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Acquired Resistance to Severe Ethanol Stress in *Saccharomyces cerevisiae* Protein Quality Control

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ABSTRACT Acute severe ethanol stress (10% [vol/vol]) damages proteins and causes the intracellular accumulation of insoluble proteins in *Saccharomyces cerevisiae*. On the other hand, a pretreatment with mild stress increases tolerance to subsequent severe stress, which is called acquired stress resistance. It currently remains unclear whether the accumulation of insoluble proteins under severe ethanol stress may be mitigated by increasing protein quality control (PQC) activity in cells pretreated with mild stress. In the present study, we examined the induction of resistance to severe ethanol stress in PQC and confirmed that a pretreatment with 6% (vol/vol) ethanol or mild thermal stress at 37°C significantly reduced insoluble protein levels and the aggregation of Lsg1, which is prone to denaturation and aggregation by stress, in yeast cells under 10% (vol/vol) ethanol stress. The induction of this stress resistance required the new synthesis of proteins; the expression of proteins comprising the bichaperone system (Hsp104, Ssa3, and Fes1), Sis1, and Hsp42 was upregulated during the pretreatment and maintained under subsequent severe ethanol stress. Since the pretreated cells of deficient mutants in the bichaperone system (*fes1Δ hsp104Δ* and *ssa2Δ ssa3Δ ssa4Δ*) failed to sufficiently reduce insoluble protein levels and Lsg1 aggregation, the enhanced activity of the bichaperone system appears to be important for the induction of adequate stress resistance. In contrast, the importance of proteasomes and aggregases (Btn2 and Hsp42) in the induction of stress resistance has not been confirmed. These results provide further insights into the PQC activity of yeast cells under severe ethanol stress, including the brewing process.

IMPORTANCE Although the budding yeast *S. cerevisiae*, which is used in the production of alcoholic beverages and bioethanol, is highly tolerant of ethanol, high concentrations of ethanol are also stressful to the yeast and cause various adverse effects, including protein denaturation. A pretreatment with mild stress improves the ethanol tolerance of yeast cells; however, it currently remains unclear whether it increases PQC activity and reduces the levels of denatured proteins. In the present study, we found that a pretreatment with mild ethanol upregulated the expression of proteins involved in PQC and mitigated the accumulation of insoluble proteins, even under severe ethanol stress. These results provide novel insights into ethanol tolerance and the adaptive capacity of yeast. They may also contribute to research on the physiology of yeast cells during the brewing process, in which the concentration of ethanol gradually increases.

KEYWORDS *Saccharomyces cerevisiae*, acquired stress resistance, severe ethanol stress, proteostasis, protein quality control, insoluble proteins, bichaperone system, aggregase, Lsg1

The budding yeast *Saccharomyces cerevisiae* efficiently produces ethanol through alcoholic fermentation, and ethanol concentrations ultimately increase to higher than 10% (vol/vol) during the typical brewing process of wine and Japanese sake. However, high concentrations of ethanol are toxic, even for yeast cells, and exert a number of adverse effects on yeast cells as severe ethanol stress. More than 10% (vol/

vol) ethanol inhibits the transport systems of glucose and amino acids (1, 2), prevents the nuclear export of bulk poly(A)$^+$ mRNA (3), and induces the strong repression of overall protein synthesis (4). Additionally, long-term exposure to severe ethanol stress leads to persistent actin depolarization and the aberrant localization of septins (5). We recently identified the intracellular accumulation of denatured proteins as another adverse effect of severe ethanol stress on yeast cells; severe ethanol stress (10% [vol/vol]) as well as thermal stress at 42°C significantly increased the levels of insoluble proteins and ubiquitinated proteins in living yeast cells (6).

The accumulation of denatured proteins often vitiates cellular homeostatic functions and has been implicated in the pathogenesis of neurodegenerative disorders, such as Alzheimer's disease, Huntington's disease, and Parkinson's disease (7–9). The protein quality control (PQC) system in the proteostasis network plays a critical role in mitigating the toxicity of denatured proteins. The PQC system primarily suppresses or scavenges denatured proteins using molecular chaperones, the ubiquitin-proteasome system (UPS), and autophagy in eukaryotic cells (10–15). Additionally, excess denatured proteins are not scattered in cells; they assemble at intracellular deposition sites, such as CytoQ, IPODs (Insoluble PrOtein Deposits), and INQs/JUNQs (INtranuclear Quality/JUxta-Nuclear Quality control compartments) (16–19). The formation of these deposition sites is useful for the sequestration of toxic denatured proteins, the generation of denatured protein-free progeny via asymmetric inheritance, the efficient refolding/degradation of denatured proteins, and the prevention of Hsp70 overload (20–22).

In *S. cerevisiae*, Hsp104, Hsp70 (Ssa1 to -4), and Fes1 constitute the bichaperone system, which plays a role in the refolding and reactivation of aggregated proteins (10, 22, 23). Fes1 promotes the dissociation of denatured proteins from Hsp70 in the refolding process (24, 25). Hsp104 functions as a disaggregase for the dissolution of protein aggregates, together with other molecular chaperones (18, 26–29). Since Hsp104 binds to the surfaces of denatured protein aggregates and forms granules, Hsp104-GFP (green fluorescent protein) is used as a representative marker of the deposition sites of denatured proteins (19, 27, 30, 31). Btn2 and Hsp42 play important roles as aggregases (sequestrases) in the assembly of deposition sites together with Sis1 (22, 28, 30, 32–35). Since these factors play important roles in yeast proteostasis, various deficient strains have been reported to exhibit abnormal phenotypes due to defects in the PQC system (18, 22, 29). We also confirmed that *hsp104Δ* and *btn2Δ* mutants showed the delayed clearance of denatured proteins during the recovery process from severe ethanol stress (6).

Cells preexposed to mild stress conditions develop enhanced resistance to subsequent severe stress conditions. This phenomenon is referred to as "acquired stress resistance," and a well-known example is increased tolerance to heat shock (36). A pretreatment with mild thermal stress has been shown to increase resistance to subsequent lethal temperatures in yeast cells through the upregulated expression of stress-responsive genes, including PQC-related genes (36–39). Regarding acquired resistance to severe ethanol stress, genome-wide gene expression was previously examined and the findings obtained revealed that alterations in H$^+$-ATPase activity and the lipid composition of the plasma membrane caused by a mild ethanol pretreatment contributed to the acquisition of resistance to high concentrations of ethanol (40–44). However, limited information is currently available on adaptive responses to severe ethanol stress in yeast proteostasis, and it remains unclear whether the activity of the PQC system is enhanced by a pretreatment with mild ethanol stress.

Therefore, the present study investigated whether a pretreatment with mild stress affects proteostasis in yeast cells under subsequent severe ethanol stress. We confirmed that a pretreatment with 6% (vol/vol) ethanol or mild thermal stress at 37°C significantly reduced insoluble protein levels in yeast cells under subsequent 10% (vol/vol) ethanol stress, suggesting that the activity of the PQC system was enhanced by the pretreatment. The acquisition of enhanced PQC activity required the new synthesis of proteins, and the pretreated cells of the *ssa2Δ ssa3Δ ssa4Δ* and *fes1Δ hsp104Δ*
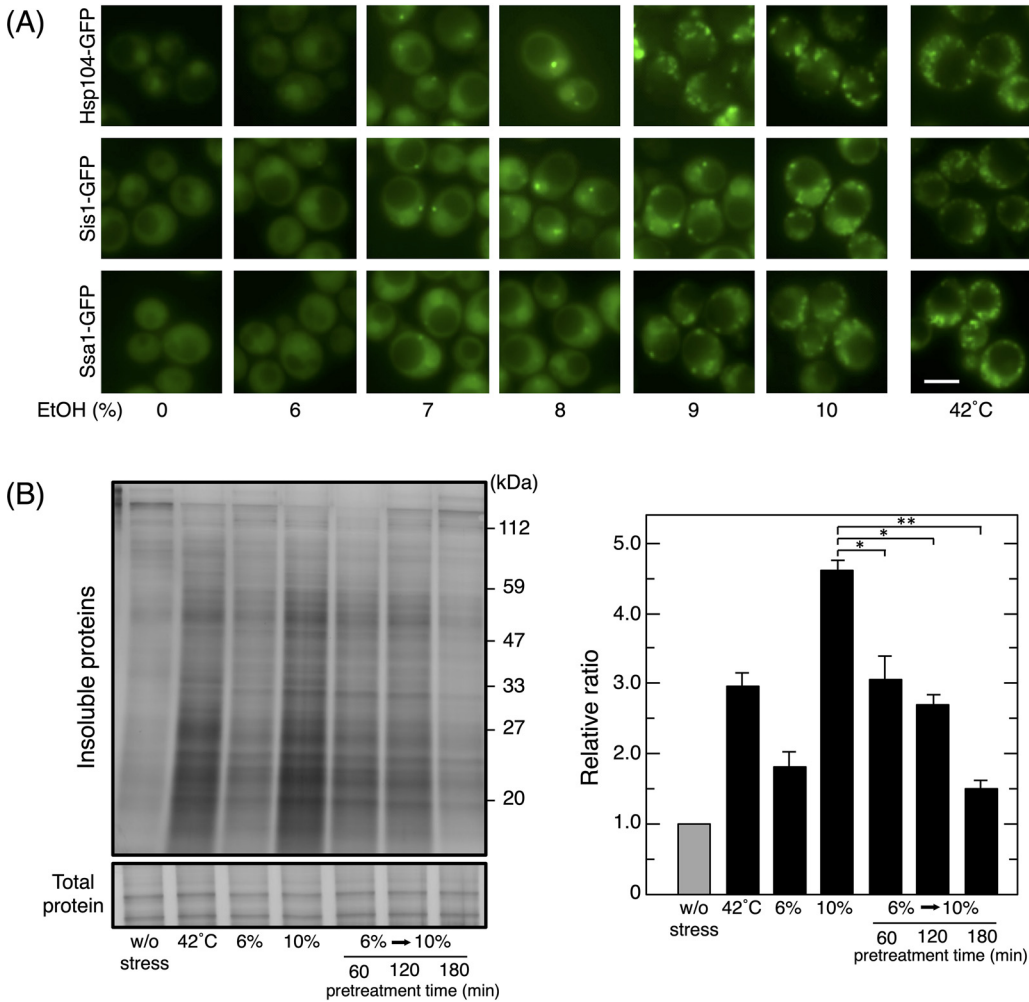
FIG 1 Reduction in denatured protein levels in cells pretreated with mild ethanol (EtOH) stress. (A) Yeast cells expressing GFP-tagged proteins were treated with various concentrations of ethanol for 180 min or thermal stress at 42°C for 60 min in SD medium. Representative images are shown. Bar, 5 μm. (B) Cells were pretreated with 6% (vol/vol) ethanol for 60 to 180 min and then exposed to 10% (vol/vol) ethanol for 180 min. Intracellular levels of insoluble proteins were assayed. Samples were separated using a 10% SDS-polyacrylamide gel and visualized by silver staining. Representative images are shown in the left panel and quantified data in the right panel. Relative ratios were calculated from three independent experiments. Each value is expressed as the mean $\pm$ SD of fold changes in the staining levels of insoluble proteins relative to those in nonstressed cells. *, $P < 0.05$; **, $P < 0.01$ by the Student t test.

mutants did not induce sufficient reductions in insoluble protein levels under subsequent severe ethanol stress. These results provide novel insights into the acquired resistance of yeast cells to severe ethanol stress.

RESULTS

The pretreatment with mild ethanol stress mitigated the accumulation of insoluble proteins. To establish the pretreatment conditions needed to enhance the activity of PQC, we initially examined the concentration-dependent effects of ethanol on the formation of the deposition sites of denatured proteins. Since Hsp104 binds to denatured protein aggregates and forms granules, Hsp104-GFP is a frequently used marker of deposition sites (27, 30, 31). The formation of Hsp104-GFP foci was induced by a treatment with thermal stress at 42°C and >7% (vol/vol) ethanol stress, but not 6% (vol/vol) ethanol (Fig. 1A). Another molecular chaperone, Ssa1 (Hsp70), and an Hsp40 cochaperone (Sis1) also formed foci under >7% (vol/vol) ethanol stress and thermal stress at 42°C.

Since 6% (vol/vol) ethanol did not induce the formation of the deposition sites of
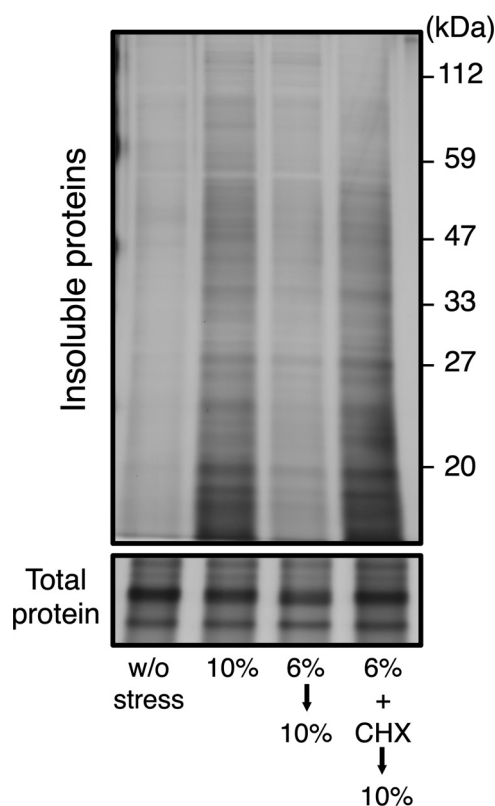
FIG 2 Acquired resistance to severe ethanol stress required new protein synthesis. Yeast cells were pretreated with 6% (vol/vol) ethanol for 180 min with or without 200 μg/ml cycloheximide (CHX) and then exposed to 10% (vol/vol) ethanol stress for 180 min. Intracellular levels of insoluble proteins were assayed by the silver staining of SDS-polyacrylamide gels.

denatured proteins, it was used as a mild dose of ethanol stress, and we investigated whether a pretreatment with 6% (vol/vol) ethanol mitigated the accumulation of denatured proteins under severe ethanol stress (10% [vol/vol]). In the present study, insoluble protein levels were measured as an indicator of denatured protein accumulation. Consistent with previous findings (6), the treatment with 10% (vol/vol) ethanol for 180 min and thermal stress at 42°C for 60 min caused the significant accumulation of insoluble proteins, whereas the treatment with 6% (vol/vol) ethanol for 180 min induced only a slight increase in insoluble protein levels (Fig. 1B). Although the pretreatment with 6% (vol/vol) ethanol for 60 or 120 min partly mitigated the accumulation of insoluble proteins during the subsequent treatment with 10% (vol/vol) ethanol, the 180-min pretreatment with 6% (vol/vol) ethanol adequately suppressed the accumulation of insoluble proteins (Fig. 1B). These results suggested that yeast cells acquired enhanced PQC activity after the pretreatment with 6% ethanol for 180 min.

New protein synthesis was essential for the acquisition of enhanced PQC activity. A previous study reported that cycloheximide (CHX), a representative inhibitor of translation elongation (45), blocked the acquisition of tolerance to severe ethanol stress (43). We also examined whether CHX blocked the acquisition of enhanced PQC activity caused by the pretreatment with 6% (vol/vol) ethanol. As shown in Fig. 2, the pretreatment with 6% (vol/vol) ethanol plus CHX did not mitigate the accumulation of insoluble proteins under subsequent 10% (vol/vol) ethanol stress, demonstrating that new protein synthesis during the pretreatment was required for enhanced PQC activity.

The pretreatment with mild thermal stress induced cross-protection against protein denaturation caused by severe ethanol stress. A pretreatment with a certain stress has been shown to induce cross-protection and enhance resistance to different types of stress (39, 46–48). We examined the effects of cross-protection induced by the
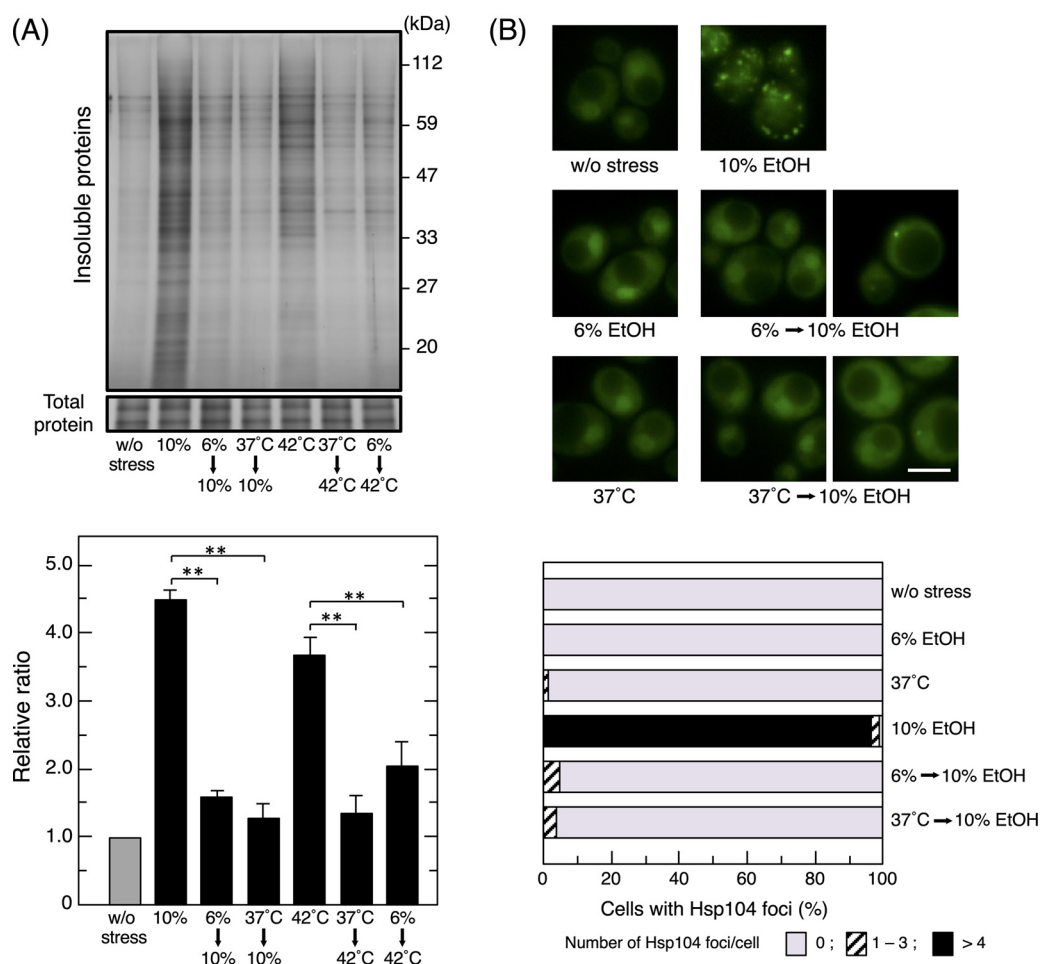
FIG 3 Cross-protection between ethanol (EtOH) stress and thermal stress. (A) Cells were pretreated with or without mild stress (6% ethanol for 180 min or 37°C for 60 min) and then exposed to severe stress (10% ethanol for 180 min or thermal stress at 42°C for 60 min). Intracellular levels of insoluble proteins were assayed. **, $P < 0.01$ by the Student t test. (B) The formation of Hsp104-GFP foci was monitored under each condition. Representative images are shown in the upper panel. The quantification of cells containing foci is shown in the lower panel. One hundred cells under each condition were examined, and experiments were repeated 3 times (300 cells in total were examined). Bar, 5 μm.

pretreatment with mild thermal stress at 37°C on protein denaturation caused by severe ethanol stress. Insoluble protein levels induced by 10% (vol/vol) ethanol were significantly lower in cells pretreated with 37°C than in those without the pretreatment (Fig. 3A). In contrast, the pretreatment with 6% (vol/vol) ethanol also reduced the levels of insoluble proteins that accumulated under severe thermal stress at 42°C.

We investigated the effects of the pretreatment on the formation of Hsp104-GFP foci. The pretreatment with 6% (vol/vol) ethanol or 37°C had a negligible effect on the formation of Hsp104-GFP foci (Fig. 3B). As shown in Fig. 1A, the majority of yeast cells directly challenged with 10% (vol/vol) ethanol stress formed multiple foci of Hsp104-GFP. In contrast, when challenged with 10% (vol/vol) ethanol stress after the pretreatment, only a small percentage of cells formed Hsp104-GFP foci, and when they did, only a few foci per cell were observed (Fig. 3B). These results clearly demonstrated that the pretreatment with 6% (vol/vol) ethanol or 37°C reduced protein insolubilization under subsequent severe ethanol stress.

The pretreatment with mild stress affected protein expression under severe stress. We examined whether pretreatments with mild stress affected the levels of proteins involved in PQC under severe ethanol stress (Fig. 4). Despite their transcriptional activation (see Fig. S1 in the supplemental material), neither Ssa3 (Hsp70) nor Sis1 protein levels were markedly increased by the direct exposure to 10% (vol/vol) ethanol for
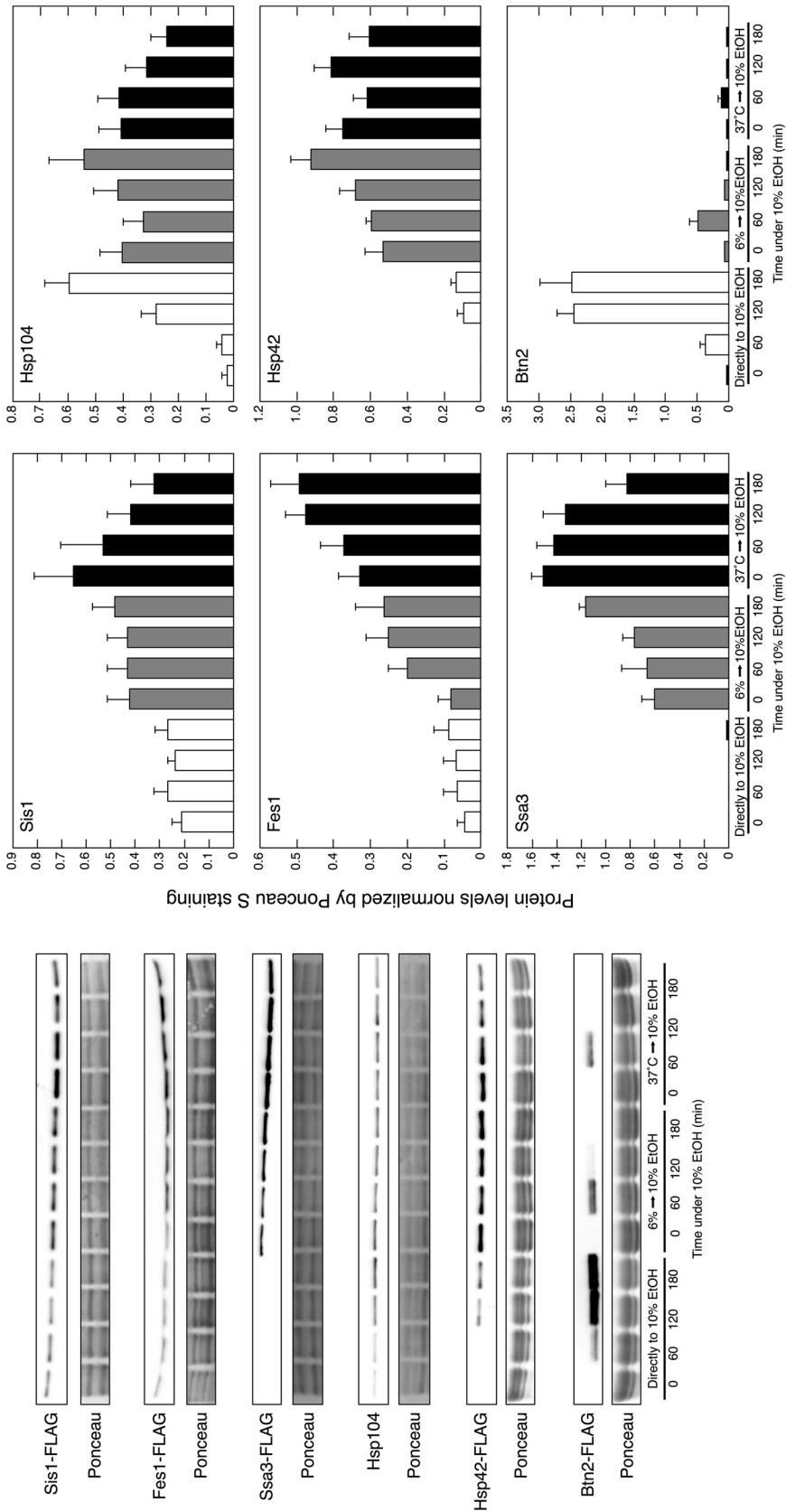
FIG 4 A pretreatment with mild stress affected protein expression under severe ethanol (EtOH) stress. Sis1-FLAG, Ssa3-FLAG, Fes1-FLAG, Hsp42-FLAG, Hsp104, and Btn2-FLAG levels under stress conditions were assayed by Western blotting. Yeast cells were pretreated with or without mild stress (6% ethanol stress for 180 min or thermal stress at 37°C for 60 min) and then exposed to 10% (vol/vol) ethanol stress for 0, 60, 120, or 180 min. Ponceau S staining was performed to confirm equal loading and the transfer of all proteins. Protein levels were normalized by Ponceau S staining, and data are expressed as the mean ± SD (n = 3).

180 min. However, these levels were markedly elevated during the pretreatment with 6% (vol/vol) ethanol or 37°C, and Ssa3 levels continued to increase during the subsequent treatment with 10% (vol/vol) ethanol in cells pretreated with 6% (vol/vol) ethanol.

Fes1, Hsp104, and Hsp42 protein levels were gradually increased by the direct exposure to 10% (vol/vol) ethanol. The synthesis of these proteins was also promoted during the pretreatment with 6% (vol/vol) ethanol or 37°C, allowing the pretreated cells to confront subsequent 10% ethanol stress with significantly enhanced levels of Fes1, Hsp104, and Hsp42.

In contrast, Btn2 showed a different expression pattern from the proteins described above under severe ethanol stress. Consistent with previous findings (4), Btn2 protein levels markedly increased with time when cells were directly exposed to 10% (vol/vol) ethanol. On the other hand, Btn2 levels in cells pretreated with 6% (vol/vol) ethanol or 37°C were transiently elevated and then disappeared after 120 min of the subsequent treatment with 10% (vol/vol) ethanol, while the protein levels of Ssa3, Sis1, Fes1, Hsp104, and Hsp42 were strongly maintained during the subsequent treatment with 10% (vol/vol) ethanol. The turnover of Btn2 by the UPS is generally rapid but is suppressed by stress (32, 49). Therefore, the proteasomal turnover of Btn2 may have been restored, even under severe ethanol stress, in pretreated cells.

To verify the importance of the induced expression of PQC-related proteins, we used various mutants deficient in PQC-related genes to examine their acquired resistance to severe ethanol stress. Regarding the reductions observed in insoluble protein levels by the pretreatments, *btn2Δ hsp42Δ* cells behaved similarly to wild-type cells. On the other hand, the accumulation of insoluble proteins was not fully mitigated by the pretreatments in *fes1Δ hsp104Δ* cells and *ssa2Δ ssa3Δ ssa4Δ* cells (22, 50, 51) (Fig. 5). Since insoluble protein levels were similar among *fes1Δ hsp104Δ*, *ssa2Δ ssa3Δ ssa4Δ*, and wild-type cells upon a direct challenge with 10% (vol/vol) ethanol, the induced expression of Fes1, Hsp104, and Hsp70 during the mild stress pretreatment appeared to be important for reducing insoluble protein levels under subsequent 10% (vol/vol) ethanol stress.

The pretreatment with mild stress prevented Lsg1 aggregation under severe ethanol stress. The denaturation of a specific protein was also examined to clarify whether it was consistent with the results of the silver staining of insoluble proteins. Lsg1 was previously identified as a cytosolic protein that is prone to denaturation and aggregation by thermal stress at 42°C (52). We verified that the treatment with 42°C induced the formation of multiple foci of Lsg1 and found that 10% (vol/vol) ethanol stress exerted similar effects (Fig. 6A). Furthermore, Lsg1-mRFP (monomeric red fluorescent protein) foci showed very similar localization with Hsp104-GFP foci after the treatments with 10% (vol/vol) ethanol and 42°C. These results suggested that severe ethanol stress also caused the aggregation of Lsg1, similar to thermal stress at 42°C.

Although the mild stress treatment with 6% (vol/vol) ethanol or 37°C did not induce the aggregation of Lsg1, it significantly mitigated the formation of Lsg1 foci under subsequent 10% (vol/vol) ethanol stress in wild-type cells (Fig. 6B and C). This mitigating effect was also noted in *btn2Δ hsp42Δ* cells, but not in *ssa2Δ ssa3Δ ssa4Δ* or *fes1Δ hsp104Δ* cells (Fig. 6C). These results were consistent with those obtained from the silver staining of insoluble proteins (Fig. 5).

Cells pretreated with mild stress strongly inhibited the formation of insoluble proteins. Insoluble protein levels may be reduced by the activation of insoluble protein removal and the suppression of insoluble protein generation. However, it is currently unclear whether the removal efficiency of insoluble proteins was enhanced in pretreated cells. To clarify the contribution of insoluble protein removal, proteasome activity was inhibited by MG132, an efficient cell-permeative proteasome inhibitor (12, 53), after the pretreatment with 6% (vol/vol) ethanol or 37°C. We confirmed that the treatment with 100 μM MG132 blocked proteasome activity under our experimental conditions based on the increases observed in Btn2 levels in cells at 37°C (see Fig. S2 in the supplemental material). In pretreated wild-type cells, the MG132 treatment did not
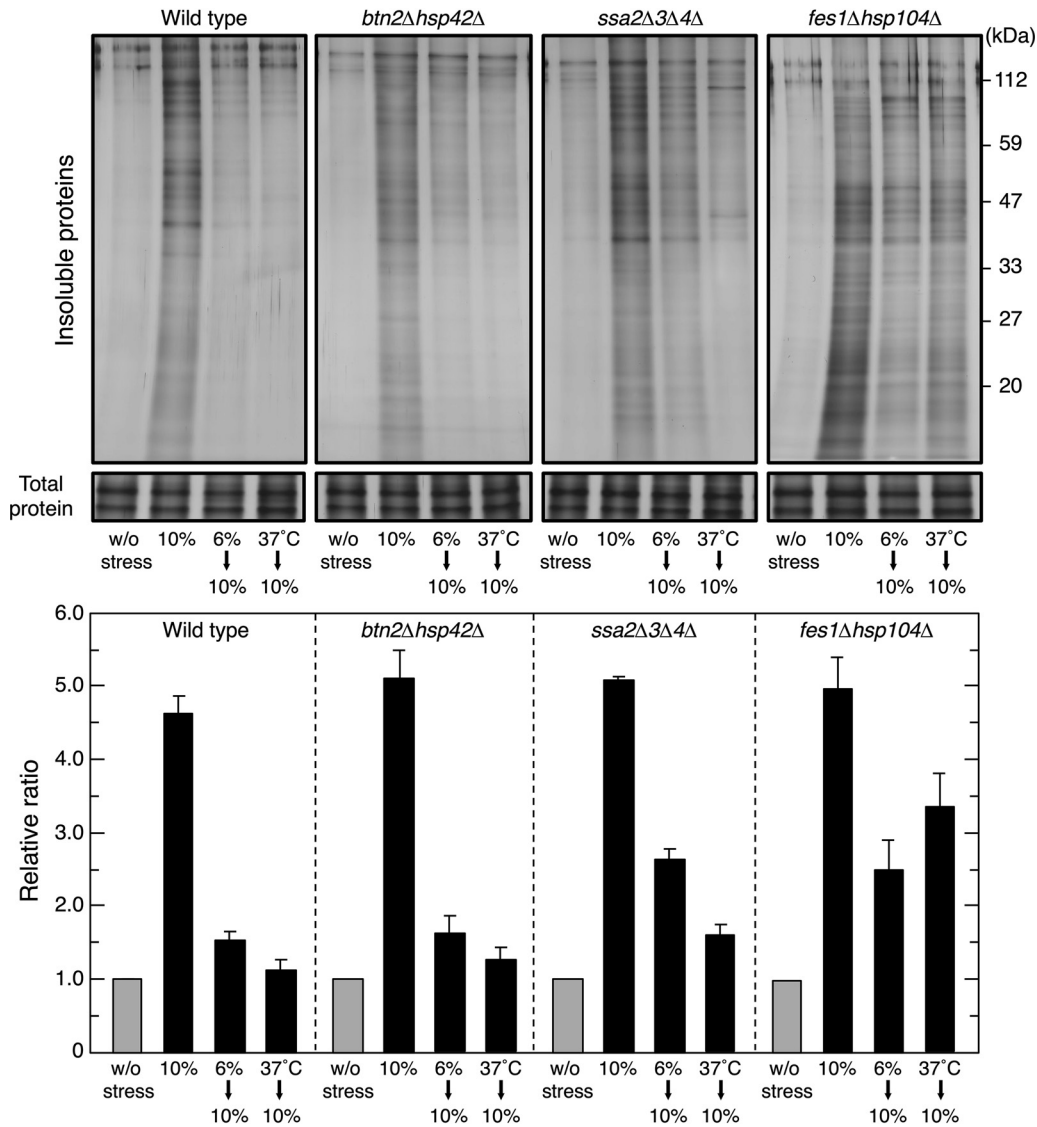
FIG 5 PQC activity in knockout mutants. Insoluble aggregated protein levels in various strains were assayed. Cells were treated with 10% (vol/vol) ethanol for 180 min after the pretreatment with or without mild stress (6% ethanol stress for 180 min or thermal stress at 37°C for 60 min). Representative images are shown in the upper panel, and quantified data are shown in the lower panel. Data are expressed as the mean ± SD ($n = 3$).

markedly affect the levels of insoluble proteins or Lsg1 aggregation under subsequent severe ethanol stress (Fig. 7A and B). We confirmed that MG132 did not significantly affect the levels of insoluble proteins or Lsg1 aggregation using *pdr5Δ* cells, which are less likely to excrete MG132 and are commonly used in proteasome inhibition assays (54, 55). These results suggested that the contribution of proteasomes to reducing the accumulation of insoluble proteins in pretreated cells was not significant.

Furthermore, to clarify whether insoluble protein generation is suppressed in pretreated cells, we examined intracellular insoluble protein levels during the early stages of the treatment with 10% (vol/vol) ethanol. As shown in Fig. 8A, insoluble proteins gradually accumulated with time in cells directly exposed to 10% (vol/vol) ethanol stress. In contrast, insoluble protein levels did not increase in pretreated cells during the early stages. Although the initial levels of insoluble proteins were higher in pretreated cells than in nonpretreated cells (time zero min under 10% ethanol stress), insoluble protein levels in pretreated cells slightly decreased over time. Similar results
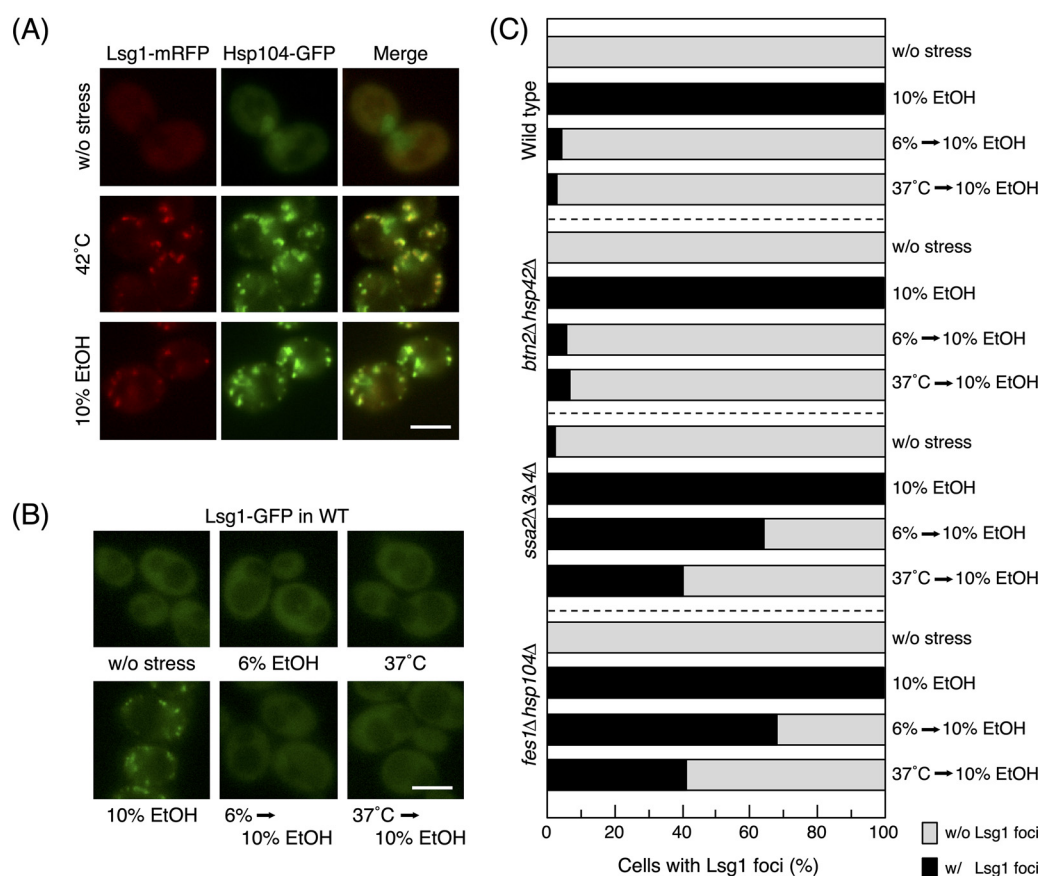
FIG 6 Aggregation of Lsg1 in response to severe ethanol (EtOH) stress. (A) Cells expressing Lsg1-mRFP and Hsp104-GFP were treated with thermal stress at 42°C for 60 min or 10% (vol/vol) ethanol stress for 180 min. Bar, 5 μm. (B and C) Cells expressing Lsg1-GFP were pretreated with or without mild stress (6% ethanol for 180 min or 37°C for 60 min) and then exposed to 10% (vol/vol) ethanol for 180 min. Representative images of wild-type cells under each condition are shown in panel B. The quantification of cells containing Lsg1-GFP foci in various mutants is shown in panel C. One hundred cells under each condition were examined, and experiments were repeated 3 times (300 cells in total were examined).

were observed for the formation of Lsg1 foci (Fig. 8B). Therefore, the pretreatment with mild stress, either 6% (vol/vol) ethanol or thermal stress at 37°C, repressed the generation of insoluble proteins throughout the subsequent treatment with 10% (vol/vol) ethanol.

DISCUSSION

We here demonstrated that the accumulation of insoluble proteins and Lsg1 aggregation in yeast cells pretreated with 6% (vol/vol) ethanol or mild thermal stress at 37°C was mitigated under subsequent severe ethanol stress, clearly indicating that PQC activity was enhanced by the pretreatment. This enhancement in PQC activity by the pretreatment is regarded as a type of acquired resistance by yeast cells to severe ethanol stress.

The induction of enhanced PQC activity required the novel synthesis of proteins during the pretreatment, and pretreated cells maintained elevated expression levels of various proteins under subsequent 10% (vol/vol) ethanol stress. Since 10% (vol/vol) ethanol markedly inhibits translation activity, the synthesis of most proteins is strongly suppressed, except for several exceptions, such as Btn2 (4, 56). Despite the significant increases observed in the mRNA levels of *SIS1* and *SSA3*, the respective protein levels were not markedly affected when cells were directly exposed to 10% (vol/vol) ethanol stress. On the other hand, the treatment with 6% (vol/vol) ethanol or 37°C did not
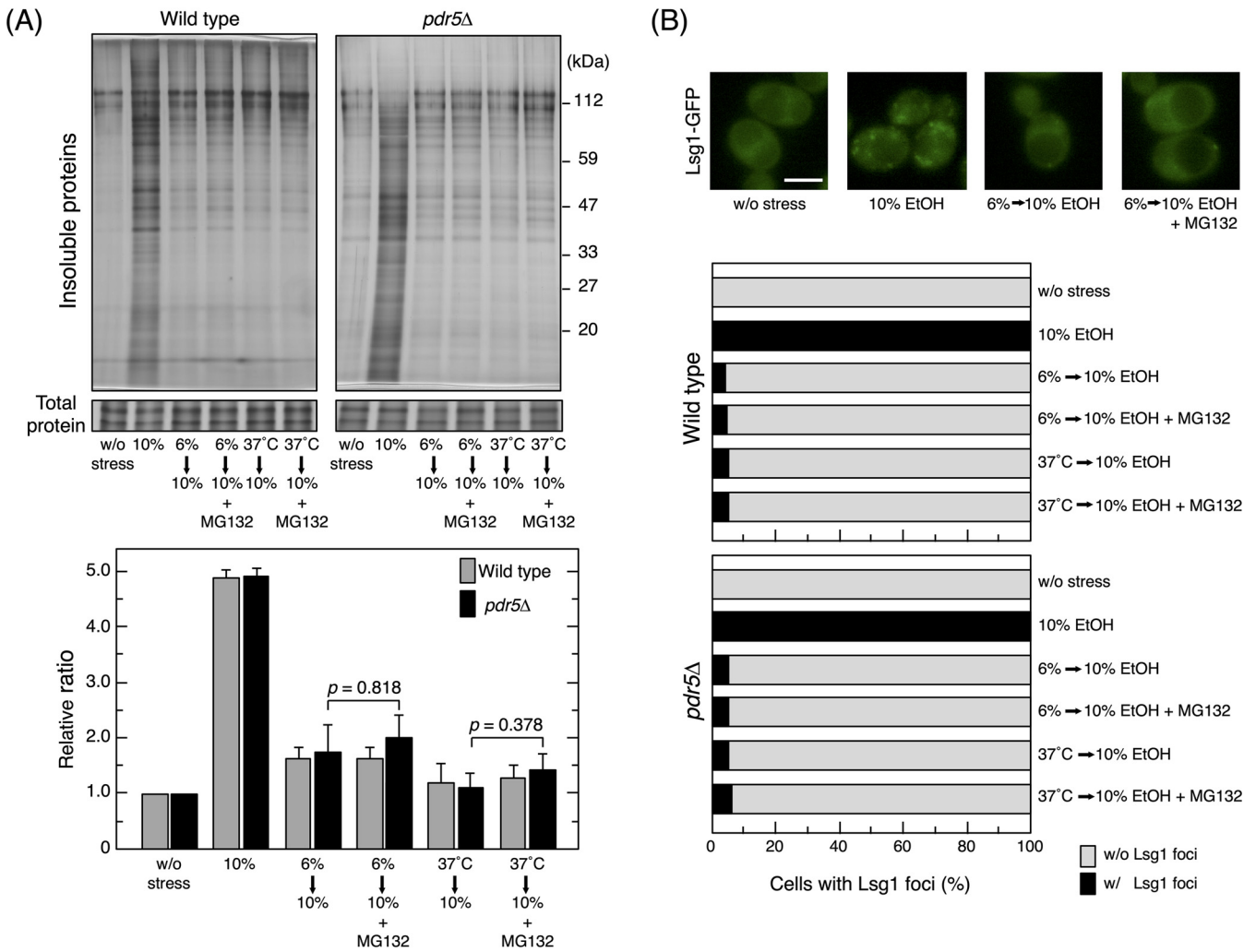
FIG 7 Effects of MG132 on insoluble protein levels in pretreated cells. Pretreated cells (wild-type and *pdr5*Δ) with 6% (vol/vol) ethanol (EtOH) for 180 min or at 37°C for 60 min were exposed to 10% (vol/vol) ethanol for 180 min in the presence or absence of 100 μM MG132, a potent cell-permeative inhibitor of proteasomes. (A) The intracellular levels of insoluble proteins were analyzed. Representative images are shown in the upper panel and quantified data in the lower panel. Relative ratios were calculated from three independent experiments. Each value is expressed as the mean ± SD ($n = 3$). (B) The quantification of cells containing Lsg1-GFP foci under each condition is shown. One hundred cells under each condition were examined, and experiments were repeated 3 times (300 cells in total were examined).

strongly inhibit translation and increased the expression levels of various proteins (57–60). The levels of Fes1, Hsp104, and Ssa3, which constitute the bichaperone system (10, 22), were markedly upregulated in pretreated cells. Therefore, pretreated cells may tolerate severe ethanol stress due to an enhanced bichaperone system via newly synthesized proteins, whereas cells directly exposed to 10% (vol/vol) ethanol have to cope with protein denaturation using a weaker bichaperone system. Additionally, the insufficient acquired stress resistance of *fes1*Δ *hsp104*Δ and *ssa2*Δ *ssa3*Δ *ssa4*Δ cells indicated the importance of enhanced bichaperone systems for increased PQC activity in pretreated cells. The enhancement of the bichaperone system may have contributed to a reduction in insoluble protein accumulation in pretreated cells.

We cannot exclude the possibility that other mechanisms besides the bichaperone system were enhanced in pretreated cells to prevent the accumulation of insoluble proteins. Previous studies demonstrated that 6% (vol/vol) ethanol and thermal stress activated general stress pathways, resulting in increased levels of intracellular trehalose (39, 57, 61, 62). Since trehalose stabilizes protein structures and prevents protein denaturation and aggregation (63, 64), increased levels of intracellular trehalose due to the pretreatments may also contribute to the suppression of insoluble protein generation.
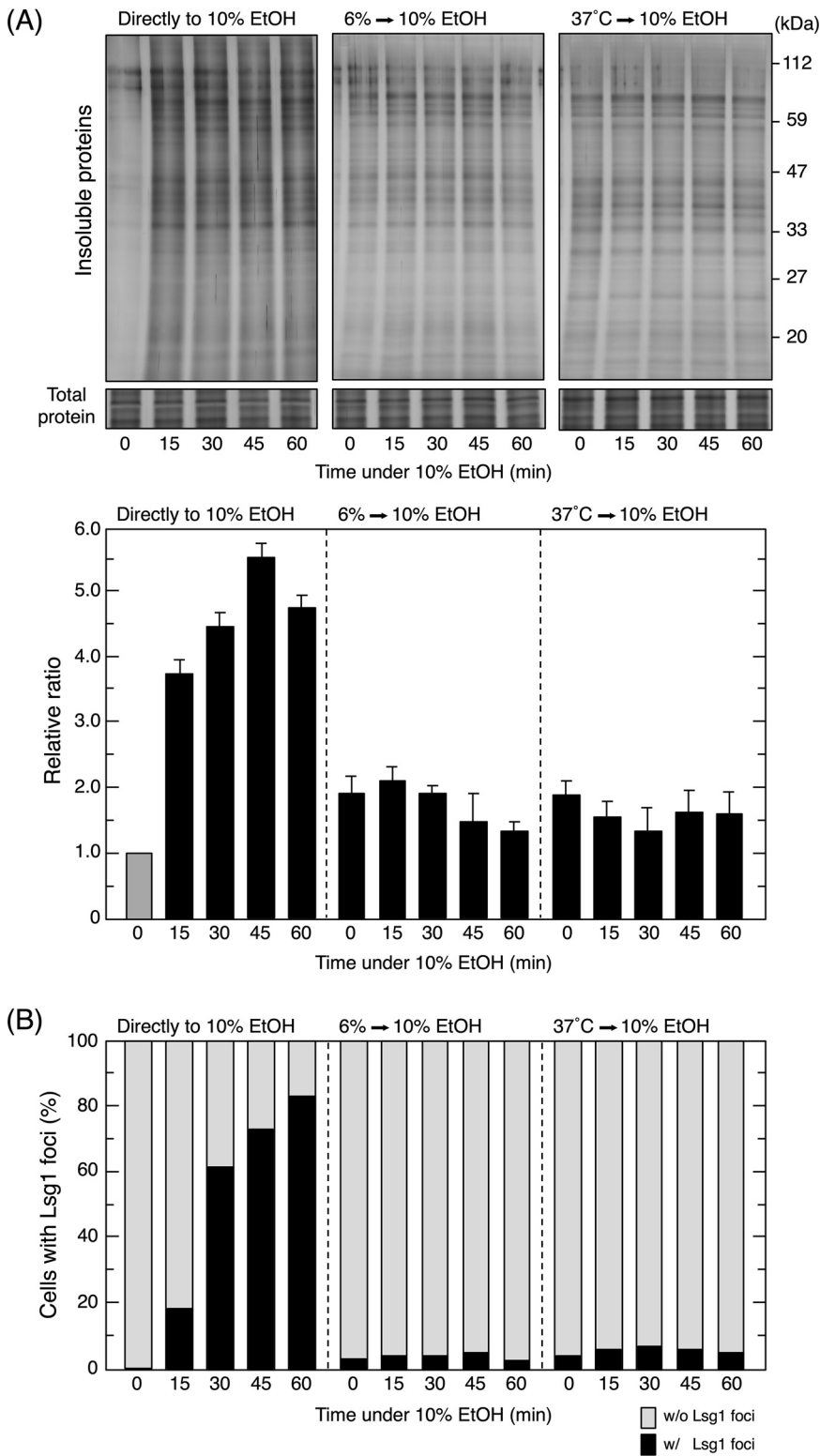
FIG 8 The generation of insoluble proteins was suppressed in pretreated cells. The intracellular levels of insoluble proteins (A) and Lsg1-GFP foci (B) were analyzed every 15 min for the first 60 min after exposure to severe ethanol (EtOH) stress (10% [vol/vol]). (A) The intracellular levels of insoluble proteins were analyzed. Representative images are shown in the upper panel and quantified data in the lower panel. Relative ratios were calculated from three independent experiments. Each value is expressed as the mean ± SD ($n = 3$). (B) The quantification of cells containing Lsg1-GFP foci under each condition is shown. One hundred cells under each condition were examined, and experiments were repeated 3 times (300 cells in total were examined).

Other molecular chaperones may also contribute to the mitigation of insoluble proteins in pretreated cells (24, 55).

In pretreated cells, insoluble protein levels did not increase immediately after the exposure to secondary stress, and the effects of proteasome inhibition by MG132 were very limited. These results suggest that the reduction observed in insoluble protein levels in pretreated cells was not due to the more efficient degradation of insoluble proteins. This interpretation appears to be supported by Rpn4, a transcription activator of proteasome-related genes, not markedly affecting the acquisition of increased resistance to severe ethanol (43). Severe protein denaturation, i.e., the generation of heavily damaged proteins that cannot be reactivated and refolded, may have been less likely to occur in pretreated cells due to the activation of the bichaperone system and other mechanisms. Since the generation of heavily damaged proteins was effectively suppressed, proteasomes appeared to be less important in pretreated cells. Additionally, a previous study demonstrated that in contrast to many exogenous thermolabile proteins, endogenous proteins are not destined to be degraded when severely aggregated by heat shock (65). Even under severe ethanol stress, denatured endogenous proteins may not be readily degraded.

Regarding aggregases, we were unable to confirm their distinct importance for enhanced PQC activity because *btn2Δ hsp42Δ* cells and wild-type cells were able to suppress the accumulation of insoluble proteins induced by the pretreatment. However, the contribution of aggregases to acquired stress resistance did not appear to be entirely absent. Hsp42 was maintained at high levels in pretreated cells during the exposure to 10% (vol/vol) ethanol. Hsp42 also functions as a molecular chaperone and interacts with partially damaged proteins to prevent protein aggregation (14, 22, 66); therefore, it may contribute to the prevention of severe protein denaturation, similar to the bichaperone system and trehalose. In contrast to Hsp42, Btn2 levels were not maintained under the subsequent 10% (vol/vol) ethanol stress in pretreated cells but were markedly elevated in cells directly exposed to 10% ethanol stress (Fig. 4). Since the turnover of Btn2 by the UPS is very effective (32, 49), our results suggest that the function of the UPS was maintained, even under 10% ethanol stress, in pretreated cells but not in nonpretreated cells.

Due to a lack of information, it currently remains unclear whether insoluble proteins accumulate in yeast cells during the brewing process of wine and Japanese sake. In typical wine and Japanese sake brewing processes, ethanol concentrations gradually increase and finally exceed 10% (vol/vol). Therefore, yeast cells are progressively exposed to mild to severe ethanol stress in the wine must or sake mash. Since the pretreatment with mild ethanol stress enhanced PQC activity in the present study, the accumulation of insoluble proteins may be suppressed due to the enhancement of PQC activity during the brewing process. A previous study reported that the expression of Btn2 was not induced during the wine-making process until the final stage (67). This may also reflect the long-term prevention of the accumulation of insoluble proteins during the wine-making process. We plan to collaborate with brewers in order to obtain a more detailed understanding of protein damage in yeast cells during the wine-making and sake brewing processes.

A mild ethanol pretreatment was previously shown to induce markedly stronger resistance to severe ethanol stress in the majority of wild yeast strains, including wine and sake yeasts, than in S288c-derived common laboratory strains, including BY4742, which was used in the present study (43). On the other hand, modern sake yeast strains commonly show defective stress responses due to mutations in *MSN4* (*msn4*[C15540A]), *PPT1* (*Δppt1::Ty2*), and *RIM15* (*rim15*[5055insA]) (68–70). Differences in the capacity to increase PQC activity between laboratory and brewer's strains are an interesting subject for future research.

MATERIALS AND METHODS

Strains and medium. The parental wild-type strain BY4742 (*MATα his3Δ1 ura3Δ0 leu2Δ0 lys2Δ0*) and its isogenic gene-deletion mutants, *hsp42Δ::kanMX*, *hsp104Δ::kanMX*, and *pdr5Δ::kanMX* were obtained from Open Biosystems Inc. (AL, USA). To disrupt the *BTN2* gene, a DNA fragment (2.0 kb)

TABLE 1 List of primers used in knockout mutant and plasmid construction

Name	Sequence
FES1-F1	5'-AAATATCTAGAGACAAGACAAAGCCACTCG-3'
FES1-R1	5'-CGGACCTCGAGATAATACATACTTTACGGC-3'
FES1-F2	5'-ATTATCTCGAGCTGACTACAAGGATGACGATGACAAGTGATTACGTCCGTAGAAATAAAATGATA-3'
FES1-R2	5'-AAGATGGTACCAACTGTTTACTTGTACTGA-3'
SIS1-F1	5'-GGTCCGAGCTCTGGTCCTGGTGGTCCTGGC-3'
SIS1-R1	5'-GGATTCTCGAGAAAAATTTTCATCTATAGC-3'
SIS1-F2	5'-TTTTTCTCGAGCTGACTACAAGGATGACGATGACAAGTAATAGTAATCCTAAGCAAATATAATTA-3'
SIS1-R2	5'-TTTGAGGATCCCTACGTAATGACTGTTCCT-3'
SSA1-F1	5'-TGCTGGTGGTGTCTAGACCAAGTTGATTCC-3'
SSA1-R1	5'-CACCAATTGGCTCGAGCAACTTCTTCAACG-3'
SSA3-F1	5'-GGATGCAGGAACTAGTGCAGGGATGAACGT-3'
SSA3-R1	5'-AGAAGAATACTCGAGCAACCTCTTCCACTG-3'
SSA3-F2	5'-AGTTGCTCGAGCCGACTACAAGGATGACGATGACAAGTGATTATTCTTCTATAGTGTTCT-3'
SSA3-R2	5'-ACGTCATCTTTACCCGTGGTACCTAATTTC-3'
LSG1-F1	5'-ACCGCGGTGGCGGCCGCTCTAGAGCGCTTGCTAAGGATTTGATTGTTCCA-3'
LSG1-R1	5'-CTCCTTTGCTAGCCATAGCTCGAGAATTATTTTCAATGCTAAAAACTTTGCTTTTCGCATTTTTAC-3'
btn2-F	5'-AGTTCTTGGCGAAGTAAAGTGGCAAAACAAATGGAAGATCTATTGCATTAGTTGTAAAACGACGGCCAGT-3'
btn2-R	5'-TCCCTTGGGAGATCTGCTTAGGGACTCGTTGTATCTGTCAACTTCCTATCACAGGAAACAGCTATGACC-3'
fes1-F	5'-AGAGCACTCATCGTCAGTCAGAAAGCCATTACCTTTCAACGAAAGAGTGTTGTAAAACGACGGCCAGT-3'
fes1-R	5'-TGGTTTGGCGGTGTTATCACTTAATACAGGTGCTATGCAGTCGAGCCCCACAGGAAACAGCTATGACC-3'

containing *btn2Δ::CgHIS3* was amplified using the primers *btn2*-F/R (Table 1) and pCgHIS3 as a template (71). The amplicon was introduced into *hsp42Δ::kanMX* to construct the *btn2Δ hsp42Δ* double-knockout mutant. To disrupt the *FES1* gene, a DNA fragment (1.8 kb) encoding *fes1Δ::CgURA3* was amplified using the primers *fes1*-F/R (Table 1) and pCgURA3 as a template (71). The amplicon was introduced into *hsp104Δ::kanMX* to construct the *fes1Δ hsp104Δ* double-knockout mutant. pCgHIS3 and pCgURA3 were provided by the National BioResource Project, Japan. JN516 (*ssa2Δ ssa3Δ ssa4Δ*) was provided by E. A. Craig (51).

Stress treatment. Yeast cells were cultured in SD medium (2% glucose, 0.67% yeast nitrogen base without amino acids, 20 mg/liter uracil, 30 mg/liter L-lysine HCl, 100 mg/liter L-leucine, and 20 mg/liter L-histidine HCl) with reciprocal shaking (120 rpm) at 28°C. The seed culture was prepared by inoculating cells from agar plates in 3 ml SD medium. After cultivation for 12 h, cells from the seed culture were diluted in 250 ml SD medium (the initial optical density was adjusted to an optical density at 600 nm [OD_{600}] of 0.05) in an Erlenmeyer flask (500 ml) and cultured further for more than eight generations to reset the cellular memory of acquired stress resistance (72). Exponentially growing cells were harvested at an OD_{600} of 0.5 to 0.6. Regarding the treatment with ethanol, harvested cells were transferred into fresh SD medium containing ethanol and incubated in a water bath at 28°C with shaking (120 rpm). In the thermal stress treatment, 25 ml of a cell culture in a 50-ml plastic centrifuge tube (catalog no. 227261; Greiner Bio-One International GmbH, Kremsmünster, Austria) was incubated in a water bath (37 or 42°C) with shaking (120 rpm). Proteasome activity was blocked by 100 μM MG132 (A11043; AdooQ Bioscience, CA, USA) (53).

Plasmids. The primers used for plasmid construction are listed in Table 1. All FLAG tag-fused proteins were expressed using integrative plasmids, and the transcription of each gene was under the control of its own promoter.

(i) YIp-*FES1*-FLAG. A 0.4-kbp fragment encoding a part of the open reading frame (ORF) of *FES1* was amplified by PCR using the primers *FES1*-F1/R1 and cloned into the XbaI/XhoI sites of pJK67 (73) to construct YIp-*FES1*-GFP. A 0.3-kbp fragment encoding a FLAG tag sequence, stop codon, and the 3'-flanking region of *FES1* was amplified using the primers *FES1*-F2/R2 and cloned into the XhoI/KpnI sites of YIp-*FES1*-GFP to construct YIp-*FES1*-FLAG. To integrate *FES1*-FLAG at the chromosomal *FES1* locus, YIp-*FES1*-FLAG was linearized by HindIII and then introduced into yeast cells. This plasmid allowed the expression of FLAG-tagged Fes1 short form (Fes1 S) (74) to be visualized by Western blotting.

(ii) YIp-*SIS1*-GFP and YIp-*SIS1*-FLAG. A 0.8-kbp fragment encoding a part of the ORF of *SIS1* was amplified by PCR using the primers *SIS1*-F1/R1 and cloned into the SacI/XhoI sites of pJK67 to construct YIp-*SIS1*-GFP. A 0.3-kbp fragment encoding a FLAG tag sequence, stop codon, and the 3'-flanking region of *SIS1* was amplified using the primers *SIS1*-F2/R2 and cloned into the XhoI/BamHI sites of YIp-*SIS1*-GFP

TABLE 2 List of primers used in qRT-PCR

Name	Sequence
FES1-F	5′-AGAACTACGTGCTGCTGCTT-3′
FES1-R	5′-ACGTCGAGTGGCTTTGTCTT-3′
HSP42-F	5′-GGACCAACCAACAGGCAAAC-3′
HSP42-R	5′-GTGGTCTCGACGATTCCTCC-3′
HSP104-F	5′-GGCCATCAAGCAACAAGCTC-3′
HSP104-R	5′-GCGGTCTTACCGATACCTGG-3′
SIS1-F	5′-GCATCAAGCTCTCCCACGTA-3′
SIS1-R	5′-CATGTGGGCCCTTTCTTCCA-3′
SSA3-F	5′-ATGTTGCGCCATTGTCCCTA-3′
SSA3-R	5′-ATTTGAGGCACACCTCTGGG-3′
BTN2-F	5′-TTTCCGAAGGTGGCATCAAC-3′
BTN2-R	5′-CTTTCGCTTTCTCCGCTTCTTC-3′
ACT1-F	5′-TTGGATTCCGGTGATGGTGTTACT-3′
ACT1-R	5′-TGAAGAAGATTGAGCAGCGGTTTG-3′

to construct YIp-*SIS1-FLAG*. To integrate *SIS1-GFP* and *SIS1-FLAG* at the chromosomal *SIS1* locus, YIp-*SIS1-GFP* and YIp-*SIS1-FLAG* were linearized by XbaI and then introduced into yeast cells.

(iii) YIp-*SSA1-GFP*. A 0.7-kbp fragment encoding a part of the ORF of *SSA1* was amplified by PCR using the primers *SSA1*-F1/R1 and cloned into the XbaI/XhoI sites of pJK67 to construct YIp-*SSA1-GFP*. To integrate *SSA1-GFP* at the chromosomal *SSA1* locus, YIp-*SSA1-GFP* was linearized by SalI and then introduced into yeast cells.

(iv) YIp-*SSA3-FLAG*. A 1.5-kbp fragment encoding a part of the ORF of *SSA3* was amplified by PCR using the primers *SSA3*-F1/R1 and cloned into the SpeI/XhoI sites of pJK67 to construct YIp-*SSA3-GFP*. A 0.5-kbp fragment encoding a FLAG tag sequence, stop codon, and the 3′-flanking region of *SSA3* was amplified using the primers *SSA3*-F2/R2 and cloned into the XhoI/KpnI sites of YIp-*SSA3-GFP* to construct YIp-*SSA3-FLAG*. To integrate *SSA3-FLAG* at the chromosomal *SSA3* locus, YIp-*SSA3-FLAG* was linearized through its digestion with ClaI and then introduced into yeast cells.

(v) YIp-*LSG1-GFP* and YIp-*LSG1-mRFP*. A 1.5-kbp fragment encoding a part of the ORF of *LSG1* was amplified by PCR using the primers *LSG1*-F1/R1 and cloned into the XbaI/XhoI sites of pJK67 and YIp-*DCP2-mRFP* (56) to construct YIp-*LSG1-GFP* and YIp-*LSG1-mRFP*, respectively. To integrate *LSG1-GFP* or *LSG1-mRFP* at the chromosomal *LSG1* locus, YIp-*LSG1-GFP* and YIp-*LSG1-mRFP* were linearized by HpaI and then introduced into yeast cells. To construct YIp-*HIS3-LSG1-GFP*, the *LSG1-GFP* region of YIp-*LSG1-GFP* was cloned in pRS303, which is an integrative plasmid with the *HIS3* marker (75).

The construction of YIp-*HSP42-FLAG*, YIp-*HSP104-GFP*, and YIp-*BTN2-FLAG* was described in our previous studies (4, 31, 76). To construct YIp-*LEU2-HSP104-GFP*, the *HSP104-GFP* region of YIp-*HSP104-GFP* was cloned in pRS305, which is an integrative plasmid with the *LEU2* marker (75).

qRT-PCR. Quantitative reverse transcription-PCR (qRT-PCR) was conducted to assess the mRNA expression levels of the *BTN2*, *FES1*, *HSP42*, *HSP104*, *SIS1*, and *SSA3* genes using previously described methods (4). Comparisons of mRNA expression levels were performed by normalizing the mRNA level of each gene to that of the reference gene, *ACT1*. The oligonucleotide sequences of the primers used in qRT-PCR are listed in Table 2.

Protein analysis. Insoluble aggregated protein levels were analyzed by the method of Koplin et al. (77) with a slight modification (6, 78). Briefly, after a treatment with Zymolyase-20T (2.5 mg/ml) (Nacalai Tesque, Kyoto, Japan) at 25°C for 20 min, cells were disrupted by vortexing with glass beads in lysis buffer (50 mM potassium phosphate buffer, 1.0 mM EDTA, and 5% glycerol, pH 7.0). Unbroken cells and debris were removed by centrifugation ($200 \times g$ for 20 min), and the total protein concentration of each sample was then measured and normalized. Insoluble aggregated proteins were collected by centrifugation ($16,000 \times g$ for 20 min), washed twice with lysis buffer containing 2% NP-40, and solubilized with urea buffer (50 mM Tris–HCl, 6.0 M urea, and 5% SDS, pH 7.5). Samples were subjected to 10% polyacrylamide gel electrophoresis and visualized by silver staining using Sil-Best Stain One (Nacalai Tesque).

Although the majority of proteins are resistant to glass bead-induced aggregation, some yeast proteins were aggregated during glass bead lysis (79). To investigate the potential artifacts of glass bead lysis, we performed mortar-pestle lysis to collect insoluble aggregated proteins using the method of Roth et al. (80). Since similar results were obtained by mortar-pestle lysis and glass bead lysis (see Fig. S3 in the supplemental material), the level of artifacts induced by glass bead lysis was considered to be negligible, and relative changes in insoluble protein levels were clearly observed with our method using glass bead lysis.

FLAG-tagged protein and Hsp104 levels were monitored by Western blotting using the procedure described previously (4, 6). An anti-FLAG M2 antibody (F1804; Sigma-Aldrich, MO, USA); anti-Hsp104 antibody (ADI-SPA-1040-D ENZ; Enzo Life Sciences, Inc., NY, USA); anti-mouse IgG, horseradish peroxidase (HRP)-linked antibody (7076S; Cell Signaling Technology, MA, USA); and anti-rabbit IgG, HRP-linked antibody (7074S; Cell Signaling Technology) were used for Western blotting. The bands on silver staining and Western blots were quantified using ImageJ software (http://imagej.nih.gov/ij/) and normalized by total proteins or Ponceau S staining, respectively. The significance of differences was evaluated by an unpaired two-tailed Student t test.

Fluorescence microscopic analysis. A fluorescence microscope system (Leica AF6500; Leica Microsystems Vertrieb GmbH, Wetzlar, Germany) was used in the fluorescence microscopic analysis. The percentage of cells containing Hsp104-GFP or Lsg1-GFP foci was calculated by examining 100 cells under each condition and shown as the mean $\pm$ standard deviation (SD). Experiments were repeated 3 times (300 cells in total were examined under each condition).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 4.2 MB.

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