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Genetic Evidence for a Functional Relationship between Hsp104 and Hsp70

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The phenotypes of single Hsp104 and Hsp70 mutants of the budding yeast *Saccharomyces cerevisiae* provide no clue that these proteins are functionally related. Mutation of the *HSP104* gene severely reduces the ability of cells to survive short exposures to extreme temperatures (thermotolerance) but has no effect on growth rates. On the other hand, mutations in the genes that encode Hsp70 proteins have significant effects on growth rates but do not reduce thermotolerance. The absence of a thermotolerance defect in *S. cerevisiae* Hsp70 mutants is puzzling, since the protein clearly plays an important role in thermotolerance in a variety of other organisms. In this report, examination of the phenotypes of combined Hsp104 and Hsp70 mutants uncovers similarities in the functions of Hsp104 and Hsp70 not previously apparent. In the absence of the Hsp104 protein, Hsp70 is very important for thermotolerance in *S. cerevisiae*, particularly at very early times after a temperature upshift. Similarly, Hsp104 plays a substantial role in vegetative growth under conditions of decreased Hsp70 protein levels. These results suggest a close functional relationship between Hsp104 and Hsp70.

The tolerance that an organism exhibits to high temperatures varies with its growth conditions. In the yeast *Saccharomyces cerevisiae*, log-phase cells are 100-fold more sensitive to a sudden increase in temperature (e.g., from 25 to 50°C) when grown in glucose, their preferred carbon source, than when grown in acetate or galactose. The high level of basal thermotolerance of cells in acetate and galactose is, in large part, due to the higher level of constitutive expression of heat shock proteins, particularly Hsp104. Stationary-phase cells and spores also exhibit a high level of basal thermotolerance, again in part due to the high level of constitutive expression of Hsp104 in these cell types (26). In cells with a low level of basal tolerance, brief conditioning treatments at moderately high temperatures (e.g., 30 min at 37°C) produce a dramatic increase (induced thermotolerance). Galactose- and acetate-grown cells also exhibit induced thermotolerance, but it is not as dramatic as that in glucose-grown cells because the basal tolerance levels of the former are already high. Induced thermotolerance is also dependent upon the expression of Hsp104.

For many other organisms, there is good evidence that the heat-inducible members of another protein family, the Hsp70 family, play important roles in thermotolerance. Treatments of insect and mammalian cultured cells that selectively increase or decrease the level of Hsp70 result in, respectively, faster or slower acquisition of thermotolerance upon heat stress (17, 18, 24, 29). Similarly, recent studies with transgenic *Drosophila* strains show that simply increasing the level of Hsp70 two- to threefold can significantly increase induced thermotolerance in early embryos (34b). The biochemical activities known for Hsp70 are among those that one might expect for a protein with a role in protecting cells from extreme temperatures. Hsp70 acts as a "molecular chaperone," binding to peptides and polypeptides with extended conformations (8, 9, 15). With at least some test substrates, Hsp70 prevents the aggregation of denatured proteins and facilitates their reactivation in vitro (11, 28).

The yeast *S. cerevisiae* would seem to be an ideal organism with which to study the role of Hsp70 in thermotolerance, because mutations in known genes can be readily created by site-directed mutagenesis. Surprisingly, mutations in the yeast Hsp70 genes analyzed to date do not reduce thermotolerance (3, 4, 37). *S. cerevisiae* contains a family of eight Hsp70-related genes divided into the four subfamilies *SSA* to *SSD* (stress 70 family A to D), as determined by sequence homology and genetic complementation analysis (2). The *SSA* subfamily includes the most heat-inducible Hsp70s and is therefore the most likely class to have a role in thermotolerance. The *SSA* subfamily contains four members (1, 37), three of which encode heat-inducible Hsp70 proteins. The Ssa3 and Ssa4 proteins are almost undetectable at 25°C, but their levels increase dramatically after a temperature upshift. Ssa1 is expressed at moderate levels at normal temperatures and is induced two- to threefold after heat shock. The Ssa2 protein is abundant at normal temperatures, and its expression changes very little after heat shock (35, 36). The *SSA1* and *SSA2* genes are highly related (97% identical), while the *SSA3* and *SSA4* genes are about 85% identical to each other and to *SSA1* and *SSA2*.

Genetic analysis demonstrates that at least one member of the *SSA* subfamily is required for viability at all temperatures (37). Elimination of the three heat-inducible genes (*ssa1*, *ssa3*, and *ssa4*) is deleterious for growth at moderately high temperatures (37.5°C) but does not reduce the ability of yeast cells to survive short exposures to extreme conditions (3, 37). Strains containing mutations in the two constitutive members of the Hsp70 gene family (*ssa1* and *ssa2*) are also temperature sensitive for growth (4). More surprisingly, *ssa1 ssa2* cells survive a direct shift from 25 to 50°C (basal thermotolerance) much better than wild-type cells and nearly as well as wild-type cells that have been given a conditioning pretreatment at 37°C.

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TABLE 1. Yeast strains used in this study[a]

Strain	Relevant genotype
DS10 derivatives	
DS10	*MAT***a** *lys1 lys2 leu2 ura3 his3 Δtrp1*
DS10U2	*MAT***a** *lys1 lys2 leu2 his3 ura3 Δtrp1* (*hsp104*::*URA3*)
MW336	*MAT***a** *lys1 lys2 leu2 ura3 his3 Δtrp1* (*ssa1*::*HIS3*) (*ssa3*::*TRP1*) (*ssa4*::*LYS2*)
MW336U2	*MAT***a** *lys1 lys2 leu2 ura3 his3 Δtrp1* (*ssa1*::*HIS3*) (*ssa3*::*TRP1*) (*ssa4*::*LYS2*) (*hsp104*::*URA3*)
DS10Δ26	*MAT***a** *lys1 lys2 leu2 ura3 his3 Δtrp1* (*hsp26*::*LEU2*)
DS10U2Δ26	*MAT***a** *lys1 lys2 leu2 his3 ura3 Δtrp1* (*hsp104*::*URA3*) (*hsp26*::*LEU2*)
W303 derivatives	
YS490	*MAT***a** *can1 his3 leu2 trp1 ura3 ade2*/pYS*GAL*
YS491	*MAT***a** *can1 his3 leu2 trp1 ura3 ade2* (*hsp104*::*LEU2*)/pYS*GAL*
YS496	*MAT***a** *can1 his3 leu2 trp1 ura3 ade2* (*hsp104*::*LEU2*)/p*GAL1-SSA1*
SL303 derivatives	
SL303	*MATα can1 his3 leu2 trp1 ura3 ade2 lys2*
SL311	*MATα can1 his3 leu2 trp1 ura3 ade2 lys2* (*ssa1*::*HIS3*) (*hsp104*::*URA3*)
SL314	*MATα can1 his3 leu2 trp1 ura3 ade2 lys2* (*ssa1*::*HIS3*) (*ssa2*::*LEU2*)
SL315	*MATα can1 his3 leu2 trp1 ura3 ade2 lys2* (*ssa2*::*LEU2*) (*hsp104*::*URA3*)
SL316	*MATα can1 his3 leu2 trp1 ura3 ade2 lys2* (*ssa1*::*HIS3*) (*ssa2*::*LEU2*) (*hsp104*::*URA3*)

[a] Alleles are as follows: *leu2-3, 112*; *ura3-52* (DS10); *ura3-1* (W303 and SL303); *ade2-1*; *his3-11,15*; *can1-100*; *trp1-1*; and *lys2Δ*.

That is, the double mutants display constitutive thermotolerance. This increased level of thermotolerance is presumably due to the fact that *ssa1 ssa2* mutants constitutively express other heat shock proteins, including Hsp104 (4, 31a).

Unlike Hsp70 mutants, cells carrying a deletion in *HSP104* grow as well as wild-type cells at all temperatures (25). However, *hsp104* cells show a severe defect in induced thermotolerance. They die at the same rapid rate as wild-type cells when shifted directly from 25 to 50°C. However, when given a brief tolerance-inducing treatment at 37°C before the shift to 50°C, mutant cells are killed 100- to 1,000-fold more rapidly (25). Nevertheless, *hsp104* cells do exhibit some residual thermotolerance. In particular, during the early stages of exposure to 50°C, *hsp104* cells that have been pretreated at 37°C survive much better than *hsp104* cells that have not been given a pretreatment (25). Thus, the *hsp104* mutation uncovers at least one other heat-inducible thermotolerance factor that can provide transient thermotolerance in the absence of Hsp104. It is likely that one or more of these factors is another heat shock protein.

Thus, the single mutations in *S. cerevisiae* analyzed to date demonstrate that Hsp70 proteins play an important role in growth at normal and moderately high temperatures, while Hsp104 seems specialized to provide protection during exposure to extreme conditions. This simple picture is perplexing, however, in light of evidence from other organisms that indicates that Hsp70 plays an important role in thermotolerance. To investigate the functions of the Hsp70 and Hsp104 proteins in greater detail, we examined growth and thermotolerance in isogenic strains carrying various combinations of *ssa* and *hsp104* mutations, as well as in strains that overexpress Hsp70 in the *hsp104* deletion background. The results indicate that when the level of constitutive Hsp70 is reduced, Hsp104 functionally compensates for it in growth at normal temperatures. Similarly, in the absence of Hsp104, heat-inducible members of the Hsp70 family appear to play important roles in thermotolerance. In addition to providing evidence for the importance of Hsp70 in thermotolerance in *S. cerevisiae*, these results also provide insight into the biochemical function of Hsp104.

MATERIALS AND METHODS

Yeast strains, transformations, and culture media. The yeast strains used in this study are isogenic derivatives of strains DS10, W303, and SL303 and are described in Table 1. Yeast transformations were performed by lithium acetate or electroporation methods. Cells were grown in rich glucose medium (YPDA), minimal glucose medium, and minimal galactose medium (34).

Plasmid and strain constructions. *HSP104* deletion mutations were created in DS10 and DS10 derivatives that carried mutations in one or several genes of the *SSA* family (37) as follows. The *Eco*RV-*Hin*dIII fragment from pYS-121 was blunted with the Klenow fragment and subcloned into the *Sph*I-*Bam*HI (also blunted) sites of pUCf1. A 1.2-kb fragment (*Apa*I to *Bgl*II) from this plasmid was removed and replaced with the *URA3* gene, generating pYSU2. Linear DNA from pYSU2 (the *Cla*I-*Hin*dIII fragment containing the disrupted *HSP104* gene) was transformed into haploid yeast strains. Gene conversion events, which replaced the wild-type gene with the mutation, were obtained by selecting for uracil prototrophy. The mutants were screened for the presence or absence of Hsp104 after heat shock by staining electrophoretically separated proteins with Coomassie blue.

The *HSP26* gene was disrupted in strains DS10 and DS10U2 by transformation with a 2.7-kb *Bam*HI-*Pst*I fragment of pVZ26ΔLEU, containing the *HSP26* gene disrupted with *LEU2*. The disruption removed the entire coding sequence of *HSP26*. Western blot (immunoblot) analysis with anti-Hsp26 antisera was used to confirm the disruption in both strains. pVZ26ΔLEU was made by replacing the *Bgl*II-*Nru*I fragment of pVZ26 (a gift from R. Susek) with the *LEU2* gene.

Strain SL303 was made by screening for haploid cells carrying the appropriate auxotrophic markers after sporulation of a diploid strain constructed by mating strains W303 **a** and W1088-2D α (a gift from J. Rothman). SL311 was created by first transforming SL303 with the *Sal*I-*Eco*RI fragment from pEC450 (37), containing the *HIS3* gene inserted into *SSA1*, and then selecting for histidine prototrophs. This transformation yielded *ssa1* mutant SL306. This strain was mated to SL305 (SL303 *hsp104*::*URA3*, created as described above), the

diploid was induced to sporulate, and His^+ Ura^+ prototrophs were selected and designated SL311. To create SL316, the *SSA2* gene in SL311 was disrupted by transformation with a linear fragment of pSSA2LEU2(G), containing the *LEU2* gene inserted into the *Bgl*II sites of *SSA2* (3), and leucine prototrophs were selected. SL314 was constructed by screening for Leu^+ His^+ Ura^- haploid cells after sporulation of a diploid made by mating SL311 and SL307 (SL303 *ssa2::LEU2*, created as described above). SL315 was made by disruption of *HSP104* with *URA3* (as described above) in SL307. All of the gene disruptions were confirmed by Southern blot analysis (19).

The p*GAL1-SSA1* vector, a CEN plasmid that encodes the yeast *URA3* gene and carries the *SSA1* coding sequence under the control of the *GAL1* promoter, was constructed as described previously (30).

Plasmid pYS*GAL* is a CEN vector encoding the *URA3* gene and was used as an isogenic control for plasmid p*GAL1-SSA1*. There is no coding sequence cloned behind the *GAL1* promoter in this vector.

Protein extractions and two-dimensional polyacrylamide gels. Cells were grown in rich medium (YPDA) at 25°C to 5×10^6 cells per ml. A portion of the culture was maintained at 25°C, and the remainder was shifted to 39°C for 1 h. Total cellular proteins were extracted by glass bead lysis in ethanol. Proteins from 7.5×10^7 cells were resuspended in 300 μl of urea buffer (9 M urea, 4% Nonidet P-40, 2% ampholyte [pHs 9 to 11], and 2% β-mercaptoethanol in water [pH 9.5]). The solubilized proteins were centrifuged at 100,000 rpm for 5 min at 5°C in an Optima TL ultracentrifuge, and 30 μl of the supernatant was loaded per gel. Two-dimensional polyacrylamide gel electrophoresis was done as previously described (5, 33). After electrophoretic separation, proteins were visualized by silver staining.

Thermotolerance assays. Cells were grown at 25°C to the mid-log phase (2×10^6 to 6×10^6 cells per ml) in YPDA or minimal galactose medium. For induced thermotolerance experiments, cells were preincubated at 37°C in a shaking water bath for 30 min unless otherwise noted. The culture was sonicated briefly to disaggregate cells, and equal portions of the culture were transferred to glass tubes and exposed to 50°C for various lengths of time. For basal thermotolerance experiments, cells were shifted directly from 25 to 50°C. After being heated, cells were transferred to ice, diluted in ice-cold YPDA, and plated in duplicate on YPDA to determine survival. In most cases, the variation between duplicate plates was negligible. Each experiment was performed at least three times. Because small fluctuations in temperature can have significant effects on the steepness of killing curves, absolute tolerance values differed from day to day. However, the general shapes of the curves and the relative differences between wild-type and mutant strains were highly reproducible. Tolerance curves for independent experiments performed on the same day in the same water bath with independent strain isolates were virtually superimposable. Such was the case when strains containing mutations in genes that do not affect thermotolerance were compared (isogenic *HSP26* and *hsp26*) (see Fig. 3).

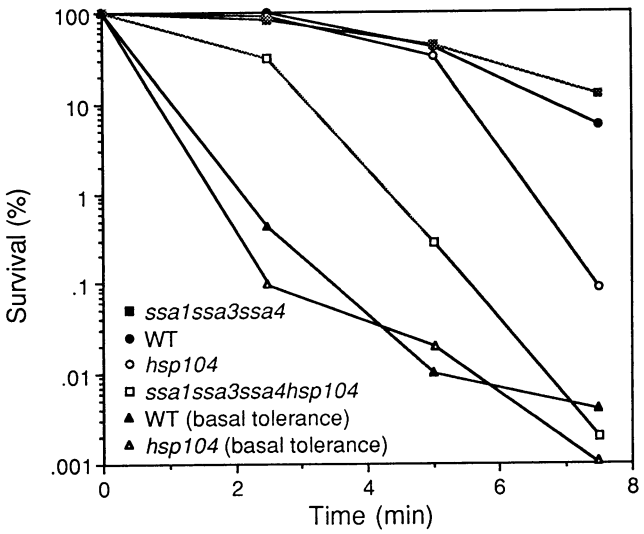

FIG. 1. Thermotolerance in cells containing mutations in *HSP70* and *HSP104* genes. Strains were labeled as follows: DS10, WT (wild type); DS10U2, *hsp104*; MW336, *ssa1 ssa3 ssa4*; MW336U2, *ssa1 ssa3 ssa4 hsp104*. Cells were grown at 25°C to the mid-log phase (2×10^6 to 6×10^6 cells per ml) in YPDA. To measure basal thermotolerance, cells were shifted directly from 25 to 50°C. To measure induced thermotolerance, cells were preincubated at 35°C for 60 min prior to exposure to 50°C. Equal portions of the culture were exposed to 50°C for various lengths of time and were plated on YPDA to assess survival.

RESULTS

Effects of multiple Hsp70 and Hsp104 mutations on induced thermotolerance. To test the importance of the heat-inducible Hsp70 proteins for induced thermotolerance, we analyzed strains carrying disruptions in the heat-inducible genes *SSA1*, *SSA3*, and *SSA4* but a wild-type copy of the major constitutively expressed Hsp70 gene, *SSA2*. In wild-type cells growing at 25°C, the expression of *SSA2* accounts for about 75% of the total *SSA* expression. To measure induced thermotolerance, cells grown at 25°C were given a conditioning pretreatment at 35°C for 60 min and then were shifted to 50°C. This pretreatment was chosen instead of the more typical 30 min at 37°C because some of these strains are temperature sensitive for growth at 37°C but not at 35°C. At various times after the shift to 50°C, survival was measured by plating aliquots of the culture to determine colony-forming ability. Several time points were used within the first 8 min of exposure to 50°C because it is during this interval that *hsp104* cells show residual induced thermotolerance (25).

In the presence of Hsp104, the *ssa1 ssa3 ssa4* triple mutation did not affect induced thermotolerance (Fig. 1). In the absence of Hsp104, however, the Hsp70 mutations greatly reduced thermotolerance. After 5 min of exposure to 50°C, the level of survival of *ssa1 ssa3 ssa4 hsp104* cells was 100-fold lower than that of *hsp104* cells. Therefore, in the absence of Hsp104, the heat-inducible Hsp70 proteins play an important role in promoting survival at high temperatures.

Although thermotolerance was greatly compromised by these heat shock protein mutations, it is notable that all of the strains showed some degree of induced thermotolerance. Without a conditioning pretreatment, survival of both wild-type and *hsp104* mutant strains was less than 0.5% after 2.5 min at 50°C (Fig. 1). Thus, even in the *ssa1 ssa3 ssa4 hsp104* quadruple mutant, other heat-inducible or heat-activated factors can provide thermotolerance, albeit very transiently.

Since it is well known that some Hsp70 mutant combinations have strong pleiotropic effects on the synthesis of other cellular proteins (4), we used two-dimensional gel electrophoresis to investigate whether the decrease in thermotolerance observed in the *ssa1 ssa3 ssa4 hsp104* mutant strain was due to global changes in protein synthesis. In Fig. 2, total cellular proteins

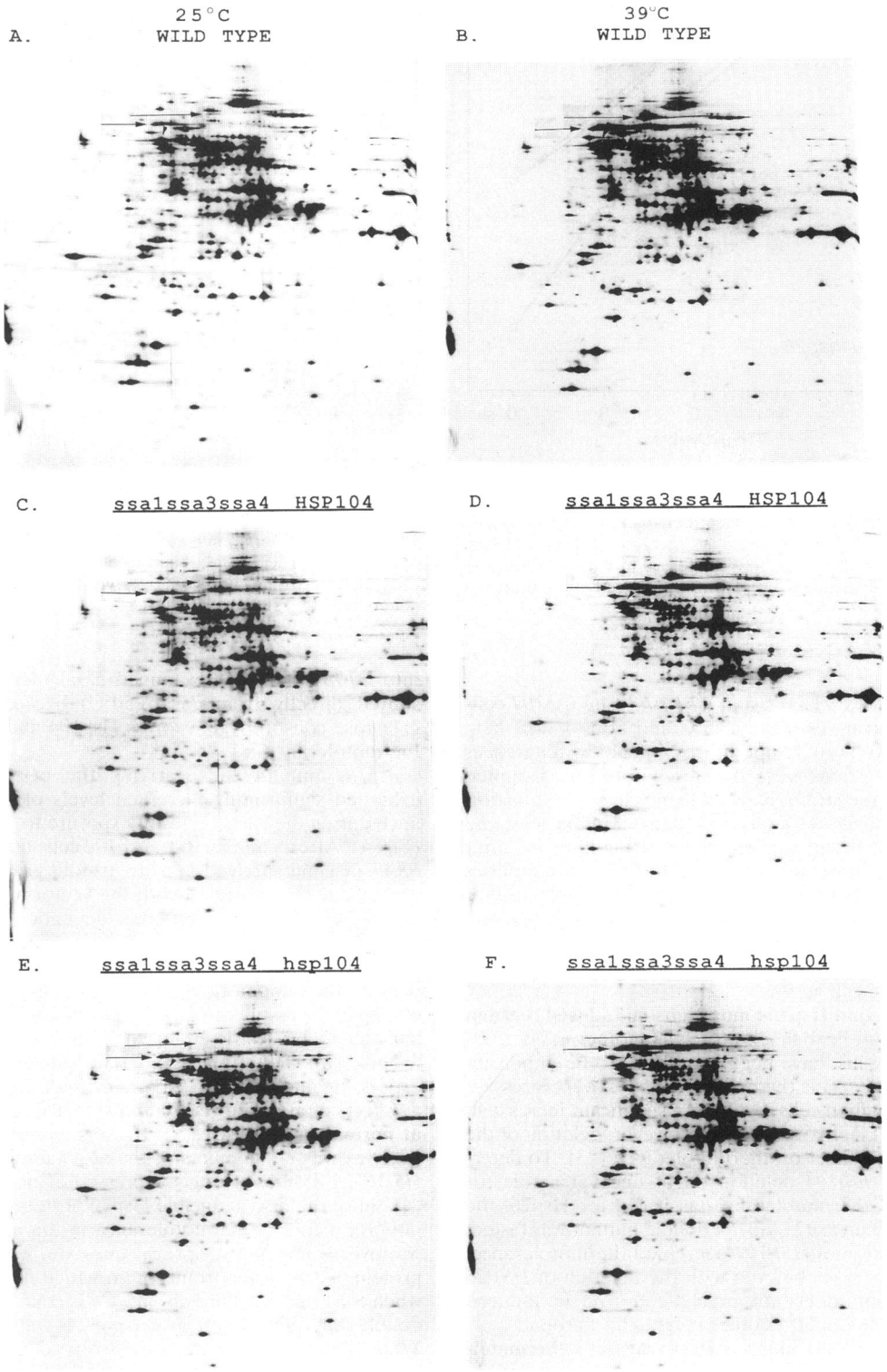

FIG. 2. Two-dimensional electrophoretic analysis of proteins extracted from wild-type and mutant cells. Cells were grown in rich medium (YPDA) at 25°C to a density of 5×10^6 cells per ml. A portion of the culture was maintained at 25°C, and the remainder was shifted to 39°C for 1 h. Total cellular proteins were prepared by glass bead lysis in ethanol. After electrophoretic separation, proteins were visualized by silver staining. The positions of some of the heat shock proteins are marked as follows: Hsp104, long arrow; Hsp/c82, short arrow; Ssa family, arrowhead.

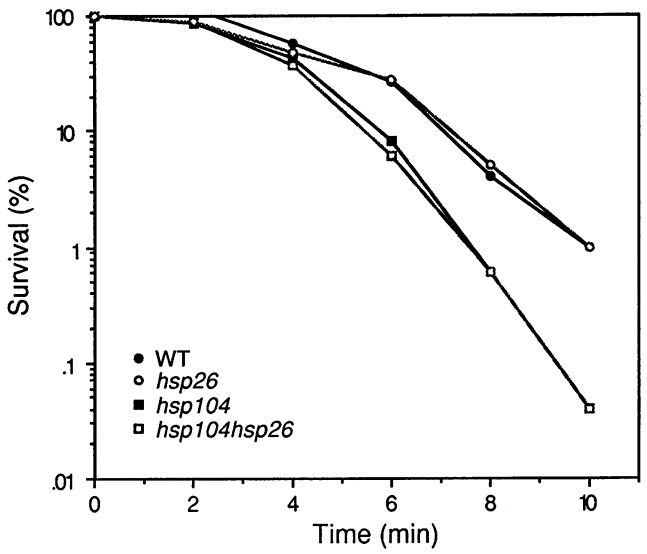

FIG. 3. Induced thermotolerance in cells carrying mutations in *HSP26* and *HSP104*. Strains were labeled as follows: DS10, WT (wild type); DS10U2, *hsp104*; DS10Δ26, *hsp26*; DS10U2Δ26, *hsp26 hsp104*. Cells were grown to the mid-log phase at 25°C in YPDA (2×10^6 to 6×10^6 cells per ml), incubated at 37°C for 30 min, and exposed to 50°C. Survival was determined by plating aliquots of the culture on YPDA.

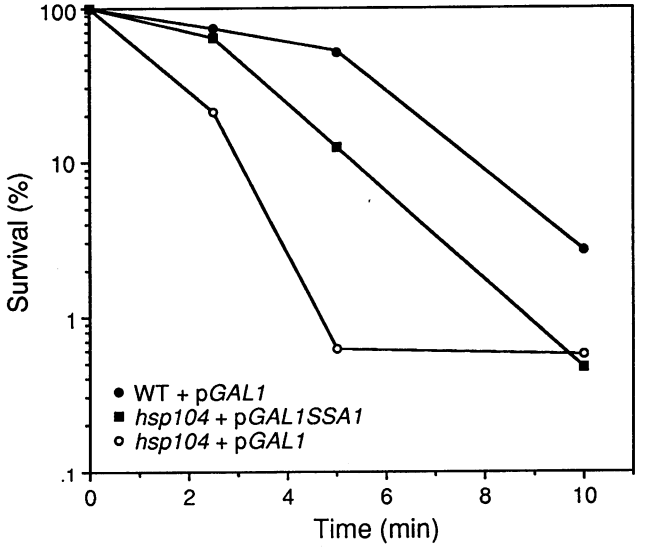

FIG. 4. Basal thermotolerance in cells overexpressing Hsp70. Strains were labeled as follows: YS490, WT + p*GAL1*; YS491, *hsp104* + p*GAL1*; YS496, *hsp104* + p*GAL1SSA1*. Cells were grown at 25°C to 10^6 cells per ml in minimal galactose medium supplemented with the required amino acids. Basal thermotolerance was tested by shifting the cells directly from 25 to 50°C. Survival was determined by plating aliquots of the culture on YPDA.

from wild-type, *ssa1 ssa3 ssa4*, and *ssa1 ssa3 ssa4 hsp104* cells were examined during growth at 25°C and after 1 h of heat shock at 39°C. At both temperatures, the only differences detected in these strains were the absence of the disrupted proteins. None of the strains showed higher levels of constitutive expression of heat shock proteins than wild-type cells, and all accumulated heat shock proteins to expected levels after heat shock. Furthermore, no changes in the levels or mobilities of any other cellular proteins could be detected. The absence of pleiotropic effects on general protein synthesis in the *ssa1 ssa3 ssa4 hsp104* strain supports the conclusion that the heat-inducible members of the Hsp70 family have an effect on thermotolerance in the absence of Hsp104.

Effects of Hsp26 and Hsp104 mutations on induced thermotolerance. The small heat shock proteins, another major class of heat shock proteins, have been postulated to be important for thermotolerance. As is the case for Hsp70, overexpression of Hsp27 in mammalian cells results in a significant increase in thermotolerance (14). In *S. cerevisiae*, however, deletion of the *HSP26* gene has no effect on thermotolerance (23). To determine whether the *hsp104* deletion might uncover a role for Hsp26 in induced thermotolerance, as it did for Hsp70, the thermotolerance of an *hsp26 hsp104* double mutant was tested. Although the deletion of *HSP104* decreased thermotolerance, no additional effect was observed with the deletion of *HSP26* (Fig. 3). This result does not preclude a role in induced thermotolerance for Hsp26 in other genetic backgrounds.

Overexpression of Ssa1 alone partially restores thermotolerance in *hsp104* cells. Since removal of the heat-inducible Hsp70 proteins demonstrated a role for these proteins in thermotolerance in an *hsp104* background, we wished to determine whether the overexpression of a single *SSA* gene could compensate for the loss of Hsp104. In these experiments, a single-copy vector carrying the *SSA1* coding sequence under the control of a galactose-inducible promoter was transformed into *hsp104* cells. We examined basal thermotolerance (cells shifted directly from 25 to 50°C) because cells grown in galactose constitutively express Hsp104 and are constitutively thermotolerant (26).

hsp104 mutant cells carrying the p*GAL1-SSA1* plasmid exhibited significantly increased levels of basal thermotolerance during the early stages of exposure to a high temperature (Fig. 4). After 5 min at 50°C, *hsp104* cells transformed with the *SSA1* plasmid survived in 5- to 10-fold greater numbers than *hsp104* cells transformed with the vector alone. The effect of the p*GAL1-SSA1* plasmid was dependent upon expression from the galactose-inducible promoter; the plasmid had no effect on thermotolerance when cells were grown in raffinose prior to the temperature upshift (data not shown). The ability of Hsp70 overexpression to compensate for the loss of Hsp104 appears to be restricted to early times after a temperature upshift. After 10 min, the difference in survival between cells expressing and cells not expressing Ssa1 dissipated.

Effects of multiple Hsp70 and Hsp104 mutations on growth at normal temperatures. In the absence of the two constitutively expressed members of the *SSA* family, *SSA1* and *SSA2*, *HSP104* becomes strongly expressed at normal temperatures (4). Since the heat-inducible Hsp70 proteins appear to play an important role in thermotolerance in the absence of Hsp104, we investigated the reciprocal question, whether the Hsp104 protein becomes important for growth at normal temperatures when Ssa1 and Ssa2 are absent. Cells containing the *ssa1 ssa2* double mutation grew nearly as well as wild-type cells at 30°C. When the *HSP104* gene was disrupted in this background, however, growth was severely impaired (Fig. 5). The growth of the *ssa1 ssa2 hsp104* strain was even more seriously impaired at moderately high temperatures (35°C) (data not shown). Hsp104 appears to be important for growth only when both constitutive Hsp70 proteins (Ssa1 and Ssa2) are absent. The colony sizes of the *ssa1 hsp104* and *ssa2 hsp104* double mutants were indistinguishable from that of the wild type (Fig. 5).

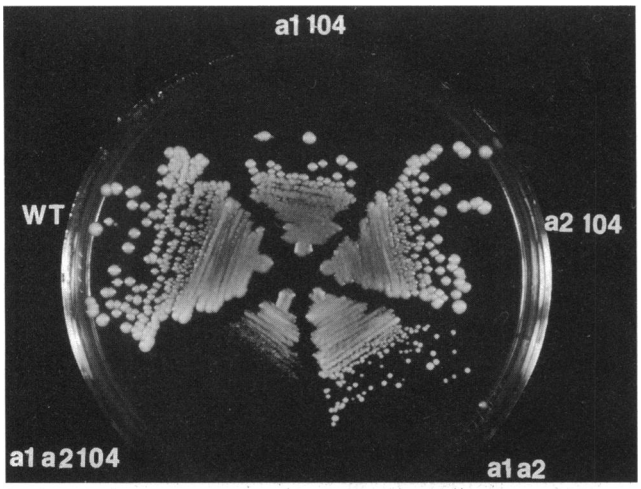

FIG. 5. Hsp104 is important for growth at normal temperatures in the absence of the constitutively expressed members of the Hsp70 family. *S. cerevisiae* strains carrying the following combinations of *hsp104* and *ssa* mutations were streaked on a YPDA plate and incubated at 30°C for approximately 40 h to assess growth: WT, SL303; a1104, SL311 (*ssa1*::*HIS3*) (*hsp104*::*URA3*); a2104, SL315 (*ssa2*::*LEU2*) (*hsp104*::*URA3*); a1a2, SL314 (*ssa1*::*HIS3*) (*ssa2*::*LEU2*); and a1a2104, SL316 (*ssa1*::*HIS3*) (*ssa2*::*LEU2*) (*hsp104*::*URA3*).

DISCUSSION

In *S. cerevisiae*, mutations in the heat-inducible *hsp70* genes have no effect on induced thermotolerance in the presence of Hsp104 (37). We have found, however, that in the absence of Hsp104, these mutations severely impair induced thermotolerance. Thus, the *hsp104* mutation uncovers a role for Hsp70 in the thermotolerance of yeast cells. Similarly, in cells carrying wild-type copies of the constitutively expressed *HSP70* genes, Hsp104 is not required for growth at normal temperatures (25). We have found, however, that in the absence of the *HSP70* genes, Hsp104 is very important for growth. Thus, *hsp70* mutations uncover a role for Hsp104 in growth at normal temperatures. To further investigate the relationship between these two proteins, one member of the essential Hsp70 protein family, Ssa1, was overexpressed in the *hsp104* mutant background. At early times after a shift to a high temperature, the overexpression of *SSA1* partially suppressed the thermotolerance defect of the *hsp104* mutation.

Thus, with respect to thermotolerance, the yeast Hsp70 proteins are not fundamentally different from those of other organisms. Rather, the function of Hsp70 in thermotolerance in yeast cells was elusive simply because Hsp104 plays such a dominant role. These and other observations (17, 18, 24, 29) demonstrate that different organisms use different constellations of heat shock proteins for thermotolerance. In yeast cells, Hsp104 is of primary importance. The induction of Hsp70 becomes important only in the absence of Hsp104. In mammalian cells, induction of Hsp70 and Hsp27 is important for thermotolerance, but it is not yet known what role Hsp110 plays. Another story is unfolding for *Drosophila* cells. This organism does not synthesize a heat shock protein in the 100-kDa size range at all. Instead, it produces extremely high levels of Hsp70 in response to increased temperatures, and in both cultured cells and early embryos, Hsp70 plays a crucial role in thermotolerance. The high levels of Hsp70 in *Drosophila* cells are due to an amplification of the *HSP70* genes in this organism. Five virtually identical genes are present per haploid genome. Our results with yeast cells suggest that this amplification in *Drosophila* cells compensates for the loss of Hsp104 in this organism. As the roles of these proteins are clarified, the next major challenge will be to understand how differences in the biology of organisms dictate different deployment strategies for heat shock proteins in thermotolerance.

Our results also provide insight into the function of Hsp104 in thermotolerance. Hsp104 and Hsp70 are both highly conserved, prominent heat-inducible proteins that share some biochemical similarities. Hsp70 binds ATP, and ATP hydrolysis is required for its function in vitro (16, 28). Hsp104 also functions in vitro as an ATPase (19a). It contains two ATP-binding sites, both of which are required for thermotolerance in vivo (20). In other respects, Hsp70 and Hsp104 appear very different. They show no similarity in amino acid sequence, and the phenotypes of the separate mutations suggest no similarity in function. The present analysis, however, strongly suggests that an important relationship exists between the functions of Hsp104 and Hsp70. Not only do the multiple mutations uncover related phenotypes, but also the overexpression of each protein partially compensates for the loss of the other (Fig. 4 and 5).

It is possible that Hsp70 and Hsp104 serve virtually identical functions but that differences in their patterns of expression and modification at 37 and 50°C lead to differences in their deployment and thus to differences in their mutant phenotypes. It seems more likely, however, that the two proteins serve different functions that are partially complementary. For example, if Hsp70 protects proteins from denaturation and Hsp104 repairs proteins once they have been denatured, overexpression of each protein could partially compensate for the loss of the other.

One observation supporting a functional difference between the two proteins is that the role of Hsp70 in thermotolerance appears restricted to the early stages of exposure to 50°C, while Hsp104 enhances survival for much longer periods. However, this result may be due to a difference in the efficiency or stability of the two proteins at extreme temperatures rather than to a true difference in biochemical mechanisms.

A more compelling argument for distinct differences in function between Hsp70 and Hsp104 can be made from the effects of overexpression of the two proteins. In several organisms, Hsp70 overexpression is difficult to achieve at normal temperatures, because the protein is detrimental to growth (7, 17). Indeed, while our Hsp70-overexpressing yeast cells grew as well as wild-type cells, the plasmid used was a single-copy (CEN) vector that increased Hsp70 expression only a few fold. Cells carrying high-copy-number Hsp70-expressing plasmids have reduced growth rates and lose the plasmids with high frequency (30). In *S. cerevisiae*, however, a >50-fold overexpression of Hsp104 has no detectable effect on cell growth (25a). Furthermore, Hsp70 affects the regulation of other heat shock proteins, while Hsp104 does not. Hsp70 regulates heat shock protein expression in bacteria (31, 32), yeast cells (3, 30), and *Drosophila* cells (6, 29). Hsp104, on the other hand, plays no role in the regulation of other heat shock proteins. Neither the overexpression of the protein nor its complete loss has any effect on the synthesis or accumulation of other heat shock proteins (19a, 21, 25).

Considerable progress has been made in defining the biochemical functions of Hsp70. Constitutively expressed members of the Hsp70 family play a general role in chaperoning unfolded proteins and in promoting the assembly and disassembly of multiprotein complexes at normal temperatures (18). At high temperatures, Hsp70 can prevent protein aggre-

gation and promote renaturation in vitro (27, 28) and presumably does so in vivo (22).

The biochemical function of Hsp104 is unknown, but *hsp104* mutations do have a specific temperature-related effect on the splicing of mRNA precursors (38). Splicing is disrupted by heat shock in wild-type and *hsp104* mutant cells, and in both, a short preincubation at 37°C provides protection from this disruption. However, when splicing is disrupted by a sudden heat shock and cells are returned to normal temperatures, splicing recovers much more slowly in *hsp104* cells than in wild-type cells. Apparently, Hsp104 is not required to protect the splicing machinery from disruption by high temperatures but is required to promote recovery once this machinery has been damaged. These results are consistent with the suggestion that Hsp104 may be specialized to repair heat-induced damage rather than to prevent it. This model is supported by recent experiments in vitro. Supplementing yeast lysates with Hsp70 protects mRNA splicing from inactivation by heat, while supplying them with Hsp104 promotes recovery from heat-induced damage (34a).

Cells cope with the accumulation of stress-damaged proteins in two general ways. They use molecular chaperones to renature them and proteases to degrade them. The existence of these two pathways points to another way in which Hsp104 might compensate for the loss of Hsp70. Perhaps Hsp104 facilitates the degradation of aberrant proteins that Hsp70 cannot salvage. This model is appealing in light of the fact that Hsp104 is a member of a highly conserved heat shock protein family (the Hsp100/Clp family), which includes the *Escherichia coli* ClpA protein (10, 20). ClpA binds to and regulates the activity of the dodecameric ClpP protease in vitro (12, 13). Hsp104 might interact directly with a protease to assist in the degradation of denatured proteins. Alternatively, it might affect proteolysis indirectly, by interacting with proteolytic substrates, preventing their aggregation, and maintaining them in a conformation susceptible to proteases. In this case, it would be acting as a chaperone.

Understanding the mechanisms by which cells protect themselves from heat will require more than the elucidation of Hsp70 and Hsp104 functions. Even in the absence of Hsp104 and all of the heat-inducible members of the Hsp70 family, a substantial, although extremely transient, increase in survival is observed at 50°C when cells have been pretreated at 35°C. Thus, other heat-inducible thermotolerance factors must exist in cells. Candidates include other newly synthesized proteins, the activation of preexisting proteins (perhaps Ssa2), the modification of thermosensitive targets, and the synthesis of thermoprotectants, such as trehalose. The multiple yeast mutations described here will provide a resource for defining these other factors.

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