Isolation and Characterization of High-Osmolarity-Sensitive Mutants of Fission Yeast

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For the fission yeast Schizosaccharomyces pombe, adaptation to high-osmolarity medium is mediated by a mitogen-activated protein (MAP) kinase cascade, involving the Wis1 MAP kinase kinase and the Sty1 MAP kinase. The MAP kinase pathway transduces an osmotic signal and accordingly regulates the expression of the downstream target gene (gpd1+) that encodes NADH-dependent glycerol-3-phosphate dehydrogenase, in order to adaptively accumulate glycerol inside the cells as an osmoprotectant. We previously characterized a set of high-osmolarity-sensitive S. pombe mutants, including wis1, sty1, and gpd1. In this study, we attempted to further isolate novel osmolarity-sensitive mutants. For some of the mutants isolated, profiles of glycerol production in response to the osmolarity of the growth medium were indistinguishable from that of the wild-type cells, suggesting that they are novel types. They were classified into three distinct types genetically and, thus, were designated hos1, hos2, and hos3 (high osmolarity sensitive) mutants. One of them, the hos1 mutant, was characterized in detail. The hos1 mutant was demonstrated to have a mutational lesion in the known ryh1+ gene, which encodes a small GTP-binding protein. Disruption of the ryh1+ gene results not only in osmosensitivity but also in temperature sensitivity for growth. It was also found that the Δryh1 mutant is severely sterile. These results are discussed with special reference to the osmoadaptation of S. pombe.

Exposure of cells to high-osmolarity conditions in their environment led to dehydration and a decrease in cell viability. Accordingly, the ability of cells to adapt to external osmotic stress is a fundamental biological process that protects the organism against fluctuation in the water activity and solute content of their environment. In fact, many types of both prokaryotic and eukaryotic cells have developed mechanisms to adapt to severe osmotic stresses in their environment (often called osmoregulation) (4, 7). Recently, much attention has been focused on osmoregulation, with special emphasis on the downstream region of the MAP kinase-Atf1-Gpd1 signaling pathway. In fact, a pair of histidine kinases (Mak1 and Mak2) and a response regulator (Msc4) were suggested to be involved in the osmosensing pathway (22). It should be noted that a similar osmoregulation scenario has been well documented for the budding yeast Saccharomyces cerevisiae, in which the Snf1p-Ypd1p-Ssk1p phosphotransfer signaling pathway and the Hog1 MAPK cascade are crucially involved (6, 15, 20, 30, 32). In S. pombe, the downstream region of the MAP kinase cascade is less clear at present. However, recent studies have uncovered a basic leucine zipper (bZIP) type of transcription factor, Atf1 (also known as Gad7), which is a direct target of the Sty1 kinase (11, 25, 27, 31). In short, the osmoregulated transcription of the gpd1+ gene is greatly reduced in the atf1-, sty1-, and atf1 mutants, and consequently these mutants, as well as gpd1 mutants, exhibit an osmosensitive phenotype.

As mentioned above, we have been extensively studying osmoregulation in S. pombe (1, 18, 19, 33, 34). In this study, to gain new insight into the molecular mechanisms underlying osmoregulation in S. pombe, we attempted to isolate novel types of mutants, each of which show a osmosensitive phenotype. Here we isolated a set of S. pombe mutants that were found to be novel in that the mutational events are not apparently linked to the well-characterized MAP kinase-Atf1-Gpd1 pathway. Furthermore, one of the novel osmosensitive S. pombe mutants was characterized in detail.
**RESULTS**

Isolation of a number of high-osmolarity-sensitive mutants.

An attempt was made to isolate osmoregulation-defective mutants of *S. pombe* by screening mutagenized cells for failure to grow on a high-osmolarity medium supplemented with 2 M sorbitol (i.e., YPD agar plates plus 2 M sorbitol). Of 40,000 colonies thus screened, 30 candidates were selected as putative high-osmolarity-sensitive mutants. They were then analyzed extensively by means of standard yeast genetics, including complementation analyses. Based on the results, they were classified into seven complementation groups. Among them, four groups were shown to be *gpd1*, *afl1*, *sty1*, and *wis1* mutants, as anticipated (see the introduction). Others were clearly different from those ascribed alleles. Since we intended in this study to isolate novel high-osmolarity-sensitive mutants, we decided to further characterize these apparently novel ones, which were designated as *hos* (high-osmolarity-sensitive) mutants (namely, *hos1*, *hos2*, and *hos3*).

For further analyses, three strains, M6 for *hos1*, M10 for *hos2*, and M26 for *hos3*, were selected as representatives of strains with mutations in these alleles. It should be noted that these mutants have been purified genetically by repeated backcross with the wild-type genetic background (YJ333, see Table 1). That the osmosensitive phenotype of these mutants was recessive was also confirmed (data not shown). First, their osmosensitivity phenotypes were verified, as shown in Fig. 1. These three mutants as well as two well-characterized osmosensitive mutants (i.e., *Aagpd1* and *Δwis1*) were streaked on YPD plates containing either 2 M glucose, 2 M sorbitol, or 0.9 M KCl. All of these mutants showed a growth defect in these
high-osmolarity media (Fig. 1B to D). The mutant M6 exhibited a relatively low growth rate even on the standard YPD agar plate (Fig. 1A). The sensitivity of the mutant M26 to 0.9 M KCl was less evident (Fig. 1D). It was also found that the mutant M6 clearly exhibited a temperature sensitivity for growth at 37°C (Fig. 1E) (it is important to also note that a temperature-sensitive phenotype has been reported for the Δwis1 mutant, as shown in Fig. 1E).

**hos1 mutants are novel types.** In the previous study, we have presented evidence that the expression of gpd1 mRNAs and the accumulation of intracellular glycerol are important for cells to grow on high-osmolarity medium (1, 19). To clarify whether the osmosensitive phenotypes of the isolated mutants were due to the defect in production of the osmoprotectant glycerol, we first examined the expression of gpd1 mRNA in these mutants. As shown in Fig. 2, hos1 mutant cells growing exponentially in YPD medium were transferred into fresh medium supplemented with 0.9 M KCl, and total RNA was isolated from the cells harvested after the times indicated. Each RNA fraction was subjected to Northern hybridization analysis with an appropriate gpd1 probe. Upon the shift to the high-osmolarity medium, for each hos1 mutant, the amount of gpd1 mRNA increased substantially within 0.5 h as in the case of the wild type. In marked contrast, no or very small amounts of gpd1 mRNA were detected in the Δgpd1 and Δwis1 mutants, respectively, regardless of the medium osmolarity. The latter observations are highly consistent with previous results (1, 18). Essentially, the same results were obtained even when 2 M sorbitol was used as an alternative osmotic solute (data not shown). It was thus found that the osmoregulated expression of gpd1 mRNA appears not to be impaired in these hos1 mutants.

We then needed to measure directly the level of intracellular glycerol, since the result described above does not necessarily mean that glycerol is normally accumulated in the mutant cells in response to high-osmolarity stress. To examine this, the mutant cells were grown in YPD medium and then transferred into the same medium supplemented with 0.9 M KCl or unsupplemented. After incubation for 2 h, the intracellular accumulation of glycerol was measured for these cells (Fig. 3). In the case of the wild type, a marked accumulation of intracellular glycerol was observed upon the upshift to the high-osmolarity medium, as has been well documented (1, 19). In the Δgpd1 and Δwis1 mutant cells, the osmoregulated intracellular accumulation of glycerol was greatly reduced, as described previously (1, 19). In the mutant cells isolated in this study, however, the intracellular accumulation of glycerol was found to occur as normally as it did in the wild type.

From these results, we confirmed that the hos1, hos2, and hos3 mutants are novel and that their osmosensitive phenotypes are not simply explained by the defect in production of the osmoprotectant glycerol. Therefore, extensive characterization of these hos mutants should shed light on the molecular mechanisms underlying the osmoregulation in this particular eukaryotic microorganism. This view encouraged us to further characterize these hos mutants and, with this end in mind, we selected the hos1 mutant strain (M6) for such detailed analyses.

**Isolation of a gene that complements hos1.** The hos1 mutant is unable to grow on plates containing 2 M glucose and exhibits a temperature sensitivity for growth at 37°C (Fig. 1). In the hope of finding S. pombe genes that are relevant to the mutation, we screened a genomic DNA library to look for such clones on a multicopy plasmid that can suppress both of the phenotypic characteristics (i.e., osmosensitivity and temperature sensitivity). A number of plasmid clones were isolated as candidates, each of which carried a certain DNA insert with different lengths relative to each other. However, as judged from the results of restriction analyses and hybridization analyses, it was found that they all contain a common genomic DNA region. The simplified result is shown in Fig. 4A, in which DNA regions are schematically shown. As shown in Fig. 4B, the 1.9-kb insert in pNo20 has the ability to complement both of the mutational lesions of M6. The nucleotide sequence of this insert was determined, and it was revealed that this region encompasses a known open reading frame, which was previously designated as the ryh1 gene that encodes a small GTP-binding protein (10). To verify that the ryh1 gene is indeed responsible for the observed complementation ability, the SpeI-SpeI region was replaced by the ura4+ marker on pNo1 to yield pHA1200. This plasmid had lost the complementation ability, as shown in Fig. 4B. From these results, we concluded that the ryh1+ gene is responsible for the complementation ability observed for the hos1 mutant.

We then wanted to determine whether hos1 is a mutant allele of the ryh1 gene. To clarify this, pHA1201, in which the ura4+ marker was inserted at the HpoI site upstream of the ryh1 gene, was also constructed, as shown in Fig. 4A. We confirmed that this particular clone still has the ability to complement hos1 (Fig. 4B). A Ura− derivative of M6 was transformed by the NheI-NheI fragment encompassing the ura4+ gene.
marker as well as the rhy1+ gene, and the stable Ura4+ transformants were selected. It was revealed that all of them grew on YPD plates containing 2 M glucose and at 37°C. It should be noted that for several such Ura4+ Osra1 transformants, we confirmed by Southern hybridization that the NheI-NheI fragment was inserted into the right place, not elsewhere on the chromosome, via homologous recombination (data not shown). These results supported the idea that the hos1 mutation is in the rhy1+ gene.

Construction of Δrhy1. Since the rhy1+ gene is known to be dispensable for growth (10), a one-step gene disruption method utilizing a haploid strain (JY741) and the ura4+ marker was adopted to construct a Δrhy1 mutant allele, in order to characterize the gene with special reference to osmoregulation, as shown in Fig. 5A. The resulting mutant (named HA1001) was confirmed by Southern hybridization to contain the disrupted gene, rhy1::ura4+, as expected (Fig. 5B). The phenotypic characteristics of the Δrhy1 mutant was confirmed by showing that it exhibits osmosensitivity and temperature sensitivity for growth (Fig. 5C). Furthermore, these phenotypic characteristics were reverted to those of the wild type by introducing the rhy1+ gene on pNo20 (Fig. 5C). We then concluded that the rhy1+ gene is somehow implicated in the osmotic adaptation of S. pombe.

Disruption of the rhy1+ gene results in sterility. To gain further insight into the function of the rhy1+ gene in terms of osmotic adaptation, several other phenotypic characteristics of the Δrhy1 mutant were explored. First, vacuole biogenesis and cell wall integrity in the Δrhy1 mutant were examined (see Discussion). We investigated the intracellular distribution of vacuoles in exponentially growing cells by visualization with a reagent (named FM4-64). We could not detect any noticeable difference with regard to the number and size of vacuoles between the Δrhy1 mutant and wild-type cells (data not shown). To examine cell wall integrity, the sensitivities of Δrhy1 mutant and wild-type cells to glucanase were compared, but no evident difference was detected in this respect (data not shown).

During the course of such examinations, however, we noticed that the Δrhy1 mutant haploid strain may be severely sterile. To confirm this intriguing finding, we constructed a homothallic h700 strain (HA1002) carrying the rhy1::ura4+ allele. Upon nitrogen starvation, the h700 wild-type strain (JY808) was able to conjugate and form spores in up to 45% of the cells, while no spores were detected in the h700 Δrhy1 mutant cells, as quantitatively shown in Fig. 6. This defect in mating was suppressed by introducing the rhy1+ gene on a plasmid into the mutant strain, as also shown in Fig. 6. It was thus suggested that the rhy1+ gene plays a role, either directly or indirectly, in the mating processes of S. pombe.

DISCUSSION

In S. pombe, glycerol appears to be the main compatible solute which is accumulated inside the cells in response to high medium osmolarity in order to maintain an osmotic homeostasis. Thus, the failure to accumulate glycerol should result in an osmosensitive phenotype for growth. In fact, a number of such osmosensitive mutants have already been isolated and characterized (e.g., gpd1, wis1, sty1, and aff1) (see the introduction). However, one can suppose that certain other mutational lesions may also result in an osmosensitive phenotype for growth. Keeping this assumption in mind, in this study we attempted to isolate novel types of high-osmolarity-sensitive
(hos) mutants to gain insight into the molecular mechanisms underlying the complicated osmotic adaptation in \textit{S. pombe}. Indeed, we succeeded in isolating such novel ones, the hos1, hos2, and hos3 mutants. Extensive characterization of these hos mutants should shed light on the relevant issues mentioned above. Studies concerned with this are currently under way in our laboratory. In the meantime, in this study we characterized the hos1 mutant at the molecular level by demonstrating that hos1 is a mutant allele of the gene known as \textit{ryh1}.

The \textit{ryh1} gene encoding a GTP-binding protein (or G protein) of 201 amino acids and belonging to the \textit{ras} superfamily was originally isolated by Hengst et al. by using the protein-coding region of the cloned \textit{S. cerevisiae} \textit{YPT1} gene as a hybridization probe (10). As is well known, members of the \textit{ras} superfamily of proteins can be further classified into five subfamilies, namely, \textit{Ras}, \textit{Rho}, \textit{Rab} (\textit{Ypt}), \textit{Ran}, and \textit{Arf}. From the entire genome sequence of \textit{S. cerevisiae}, 11 genes, whose protein products bear sequence features justifying their membership in the Rab (\textit{Ypt}) subfamily, have been recently identified by Lazar et al. (14). On the basis of this current knowledge, it would be of interest to examine the relationship between the \textit{S. pombe} \textit{Ryh1} sequence and those of the \textit{S. cerevisiae} \textit{Rab} (\textit{Ypt}) family of sequences by constructing a reliable phylogenetic tree. As shown in Fig. 7, the results confirmed that \textit{S. pombe} \textit{Ryh1} appears to belong to the \textit{Rab} (\textit{Ypt}) family and is closely related to the recently identified \textit{S. cerevisiae} \textit{Ypt6} sequence (it is important to note that, from the comparison, \textit{Ryh1} appears to be distantly related to \textit{Ypt1}). Such an inspection also revealed that among sequences in the current databases, the most homologous protein to \textit{Ryh1} is \textit{Ypt6} and \textit{Rab6}, as far as their amino acid sequences are concerned. Both \textit{Ypt6} and \textit{Rab6} were suggested to play a role in the Golgi event of vesicular transport (14). \textit{Ryh1} might play a similar role, and addressing this issue by using the \textit{hos1} (\textit{D\textit{ryh1}}) mutant is of interest. In fact, in the original study on the \textit{D\textit{ryh1}} mutant by Hengst et al. (10), the authors pointed out the possibility that an under glycosylation of invertebrate may occur in their \textit{D\textit{ryh1}} \textit{S. pombe} mutant. This is consistent with the current view that protein glycosylation processes are closely linked to vesicular transport processes. In this respect, we recently found that our \textit{D\textit{ryh1}} mutant showed a phenotype of hypersensitivity to hygromycin B (25 \mu g/ml) and vanadate (4 mM) (28a). A similar phenotype has been reported for the \textit{S. pombe} \textit{gms1} mutant that is defective in protein glycosylation (26, 28). It is thus tempting to speculate that the \textit{hos1} (\textit{D\textit{ryh1}}) mutant may have a defect in the process of vacuole biogenesis. However, this appears not to be the case, as mentioned above (data not shown). We also suspected that \textit{Ryh1} might somehow be involved in the maintenance of cell wall integrity. But, the results of our glucanase treatment experimentation could not support this idea, as also mentioned (data not shown). Therefore, another plausible explanation(s) should be considered for the function of \textit{Ryh1} in relation to the osmosensitivity.

As mentioned above, \textit{Ryh1} seems to be closely related to \textit{S. cerevisiae} \textit{Ypt6} and human \textit{Rab6}, as far as their amino acid sequences are concerned. Both \textit{Ypt6} and \textit{Rab6} were suggested to play a role in the Golgi event of vesicular transport (14). \textit{Ryh1} might play a similar role, and addressing this issue by using the \textit{hos1} (\textit{D\textit{ryh1}}) mutant is of interest. In fact, in the original study on the \textit{D\textit{ryh1}} mutant by Hengst et al. (10), the authors pointed out the possibility that an under glycosylation of invertebrate may occur in their \textit{D\textit{ryh1}} \textit{S. pombe} mutant. This is consistent with the current view that protein glycosylation processes are closely linked to vesicular transport processes. In this respect, we recently found that our \textit{D\textit{ryh1}} mutant showed a phenotype of hypersensitivity to hygromycin B (25 \mu g/ml) and vanadate (4 mM) (28a). A similar phenotype has been reported for the \textit{S. pombe} \textit{gms1} mutant that is defective in protein glycosylation (26, 28). It is thus tempting to speculate that the \textit{hos1} (\textit{D\textit{ryh1}}) mutant may have a defect in the process of protein glycosylation in such a way that the mutation affects the cell surface structure (or integrity). Such a mutational lesion might in turn result in the phenotype of high osmolarity sensitivity. Another intriguing finding, that the \textit{D\textit{ryh1}} mutant is severely sterile, may also be explained by assuming that a process of conjugation (or cell-cell contact) is impaired in the mutant (Fig. 6), which likely has an altered cell surface structure.
In short, in this study we intended to isolate novel types of high-osmolarity-sensitive S. pombe mutants and succeeded in doing so. Characterization of these mutants will provide us with clues toward understanding the molecular mechanisms underlying the osmotic adaptation in S. pombe. But also, we provide us with new insight into the functions of relevant genes that are involved in other important cellular processes, as demonstrated for the rhy1 gene encoding a GTP-binding protein. Other mutants, hos2 and hos3, are also of interest for further examination.

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