and cellular extract. A change in absorbance was monitored at 550 nm.

Statistical analysis

The results are reported as mean ± standard deviation (SD). Statistical comparisons between experimental groups were performed using unpaired Student's *t*-test. A *P* value less than 0.05 was considered to be statistically significant.

Results

Enhancement of high temperature-reduced yeast growth

The involvement of the *S. pombe* BCP in the yeast growth under high temperature stress was investigated using temperature-shift experiments. The same numbers of the *S. pombe* cells harboring pRS316 or pBCP10, grown to the early exponential phase in rich medium at 30°C, were transferred into fresh rich medium and continued to be cultured at higher incubation temperatures, such as 37°C and 42°C. As a control, the *S. pombe* cells harboring pRS316 or pBCP10 were continued to be cultured at 30°C. The yeast growths, detected by the

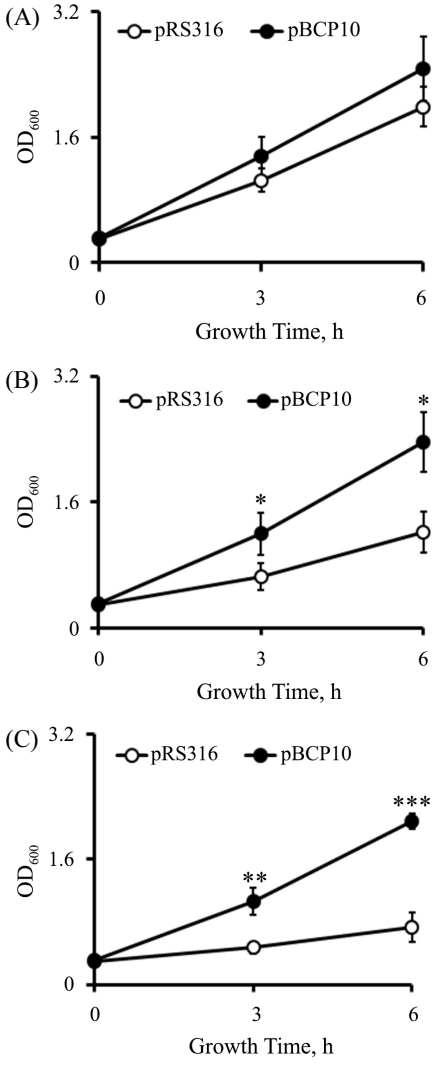

Fig. 1. Enhancing effect of BCP on the *S. pombe* growth under high temperature stress. The *S. pombe* cells harboring pRS316 or pBCP10, grown to early exponential phase at 30°C in rich growth medium, were shifted to 30°C (Control, A), 37°C (B) or 42°C (C). The yeast growth was monitored by absorbance (OD_{600}) at 3 and 6 h following the shifts. Each point shows the mean ± SD of the three independent experiments. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ versus the corresponding pRS316-containing cells.

absorbance at 600 nm, at 3 and 6 h after the shifts were compared in Fig. 1. At the incubation temperature of 30°C, the *S. pombe* cells harboring pBCP10 and the vector control cells displayed no significant difference between their growth patterns after the shifts (Fig. 1A). After the shifts to 37°C and 42°C, the *S. pombe* cells harboring pBCP10 grew much better than the vector control cells (Fig. 1B and C). Although the growth of the vector control cells tended to be arrested at 42°C, the *S. pombe* cells harboring pBCP10 could reasonably grow albeit a little delayed (Fig. 1C). When the growth rates after the shifts were compared, the enhanced growths of the *S. pombe* cells harboring pBCP10 at both 37°C and 42°C were also assessed by comparison with those of the vector control cells (Fig. 2). At the incubation temperatures of 37°C and 42°C, the growth rates of the *S. pombe* cells harboring pBCP10 were 2.2- and 4.1-fold higher than those of the corresponding vector control cells, respectively (Fig. 2). Collectively, BCP is involved in the growth of *S. pombe* cells under high temperature stress.

Attenuation of high temperature-induced reactive oxygen species (ROS) production

The intracellular ROS are continuously generated under aerobic conditions and exogenously-added oxidative stress agents, including high incubation temperature, which subsequently disrupts the integrity of various biomolecules, including DNA and proteins. Cells are equipped with a variety of defense-related components, such as antioxidant enzymes and free radical scavengers, to protect themselves from oxidative damages. However, if the cellular defense systems do not sufficiently cope with ROS generation, the cells undergo oxidative stress. Since the intracellular ROS levels elevate under various kinds of stresses, especially oxidative stress, they are thought as one of crucial cellular markers which are related with the stress levels inside cells.

The ROS levels were determined in the *S. pombe* cells harboring pRS316 or pBCP10 when the same numbers of the exponentially grown yeast cells were transferred and grown at 37°C or 42°C for 6 h. As the incubation temperature went up, the intracellular ROS levels of the vector control cells also became higher (Fig. 3). The vector control cells contained the ROS levels 1.6- and 4.3-fold higher at 37°C and 42°C, respectively, than at 30°C, proving that the yeast cells undergo enhanced oxidative stress when grown at 37°C and 42°C (Fig. 3). The ROS levels of the *S. pombe* cells harboring pBCP10 declined to 76.3% and 32.3% at 37°C and 42°C, respectively, of those from the corresponding vector control cells (Fig. 3). But, the *S. pombe* cells harboring pBCP10 and the vector control cells appeared to contain the similar levels of ROS when grown at 30°C (Fig. 3). These results imply that the ROS-attenuating effect of BCP is evident at higher incubation temperature. Collectively, BCP plays a scavenging role on ROS generation enhanced in *S. pombe* cells under high temperature stress.

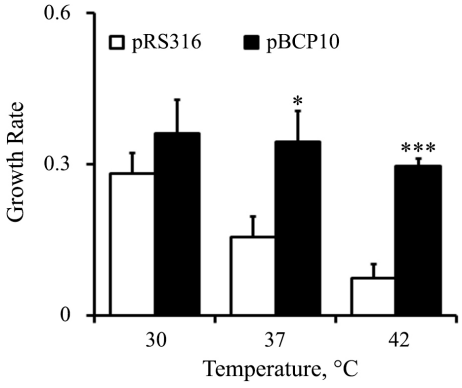

Fig. 2. Enhancing effect of BCP on the growth rates of the *S. pombe* cells under high temperature stress. The *S. pombe* cells harboring pRS316 or pBCP10, grown to early exponential phase at 30°C in rich growth medium, were shifted to 30°C, 37°C or 42°C. The growth rates were obtained as ΔOD_{600}/h. Each point shows the mean ± SD of the three independent experiments. *, $P<0.05$; ***, $P<0.001$ versus the corresponding pRS316-containing cells.

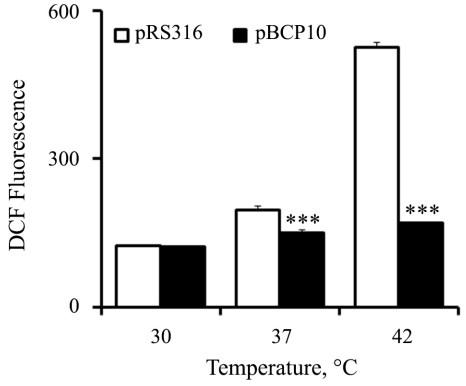

Fig. 3. Effect of BCP on the reactive oxygen species (ROS) levels of the *S. pombe* cells grown under high temperature stress. The yeast cells harboring pRS316 or pBCP10 in the early exponential phase at 30°C were subjected to 30°C, 37°C or 42°C for 6 h. The ROS levels were relatively represented as DCF fluorescence, an arbitrary unit. Each point shows the mean ± SD of the three independent experiments. ***, $P<0.001$ versus the corresponding pRS316-containing cells.

Attenuation of high temperature-induced nitric oxide (NO) production

Nitric oxide (NO•, NO), a free radical synthesized from L-arginine by nitric oxide synthase (NOS), acts as a normal physiological regulator when produced in minute quantities by constitutive NOS, while it exhibits pathologic effects when produced in excessive quantities by inducible NOS. NO can react directly with its physiological targets, while its effects also occur indirectly through the mediation of reactive nitrogen species (RNS) derived from NO metabolism (Wink *et al*., 2001). Although RNS play regulatory roles in cellular signaling, they can subject cells to nitrosative stress at high concentrations, ultimately leading to cell death. Constitutive NOS, verified by western blotting using mouse monoclonal anti-neuronal NOS, is present in the budding yeast *Saccharomyces cerevisiae*, which is activated by calmodulin and arginine (Kanadia *et al*., 1998). NO, produced by an NOS-like activity in *S. pombe*, acts as a signaling molecule which can induce both transcriptional and physiological changes (Kig and Temizkan, 2009).

As shown in Fig. 4, the vector control cells contained the gradually enhanced contents of nitrite, an index of NO, when the incubation temperature went up from 30°C to 37°C to 42°C. This finding implies that NO tends to elevate in *S. pombe* cells under high temperature stress. But, the nitrite contents of the *S. pombe* cells harboring pBCP10 remained unchanged irrespective of the incubation temperatures applied (Fig. 4). They seemed to have capabilities to resist the enhancement of NO generation under high temperature stress. Taken together, BCP tends to play a diminishing role on NO levels in the *S. pombe* cells grown under high incubation temperature.

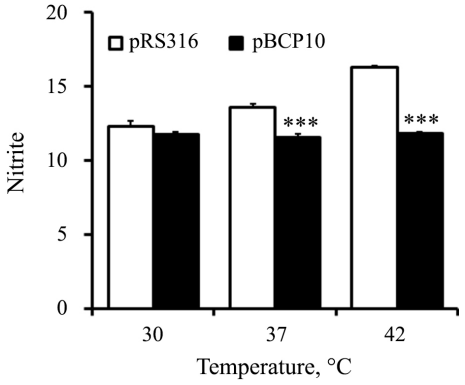

Fig. 4. Effect of BCP on the nitric oxide (NO) levels of *S. pombe* cells grown under thermal stress. The yeast cells harboring pRS316 or pBCP10 in the early exponential phase were subjected to 30°C, 37°C or 42°C for 6 h. The levels of nitrite, an index of NO, in supernatant fractions were determined based upon Griess reaction. Nitrite in the y-axis was expressed in μM. Each point shows the mean ± SD of the three independent experiments. ***, $P<0.001$ versus corresponding pRS316-containing cells.

Enhancement of high temperature-reduced total glutathione (GSH) content

Since GSH, as one of principal non-enzymatic antioxidants, plays a major role in the defense against many different kinds of stressful agents, its cellular levels are one of important factors to maintain redox homeostasis. The total GSH contents in the vector control cells became gradually lower as the incubation temperature shifted up to 37°C to 42°C (Fig. 5). As shown in Fig. 5, the total GSH contents in the vector control cells dropped to 58.5% and 23.4%, respectively, at 37°C and 42°C, compared to those at 30°C. The total GSH contents in *S. pombe* cells harboring pBCP10 were 1.1-, 1.6-, and 3.4-fold higher at 30°C, 37°C, and 42°C, respectively, than those in the corresponding vector control cells (Fig. 5). In brief, BCP plays a GSH-enhancing role in *S. pombe* cells at the enhanced incubation temperatures as well as 30°C.

Enhancement of high temperature-reduced superoxide dismutase (SOD) activity

In addition to GSH, antioxidant enzymes such as superoxide dismutase, catalase, peroxidase and peroxiredoxin are principal components of the cellular defense against diverse stresses. As

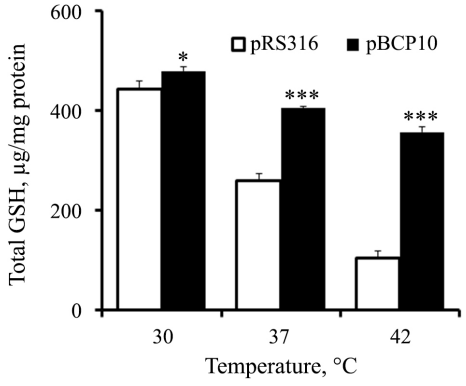

Fig. 5. Effect of BCP on the total glutathione (GSH) contents in the *S. pombe* cells grown under thermal stress. The yeast cells harboring pRS316 or pBCP10 in the early exponential phase were subjected to 30°C, 37°C or 42°C for 6 h. The total GSH levels were represented as μg/mg protein. *, $P<0.05$; ***, $P<0.001$ versus the corresponding pRS316-containing cells.

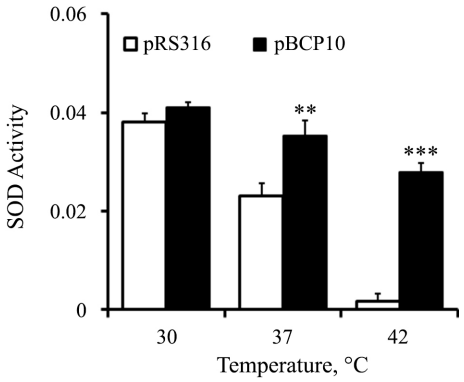

Fig. 6. Effect of BCP on total superoxide dismutase (SOD) activities in the *S. pombe* cells grown under thermal stress. The yeast cells harboring pRS316 or pBCP10 in the early exponential phase were subjected to 30°C, 37°C or 42°C for 6 h. Total SOD activity was represented as Δ_{550}/min/mg protein. **, $P<0.01$; ***, $P<0.001$ versus the corresponding pRS316-containing cells.

shown in Fig. 6, total SOD activities in the vector control cells gradually became lower at the higher incubation temperatures. Although total SOD activities in the *S. pombe* cells harboring pBCP10 also became gradually lower at the higher incubation temperatures, their levels were maintained to be higher than those in the vector control cells. The total SOD activity levels in the *S. pombe* cells harboring pBCP10 were 1.1-, 1.5-, and 16.8-fold higher at 30°C, 37°C, and 42°C, respectively, than those in the corresponding vector control cells (Fig. 6). Taken together, BCP is capable of enhancing total SOD activities in the *S. pombe* under high temperature stress.

Discussion

Since BCP was initially identified from *E. coli*, BCPs have been isolated and characterized from various organisms, including eukaryotic cells as well as other prokaryotic cells. The plant pathogen *Xanthomonas campestris* BCP, belonging to a subfamily of atypical 2-Cys Prxs, carries out its oxidoreduction activity through the alternate opening and closing of the substrate entry channel and the disulfide-bond pocket (Liao *et al*., 2009). Among the three oligomeric forms, such as 700 kDa, 70 kDa, and 20 kDa, of the hyperthermophilic *Thermococcus kodakaraensis* KOD1 BCP, the monomeric form contains high levels of peroxidase activity, whereas the higher-molecular weight, oligomeric forms have the molecular chaperone-like function (Pham *et al*., 2015). Interestingly, oxidative agents cause the protein structure of *T. kodakaraensis* KOD1 BCP to shift from low-molecular weight form to high-molecular weight complexes, while reducing agents cause a shift in the reverse direction (Pham *et al*., 2015). The molecular chaperone-like function of BCP may relate with the thermoresistant properties of the *S. pombe* BCP identified in the present work.

Several BCPs have already been reported to participate in the cellular response against different kinds of stresses. A BCP homolog of the plant *Sedum lineare* reveals thioredoxin-dependent peroxidase activity, the hallmark of Prx family, and has two cysteine residues well conserved among proteins of the BCP family (Kong *et al*., 2000). It can functionally complement an *E. coli bcp* mutant and is able to suppress its hypersensitivity to peroxides, implying its *in vivo* antioxidant activity (Kong *et al*., 2000). In the Trx- but not Grx-dependent manner, poplar peroxiredoxin Q, as one of the four plant subtypes and a homologue of BCP, reduces various alkyl hydroperoxides, but with a better efficiency for cumene hydroperoxide than hydrogen peroxide and *t*-butyl hydroperoxide (Rouhier *et al*., 2004). Peroxiredoxin Q, localized in chloroplast, participates in the response to an infection of poplar by the rust fungus *Melampsora larici-populina* (Rouhier *et al*., 2004). In *Synechococcus elongatus*, BCP is considered as one of antioxidant enzymes which are responsible for the detoxification of peroxide derived by bentazone, often applied as a substitute of atrazine to control weeds and sedges in post-emergence crops (Das and Bagchi, 2012). BCP was found to be highly expressed even without bentazone in the *S. elongatus* PCC7942 mutant capable of growing at its five times higher concentration than wild type, whereas in the corresponding wild type, its level is enhanced only upon bentazone exposure (Das and Bagchi, 2012). The *Helicobacter pylori* BCP, identified as a thiol peroxidase depending on the reducing activities of thioredoxin and thioredoxin reductase, contributes significantly to the bacterium's ability to colonize the host stomach, and the *bcp* mutant exhibits enhanced sensitivity to the superoxide-generating paraquat and to organic hydroperoxides, implying its contribution to oxidative stress resistance (Wang *et al*., 2005). Until recently, the participation of BCPs in the cellular protection against high temperature stress has not been assessed. This work for the first time demonstrates that the *S. pombe* BCP plays a protective role

against high temperature stress. Although we focused the influences of the *S. pombe* BCP on the high temperature stress during exponential growth, its thermoresistant effects in slow-growing phases would be elucidated in future approaches.

In conclusion, the BCP analog is able to attenuate the ROS and NO elevations in the *S. pombe* cells grown under high temperature stress. On the contrary, it is capable of maintaining the high levels of the total GSH contents and SOD activities in the *S. pombe* cells under the same stress conditions. These findings suggest that the BCP analog plays a thermoresistant role in *S. pombe* via maintaining thermal stability.

적 요

이전의 연구에서, bacterioferritin comigratory protein (BCP)을 인코딩하는 *Schizosaccharomyces pombe*의 구조유전자를 shuttle vector인 pRS316에 클로닝하여 BCP 과잉발현 플라즈미드인 pBCP10을 제조한 바 있다. 본 연구에서는, 플라즈미드 pBCP10을 사용하여 고온 스트레스에 대한 BCP의 열저항적 성질을 평가하였다. 대수기의 초기까지 성장시킨 *S. pombe* 세포의 배양 온도를 30°C에서 37°C나 42°C로 전이시키는 경우, pBCP10 함유 *S. pombe* 세포가 벡터 대조 세포보다 37°C와 42°C 모두에서 유의하기 더 잘 성장하였다. 높은 배양 온도로 전이한 뒤 6시간에서, pBCP10 함유 *S. pombe* 세포가 벡터 대조 세포보다 낮은 활성산소종(ROS)과 일산화질소(NO)의 지표로 측정된 아질산염(nitrite) 함량을 갖고 있음이 확인되었다. 온도 전이 뒤에, 총 글루타치온(total glutathione) 함량과 총 수퍼옥사이드 디스뮤타제(superoxide dismutase) 활성은 대응되는 벡터 대조 세포보다 pBCP10 함유 *S. pombe* 세포에서 현저하게 높다는 사실도 확인되었다. 종합하면, *S. pombe* BCP는 열저항적 역할을 보유하는 데, 활성산소종과 일산화질소에 대한 하강시키는 활성과 총 글루타치온과 수퍼옥사이드 디스뮤타제 등의 항산화 성분들을 상승시키는 활성, 즉 종합적으로 열안정성을 유지하는 활성에 근거하는 것으로 추정되었다.

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