Prokaryotic Chaperones Support Yeast Prions and Thermotolerance and Define Disaggregation Machinery Interactions

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ABSTRACT *Saccharomyces cerevisiae* Hsp104 and *Escherichia coli* ClpB are Hsp100 family AAA+ chaperones that provide stress tolerance by cooperating with Hsp70 and Hsp40 to solubilize aggregated protein. Hsp104 also remodels amyloid *in vitro* and promotes propagation of amyloid prions in yeast, but ClpB does neither, leading to a view that Hsp104 evolved these activities. Although biochemical analyses identified disaggregation machinery components required for resolubilizing proteins, interactions among these components required for *in vivo* functions are not clearly defined. We express prokaryotic chaperones in yeast to address these issues and find ClpB supports both prion propagation and thermotolerance in yeast if it is modified to interact with yeast Hsp70 or if *E. coli* Hsp70 and its cognate nucleotide exchange factor (NEF) are present. Our findings show prion propagation and thermotolerance in yeast minimally require cooperation of species-specific Hsp100, Hsp70, and NEF with yeast Hsp40. The functions of this machinery in prion propagation were directed primarily by Hsp40 Sis1p, while thermotolerance relied mainly on Hsp40 Ydj1p. Our results define cooperative interactions among these components that are specific or interchangeable across life kingdoms and imply Hsp100 family disaggregases possess intrinsic amyloid remodeling activity.

THE ability of yeast Hsp104 and *Escherichia coli* ClpB chaperones to resolubilize proteins from aggregates is critical for survival of cells exposed to stresses that cause protein aggregation. These Hsp100s, like other cellular chaperone machines, cooperate with Hsp70 chaperones and their J protein (*e.g.*, Hsp40) and nucleotide exchange factor (NEF) regulatory partners (Glover and Lindquist 1998; Goloubinoff *et al.* 1999; Lum *et al.* 2004). DnaK, DnaJ, and GrpE are *E. coli* counterparts of eukaryotic Hsp70, Hsp40, and NEF, respectively. Hsp70 and J-protein families each contain several members that have both redundant and specific functions. For example, Hsp70s within and across species complement Hsp70 functions in yeast, but to widely varying degrees (Tutar *et al.* 2006; Sharma and Masison 2008; Sharma *et al.* 2009a; Sharma and Masison 2011).

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What determines functional specificity among Hsp70s is uncertain, but the evolutionary amplification of J proteins suggests a major factor is the regulation of Hsp70 by its many cochaperones (Kampinga and Craig 2010). The influence of this regulation can be expected to extend to the functions of all cellular chaperone machineries that depend on Hsp70, including Hsp104 and Hsp90. How the functions of such machines might be influenced by the way Hsp70 is regulated by J proteins and other protein "quality control" factors is an important question.

Yeast prions are infectious forms of cellular proteins that propagate as amyloid, a highly structured fibrous protein aggregate. The yeast prions [*PSI*$^+$], [URE3], and [*PIN*$^+$] (also known as [*RNQ*$^+$]) are composed of Sup35p, Ure2p, and Rnq1p, respectively (Wickner 1994; Derkatch *et al.* 1997; Sondheimer and Lindquist 2000). Growth of prion fibers from their ends does not appear to require chaperones, but their replication depends on Hsp104 (Chernoff *et al.* 1995; Derkatch *et al.* 1997; Moriyama *et al.* 2000), which is believed to fragment prion fibers by extracting polypeptides from them (Hung and Masison 2006; Tessarz *et al.* 2008). Continual fragmentation in this manner is necessary for prions to persist in an expanding

Table 1 Plasmids used in this study

Plasmid	Construct	Backbone	Reference
pJ312	*HSP104*	pRS316 (*URA3*)	Jung *et al.* (2002)
pMR116	P_{HSP104}::MCS::T_{HSP104}	pRS314 (*TRP1*)	Miot *et al.* (2011)
pMR117	*HSP104* (4444)	pMR116 (*TRP1*)	Miot *et al.* (2011)
pMR118	*ClpB* (BBBB)	pMR116 (*TRP1*)	Miot *et al.* (2011)
pMR120	BB4B	pMR116 (*TRP1*)	Miot *et al.* (2011)
pMR121	B44B	pMR116 (*TRP1*)	Miot *et al.* (2011)
pMR122	444B	pMR116 (*TRP1*)	Miot *et al.* (2011)
pMR123	4B44	pMR116 (*TRP1*)	Miot *et al.* (2011)
pMR138	4BB4	pMR116 (*TRP1*)	Miot *et al.* (2011)
pMR140	44B4	pMR116 (*TRP1*)	Miot *et al.* (2011)
pMR136	*clpBΔN143*	pMR116 (*TRP1*)	This study
pMR144	4BBB	pMR116 (*TRP1*)	This study
pMR145	B444	pMR116 (*TRP1*)	This study
pMR159	BBB4	pMR116 (*TRP1*)	This study
pMR160	B4BB	pMR116 (*TRP1*)	This study
pGCH16W	*HSP104ΔN147*	pRS314 (*TRP1*)	Hung and Masison (2006)
pMR118-Y503D	$clpB^{Y503D}$	pMR116 (*TRP1*)	This study
pMR150LG	P_{GPD}::*DnaK*::T_{CYC1}	p415-GPD (*LEU2*)	This study
pMR150LG-R167H	$dnaK^{R167H}$	p415-GPD (*LEU2*)	This study
pMR142H	P_{GPD}::*GrpE*::T_{CYC1}	pRS313 (*HIS3*)	This study
pMR141W	P_{GPD}::*DnaJ*::T_{CYC1}	pRS314 (*TRP1*)	This study
pMR141W-D35N	$dnaJ^{D35N}$	pRS314 (*TRP1*)	This study
p109-D36N	$sis1^{D36N}$	pRS314 (*TRP1*)	This study
p314-YDJ1-D36N	$ydj1^{D36N}$	pRS314 (*TRP1*)	This study

yeast population (Chernoff *et al.* 1995; Paushkin *et al.* 1996).

Eukaryotic Hsp100 homologs from divergent species can substitute for Hsp104 in thermotolerance or prion propagation (Lee *et al.* 1994; Schirmer *et al.* 1994; Zenthon *et al.* 2006; Senechal *et al.* 2009), but ClpB supports neither process in yeast and Hsp104 does not function in *E. coli* (Tipton *et al.* 2008; Miot *et al.* 2011). A version of ClpB modified to contain only the middle region (M) of Hsp104, however, cooperates specifically with eukaryotic Hsp70 in protein refolding reactions and provides protein resolubilizing and thermotolerance functions in yeast (Miot *et al.* 2011). The interspecies restrictions are therefore determined by the specificity of interaction between Hsp100 and Hsp70, which is mediated by the M region (Glover and Lindquist 1998; Sielaff and Tsai 2010; Miot *et al.* 2011). These findings support a proposal that regulation of Hsp100 is mediated by an affect of Hsp70 on the M region (Haslberger *et al.* 2007) and imply that function of Hsp100 proteins requires specific cooperation with their cognate Hsp70s.

Because purified Hsp104 and ClpB can act independently of Hsp70 under some conditions, and essential Hsp40 and Hsp70 functions cannot be deleted in yeast, questions remain about whether components of the disaggregation machinery other than Hsp104 are required for yeast prion propagation (Shorter and Lindquist 2004; Doyle *et al.* 2007; Staniforth and Tuite 2012; Tyedmers 2012; Winkler *et al.* 2012). Here, we replaced Hsp104 with Hsp104–ClpB hybrid proteins or ClpB to address this issue and identify chaperone interactions necessary for yeast prion propagation and thermotolerance. Our findings that ClpB functioned in yeast when it was either modified to interact with yeast Hsp70 or coexpressed with *E. coli* Hsp70 system components, and that Hsp40 determines Hsp100 machinery activity in these processes, both define necessary interactions among the disaggregation machinery components and identify which interactions are species specific or interchangeable.

Materials and Methods

Yeast strains, growth conditions, and monitoring prions

Yeast strains used were 1408 (*MATα*, *kar1-1*, *SUQ5*, *ade2-1*, *his3Δ202*, *hsp104*::*kanMX*, *leu2Δ1*, *trp1Δ63*, *ura3-52*) and 1410, which is isogenic but has P_{DAL5}::*ADE2* (*ADE2* controlled by the *DAL5* promoter) in place of *ade2-1*. Both strains carry plasmid pJ312 encoding Hsp104 (Table 1). Strain MR289 is 1408, but *MAT***a**, and has *ClpB* at the *HSP104* chromosomal locus. It was made by first inserting the *HisG*::*URA3*::*HisG* (HUH) cassette (Alani *et al.* 1987; Jung *et al.* 2002) into the *Aat*II site in the *HSP104* terminator region of plasmid pMR118 to give pMR118–HUH. The *Not*I–*Kpn*I fragment of pMR118–HUH was integrated into the *HSP104* locus of a *MAT***a** version of strain 1408 lacking pJ312. Transformants lacking the HUH cassette were then isolated on FOA. Plasmid pJ312 was reintroduced and cells were made [PSI^+] by a cytoduction mating with strain 779-6A. [psi^-] strains 620 (*MAT***a**, *ade2-1*, *ura2*) and 621 (*MATα*, *ade2-1*, *ura2*) were used in crosses to assess infectivity and dominance of prions in strain 1408 and MR289, respectively. [ure-o] strain 1429, used similarly to assess prions in strain 1410, is strain 620 with P_{DAL5}::*ADE2* in place of *ade2-1*. An

hsp104::*KanMX* version of strain BY4741 was obtained from American Type Culture Collection (Manassas, VA). 1/2YPD contains 0.5% yeast extract, 2% peptone, and 2% glucose. It has a limiting but undefined amount of adenine. All other media and growth conditions were as described (Sherman 2002; Kirkland *et al.* 2011) or as indicated in *Results*.

[*PSI*$^+$] and [URE3] were monitored by their ability to promote expression of Ade2p (Cox 1965; Schlumpberger *et al.* 2001; Brachmann *et al.* 2005). Cells lacking Ade2p are Ade$^-$ and red on limiting adenine due to accumulation of a metabolite of adenine biosynthesis. Sup35p catalyzes translation termination at stop codons, thereby preventing translation of *ade2-1* mRNA. Depletion of Sup35p into [*PSI*$^+$] prion aggregates causes enough read through of the nonsense mutation in this transcript to restore adenine prototrophy and white colony color. Ure2p represses transcription of nitrogen utilization genes, such as *DAL5*, when a good nitrogen source is present. On standard ammonium-containing media the *DAL5* promoter is repressed and cells with P_{DAL5}::*ADE2* are Ade$^-$ and red. Depletion of Ure2p into [URE3] prion aggregates relieves this repression so cells express Ade2p and become Ade$^+$ and white.

The presence of [*PSI*$^+$] or [URE3] was confirmed in standard assays by their dominant infectious phenotypes and ability to be "cured" when cells are grown in the presence of 3 mM guanidine hydrochloride. [*PIN*$^+$] was monitored by assessing the guanidine-curable aggregation state of Rnq1–GFP, which is punctate in [*PIN*$^+$] cells and diffuse in [*pin*$^-$] cells.

Plasmids

Plasmids used in this study are shown in Table 1. Plasmid pMR150LG is p415–GPD (Mumberg *et al.* 1995) with DnaK on a *Spe*I/*Xho*I fragment. Plasmid pMR142H is pRS313 (Sikorski and Hieter 1989) with *GrpE* flanked by the *GPD* promoter and *CYC1* terminator. *GrpE* was cloned into p415–GPD via *Xba*I/*Xho*I and the expression cassette on a *Kpn*I/*Sac*I fragment was then moved into pRS313. Plasmid pMR141W encoding *DnaJ* was made similarly except the expression cassette was subcloned into pRS314 (Sikorski and Hieter 1989). Plasmid p314–YDJ1 is pRS314 with a PCR-generated *Bam*HI fragment containing *YDJ1* and 500 bp of 5′ and 3′ flanking DNA. Point mutations were made by site-directed mutagenesis (QuickChange kit, Stratagene, La Jolla, CA). PCR generated alleles were cloned as *Sac*I/*Xho*I fragments into p414–Gal. *DnaJ*^{D35N} is under the control of the *GPD* promoter and *Ydj1*^{D36N} and *Sis1*^{D36N} are under the control of their native promoters.

Plasmid exchange

Prion containing strains 1408 or 1410 carrying pJ312 (*URA3*, *HSP104*) were transformed by plasmids encoding hybrid proteins or ClpB with and without cochaperones as indicated. Individual white transformants were grown as patches of cells on medium selecting for the chaperone plasmids and containing uracil to allow growth of cells having lost pJ312. These patches were then replica plated onto similar medium containing 5-fluoroorotic acid (FOA), which is toxic to *URA3*$^+$ cells. FOA plates lacking adenine were also used to ensure we did not fail to isolate cells that had reduced capacity to support the prions.

Western analysis

Cell lysates were prepared as described (Reidy and Masison 2010). Proteins were separated in SDS–PAGE gels, transferred to PVDF membranes, and probed with the indicated antibodies. Antibodies used were: mouse monoclonal anti-DnaK (Enzo Life Sciences SPA-880), rabbit polyclonal anti-DnaJ (Enzo Life Sciences SPA-410), rabbit polyclonal anti-GrpE (LifeSpan Biosciences LS-C66627), rabbit polyclonal anti-ClpB (gift of Sue Wickner, National Institutes of Health, Bethesda, MD), and rabbit polyclonal anti-Hsp104 (gift of John Glover, University of Toronto, Toronto, ONT, Canada).

Thermotolerance

Thermotolerance assays were performed as described (Miot *et al.* 2011). Briefly, overnight cultures were diluted to OD_{600} = 0.25 and shaken for 30 min at 30°. Cultures were pretreated at 37° for 30 min, or as indicated, and transferred to prewarmed tubes in a 50° block. Tubes were transferred to ice at indicated times. Five microliters of fivefold serial dilutions were spotted onto YPAD plates and incubated at 30°. Images were taken after 2–3 days.

Results

Hsp104/ClpB hybrids support propagation of yeast prions

We monitored [*PSI*$^+$] and [URE3] prions in strains 1408 and 1410, respectively, where Sup35p and Ure2p regulate expression of Ade2p (see *Methods*). Cells propagating [*PSI*$^+$] or [URE3] grow in the absence of adenine and are white or pink (Figure 1A, top). When they lack these prions they require adenine for growth and are red when adenine is limiting (Figure 1A, bottom). We monitored [*PIN*$^+$] in strain 1408 by observing fluorescence of an Rnq1–GFP fusion protein expressed from a plasmid. [*PIN*$^+$] and [*pin*$^-$] strains have readily distinguishable punctate or diffuse fluorescence, respectively (Figure 1A). [*PSI*$^+$] and [*PIN*$^+$] propagate compatibly in the same cells (Derkatch *et al.* 1997). Strains 1408 and 1410 lack chromosomal *HSP104* and carry *HSP104* on a plasmid. The [*PSI*$^+$] phenotype is sensitive to modestly elevated levels of Hsp104 (Chernoff *et al.* 1995), but [URE3] and [*PIN*$^+$] are not. Since the amount of Hsp104 in our strains expressing Hsp104 from a single-copy plasmid is two- to threefold higher than in wild-type cells (Reidy and Masison 2010), [*PSI*$^+$] is weakened enough to make cells look pink and to make [*PSI*$^+$] mitotically unstable, giving rise to red [*psi*$^-$] cells (Figure 1A, upper left).

ClpB-related proteins have four highly conserved structural regions (Schirmer *et al.* 1996; Lee *et al.* 2004) (Figure 1B). To identify functions of Hsp104 important for prion propagation that are lacking in ClpB, we assessed ability

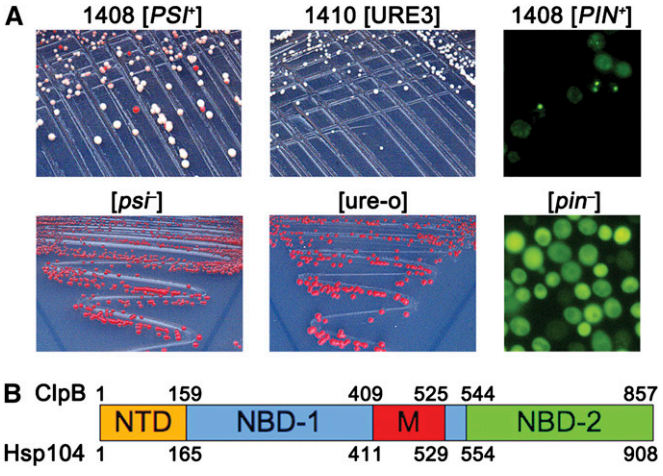

Figure 1 Prion phenotypes of parental strains and domain structure of the Hsp100 disaggregase. (A) Strains 1408 and 1410 lacking prions ([*psi*⁻], [ure-o]) are red (lower). [*PSI*⁺] and [URE3] make the cells white, and [URE3] reduces growth rate. [*PSI*⁺] is unstable in strain 1408 due to heterogeneity of expression of the plasmid-based *HSP104*. Rnq1p–GFP forms foci in [*PIN*⁺] cells, but remains diffuse in [*pin*⁻] cells. (B) Diagram of the domain structure of Hsp100 monomer. NTD, amino-terminal domain; NBD-1, nucleotide-binding domain 1; M, Middle region; NBD-2, nucleotide-binding domain 2. The approximate position of domain boundaries is indicated for ClpB (above) and for Hsp104 (below). The M region is imbedded within NBD-1.

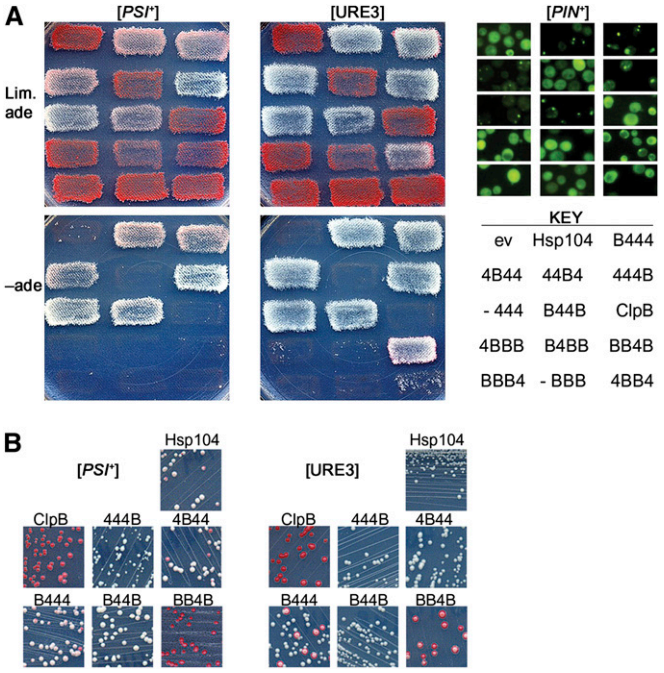

Figure 2 The M region of Hsp104 allows ClpB to support propagation of yeast prions. (A) Resident wild type Hsp104-encoding plasmid in *hsp104Δ* strains 1408 ([*PSI*⁺]) and 1410 ([URE3]) was exchanged with plasmids encoding the indicated Hsp104/ClpB hybrids (KEY). Patches of cells expressing the hybrids in place of Hsp104 were replica plated onto solid media containing limiting (upper) or no (lower) adenine to assess prion phenotypes. [*PSI*⁺] and [URE3] cells appear white or pink on limiting adenine (upper) and can grow in the absence of adenine (lower). Punctate fluorescence of cells expressing an Rnq1–GFP fusion protein (rightmost) indicates presence of [*PIN*⁺]. (B) Cells from plates in A expressing the indicated Hsp104/ClpB hybrid or control protein from *TRP1* plasmids were streaked onto plates containing limiting adenine and lacking tryptophan. [*PSI*⁺] and [URE3] cells appear white or pink, while cells lacking prions give rise to red colonies. Red sectors in white or pink colonies are progeny of cells that lost the prion during growth of the colony.

of Hsp104/ClpB hybrid proteins to support the different prions by exchanging the resident plasmid encoding Hsp104 in strains 1408 and 1410 with plasmids encoding the hybrids or the empty vector (ev). The hybrid proteins engineered previously (Miot *et al.* 2011) are named according to the source of their structural domains. For example, hybrid 4BB4 has the N-terminal and NBD2 regions of Hsp104 and the NBD1 and M regions of ClpB. None of the prions propagate in cells with the empty vector because they lack Hsp104 (Figure 2A). As anticipated, Hsp104 supported propagation of all three prions, but ClpB did not support any of them. Four hybrid proteins supported propagation of all three prions, and the only structural domain common to all of them was the M region of Hsp104. Hybrid BB4B, which contains only this domain, supported propagation of [URE3] and [*PIN*⁺], but not [*PSI*⁺]. Thus, the only Hsp104-specific function needed by ClpB to enable it to support propagation of yeast prions was contained in the M region, which mediates cooperation specifically with eukaryotic Hsp70.

The obvious pink color of [URE3] cells expressing BB4B, and the slight pigmentation of [URE3] cells expressing B444 (Figure 2A, center), reflect reduced efficiency of prion propagation. BB4B also had a weaker [*PIN*⁺] phenotype, as indicated by a reduced proportion of cells containing [*PIN*⁺] foci and by increased diffuse fluorescence even in cells with foci (Figure 2A, right). Hybrid proteins are expressed at similar levels (Miot *et al.* 2011), (Supporting Information, Figure S1) so the differences in phenotypes do not appear to be due to differences in hybrid protein abundance. BB4B has less ATPase activity than the other hybrids, however (Miot *et al.* 2011), which might explain its reduced capacity to promote propagation of [URE3] and [*PIN*⁺]. If so, then its inability to support [*PSI*⁺] might indicate [*PSI*⁺] is more sensitive to reductions in this activity than the other prions.

Variations in mitotic stability of the prions were consistent with the differences in strength of prion phenotypes. [URE3] was lost from dividing cells expressing B444, which is seen as red colonies arising in streaks of cells taken from white colonies, and it was even less stable in cells expressing BB4B (Figure 2B). The weakened strength and stability phenotypes indicate these Hsp104/ClpB hybrids promote prion propagation less efficiently than Hsp104. Interpreting effects of the hybrids on [*PSI*⁺] is less straightforward since either increasing or decreasing Hsp104 activity impairs [*PSI*⁺] propagation.

Bacterial disaggregation machinery supports propagation of [PSI⁺]

Since ClpB hybrids containing the Hsp104 M region supported prion propagation, we inferred that ClpB has all Hsp104 functions needed to support prion propagation

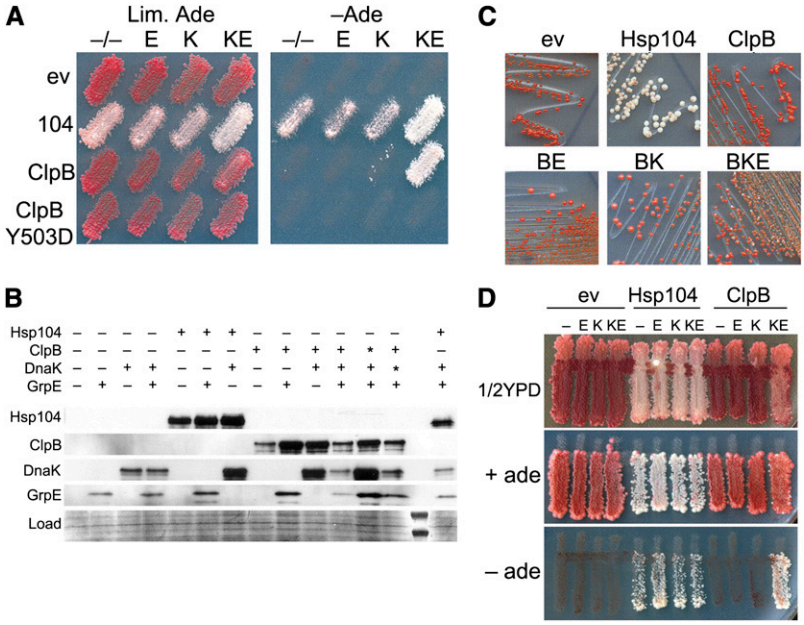

Figure 3 ClpB supports [*PSI*$^+$] propagation if DnaK and GrpE are coexpressed. (A) The Hsp104-encoding plasmid in strain 1408 [*PSI*$^+$] cells expressing combinations of GrpE (E) and DnaK (K) was exchanged with empty vector (ev) or plasmids encoding Hsp104 (104), ClpB, or ClpB–Y503D. Transformants were replica plated onto medium containing limiting (left) or no (right) adenine. (B) Identical samples of lysates of cells exogenously expressing combinations of chaperones (indicated above blots) were separated on four SDS–PAGE gels, blotted, and probed with antibodies to the indicated chaperones. Load is a representative portion of amido-black stained membrane of the Hsp104 blot. Asterisks indicate mutant proteins ClpB–Y503D or DnaK–R167H. (C) Cells expressing ClpB and combinations of DnaK and GrpE as indicated were grown with limiting adenine. We quantified mitotic loss of [*PSI*$^+$] from BKE cells by adding adenine to liquid cultures grown without adenine and growing another six generations. A total of 33% (SD ± 1.7, n = 3) of them were [*psi*$^-$], while <1% of the cells expressing wild-type Hsp104 were [*psi*$^-$]. (D) The same cells were mated to [*psi*$^-$] strain 620 (horizontal streak) to test for dominant prion phenotype. Crosses grown on rich medium (top) were replica plated onto medium containing limiting adenine that selects for [*PSI*$^+$] and [*psi*$^-$] diploids (middle) and onto similar medium lacking adenine (bottom) that allows growth of [*PSI*$^+$] diploids only.

except the ability to interact with the available Hsp70 and that it might function in yeast if its native Hsp70 system was present. We tested this hypothesis by expressing DnaK exogenously in [*PSI*$^+$] strain 1408 and then exchanged the plasmid encoding Hsp104 with one encoding ClpB. We included GrpE, but initially did not include DnaJ because the number of markers for plasmid selection is limiting and barriers preventing collaboration between eukaryotic and prokaryotic disaggregation machinery components do not appear to extend to J proteins (Glover and Lindquist 1998).

The combination of ClpB, DnaK, and GrpE (BKE), and only this combination, supported propagation of [*PSI*$^+$] (Figure 3A). The failure of the other combinations to support prion propagation was not due to reduced expression of ClpB, DnaK, or GrpE (Figure 3B). When grown with limiting adenine, BKE cells were dark pink (Figure 3A, left) and the prion was mitotically unstable (Figure 3C), again reflecting weaker than normal prion propagation. Using standard assays, we confirmed that BKE cells grown on limiting adenine had prions by their dominant prion phenotype when mated with wild-type [*psi*$^-$] cells (Figure 3D), and their sensitivity to curing by growth in the presence of millimolar amounts of guanindine, which impairs disaggregation function of Hsp104 and ClpB (Grimminger *et al.* 2004; Nowicki *et al.* 2011) (Figure S2). Nevertheless, when grown without adenine, which requires the prion for growth, [*PSI*$^+$] cells expressing BKE grew as robustly as those expressing Hsp104 (Figure 3A, see also Figure 4D, below). Thus, ClpB promoted propagation of [*PSI*$^+$] prions, but it required cooperation with its native Hsp70 partner, which in turn required its cognate NEF.

We tested whether the requirement of DnaK for ClpB to support propagation of [*PSI*$^+$] depended upon a specific involvement of the ClpB M region by using the M-region mutant Y503D. ClpB–Y503D retains DnaK-independent processes, such as ATPase activity and threading of soluble unfolded polypeptides, but is unable to refold aggregated model substrates *in vitro*, which is a DnaK-dependent process (Haslberger *et al.* 2007). ClpB–Y503D was unable to support [*PSI*$^+$] propagation alone or with DnaK and GrpE (Figure 3A, bottom row), which is consistent with the idea that ClpB requires functional interaction with a specific Hsp70 to remodel amyloid *in vivo*.

ClpB, DnaK, and GrpE cooperate with yeast Hsp40 Sis1p to promote prion propagation

The observation that BKE did not require DnaJ to promote propagation of [*PSI*$^+$] suggested BKE acted independently of a J protein or was cooperating with yeast Hsp40. To test whether a yeast Hsp40 was involved, we first shuffled ClpB into strain 1408 expressing DnaK with the R167H substitution (designated K*). This mutation causes loss of DnaK function by disrupting a physical interaction of DnaK with DnaJ (Suh *et al.* 1998). Given that the homologous mutation in yeast Hsp70 is lethal (Jones and Masison 2003), we reasoned that K* would not be able to interact functionally with any J protein. As anticipated, ClpB was unable to support [*PSI*$^+$] in a strain expressing K* and GrpE (Figure 4A, BK*E). This result agrees with the idea that J-protein function is necessary for the disaggregation machinery to act in prion propagation and that a yeast J protein interacts with DnaK to provide that function.

To identify yeast J-protein partners for DnaK, we made use of the finding that interaction of DnaK–R167H with DnaJ is restored by the compensatory D35N substitution within the highly conserved HPD (histidine-proline-aspartate) signature motif of DnaJ (Suh *et al.* 1998). We first reengineered strain

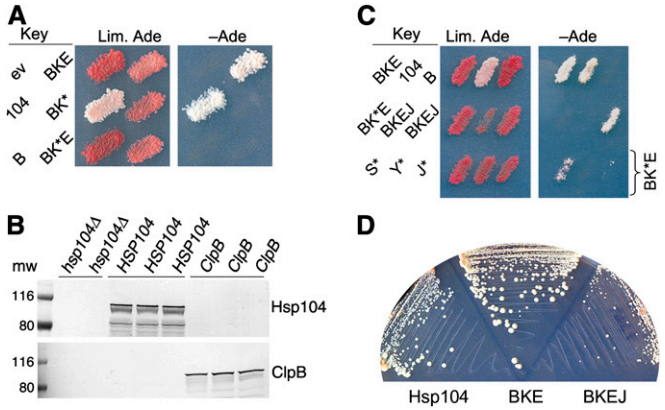

Figure 4 J proteins that support prion propagation. (A) As in Figure 3A, except DnaK–R167H (K*) was used in place of DnaK where indicated. (B) Replicate samples of lysates of cells lacking *HSP104* (*hsp104Δ*), or expressing Hsp104 (*HSP104*) or ClpB (*ClpB*) from the *HSP104* genomic locus were separated by SDS–PAGE, blotted, and probed using appropriate indicated antibodies; mw, molecular weight size markers. (C) As in A except DnaJ (BKEJ, [*psi*$^-$] and [*PSI*$^+$] versions), DnaJ–D35N (J*), Sis1–D36N (S*), or Ydj1–D36N (Y*) were expressed exogenously in cells expressing ClpB from the chromosomal *HSP104* locus. Wild-type (K) and R167H (K*) versions of DnaK are indicated. All panels are representative of at least three independent experiments. (D) Cells used in panel C were streaked for single colonies on medium lacking adenine and grown 3 days at 30°.

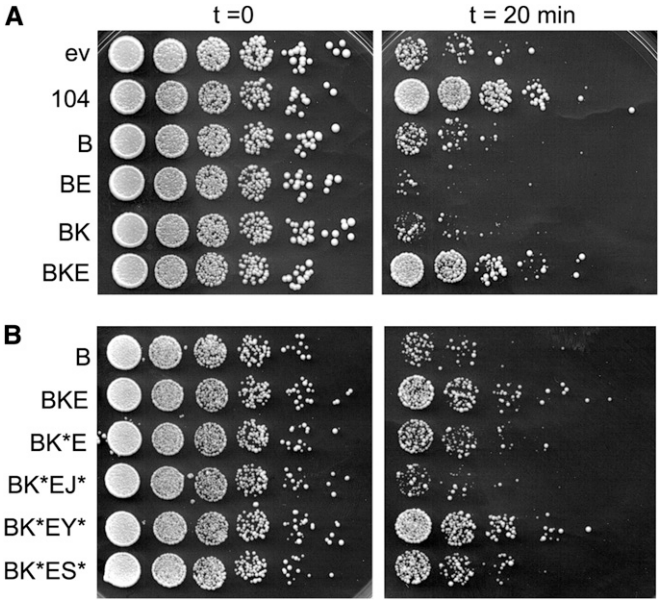

Figure 5 Thermotolerance of *hsp104Δ* yeast expressing combinations of *E. coli* proteins. (A) Cultures of the strains used in Figure 3A expressing empty vector (ev), Hsp104 (104), or the indicated combinations of ClpB (B), DnaK (K), and GrpE (E) were pretreated at 37° for 15 min and then exposed to 50° for 20 min. Fivefold serial dilutions of the cultures were transferred to rich medium and incubated 3 days. (B) Cultures of cells used in Figure 4C expressing the indicated chaperones were treated and plated similarly. Data are representative of at least three independent experiments.

1408 to express ClpB from the *HSP104* chromosomal locus (see *Materials and Methods* and Figure 4B), which allowed us to use a *TRP1* plasmid for expressing J proteins. We then modified the major yeast Hsp40s Ydj1p and Sis1p to contain the homologous D36N substitution (designated Y*, and S*, respectively) and expressed them exogenously in [*PSI*$^+$] cells expressing both Hsp104 and BK*E. Finally, we tested whether these cells were able to support [*PSI*$^+$] after they lost the plasmid encoding Hsp104. We included DnaJ–D35N (J*) as an additional control.

[*PSI*$^+$] propagated in cells expressing BK*E when S* was coexpressed, but not when either Y* or J* were present (Figure 4C). The presence or absence of prions in these cells was confirmed as described above (Figure S3). Thus, Sis1–D36N cooperates with DnaK–R167H to restore ability of ClpB to promote [*PSI*$^+$] propagation, which confirms that Hsp40 is required by the protein disaggregation machinery to promote prion propagation. These results also suggest that wild-type Sis1p acts as the J protein that cooperates with the ClpB machinery in BKE cells and that both DnaJ and Ydj1p lack a function provided by Sis1p that is important for propagation of [*PSI*$^+$].

Expressing DnaJ actually reduced ability of BKE to support prion propagation. After loss of the Hsp104 plasmid we recovered [*PSI*$^+$] in all of 10 tested candidates of cells expressing BKE, while only 3 of 10 candidates of cells expressing BKEJ propagated the prion (Figure 4C, [*PSI*$^+$] and [*psi*$^-$] examples are shown). Additionally, BKEJ [*PSI*$^+$] cells were pinker and grew more slowly than BKE [*PSI*$^+$] cells on medium lacking adenine (Figure 4D), which suggests that DnaJ competes with Sis1p for interaction with substrate or DnaK and is consistent with it lacking an activity required by the disaggregation machinery to promote prion propagation.

BKE cooperate with yeast Hsp40 Ydj1p to provide thermotolerance in yeast

Hsp104 activities required for prion propagation and thermotolerance overlap considerably, but they can be separated (Jung *et al.* 2002; Hung and Masison 2006; Kurahashi and Nakamura 2007). To explore possible distinctions of chaperone machinery function in these processes, we assessed thermotolerance of our strains. BKE cells were noticeably more tolerant to exposure to lethal heat than cells lacking Hsp104 (Figure 5A). Similar results were obtained in the BY4741 strain background (Figure S4). These results indicate that yeast Hsp40 also cooperated with the *E. coli* disaggregation machinery to provide thermotolerance in yeast. Neither BK*E nor BK*EJ provided thermotolerance, but BK*EY* protected cells like BKE (Figure 5B). In contrast, BK*EJ* caused modest hypersensitivity to heat shock, and BK*ES* provided only slightly more protection than BK*E. These results indicate that Ydj1p was a preferred J protein for the thermotolerance function of ClpB in yeast. Thus, the functions of this machinery in prion propagation and thermotolerance relied on different J proteins.

Discussion

We show that disaggregation machinery function in prion replication requires species-specific Hsp70. Additionally, the

requirement of Sis1p for the BKE machinery to support [*PSI*$^+$] agrees with data indicating that specific J-protein activity is also necessary for prion replication (Higurashi *et al.* 2008). The dependency on the species-specific nucleotide exchange factor GrpE for ClpB and DnaK to promote prion propagation further shows that NEFs, whose altered abundance or function in yeast strongly influences prions (Jones *et al.* 2004; Kryndushkin and Wickner 2007; Sadlish *et al.* 2008), also act as critical components of the Hsp70 system required by Hsp100 chaperones to promote prion replication. Thus, all of the core Hsp70 system components known to be involved in efficient protein folding and resolubilization of protein aggregates play critical roles in the amyloid fragmentation required for yeast prion replication. Our results further show that cooperation of Hsp100, Hsp70, NEF, and Hsp40 is also needed for thermotolerance.

Our findings add insight regarding the specificity of interactions among components of the disaggregation machinery. The ability of the BKE system to work with yeast Hsp40 is consistent with earlier data (Glover and Lindquist 1998) and shows that interactions between Hsp70 and J proteins are less stringent than between Hsp70 and either Hsp100s or NEFs. Yet, while ClpB possesses all the Hsp104 functions that are necessary for providing thermotolerance and supporting prion propagation, our data showing BKE prefers Sis1p to promote prion propagation and Ydj1p to support thermotolerance uncover a role for J proteins in specifying action of the disaggregation machinery in these different processes.

Earlier we showed that [*PSI*$^+$] and [URE3] depend on distinct Hsp70 activities and that propagation of both prions varies, depending on the Hsp70 available (Sharma and Masison 2008). Additionally, depleting Sis1p impairs [URE3] considerably more than [*PSI*$^+$], and elevating expression of Ydj1p impairs [URE3], but not [*PSI*$^+$] (Moriyama *et al.* 2000; Higurashi *et al.* 2008). The importance of Sis1p for [*PSI*$^+$] is related to how it regulates Hsp70 (Kirkland *et al.* 2011), and the extent that Ydj1p inhibits [URE3] is Hsp70 dependent and specific (Sharma *et al.* 2009a,b). Inhibition of [URE3] and [*PSI*$^+$] propagation caused by alterations of NEFs also depends on their ability to regulate Hsp70 (Kryndushkin and Wickner 2007). Moreover, subtle differences in Hsp70 structure that marginally affect enzymatic activity can considerably affect prion propagation and protein turnover (Sharma and Masison 2011). Taken together, the data are consistent with a notion that Hsp70 plays a major role in distinguishing how the disaggregation machinery influences propagation of different prions, and that differences in Hsp70 function in turn are specified by the efficiency or specificity of interactions of Hsp70 with cochaperones.

Considering the collective data and building on earlier work showing that J proteins recruit Hsp70 to sites of action and that Hsp70/40 act upstream of Hsp100 (Weibezahn *et al.* 2004; Zietkiewicz *et al.* 2004, 2006; Acebron *et al.* 2008), our findings lead us to infer that optimal activity of the protein disaggregation machinery *in vivo* requires pairing of Hsp100 with a specific Hsp70 that depends on appropriate regulation by a J protein and a cognate NEF. Although Hsp70 might contribute to substrate specificity, the functions of this machinery in different cellular processes seem to be directed by these Hsp70 cochaperones.

Our data also suggest a way to explain how certain mutations of Hsp104 affect prion propagation and thermotolerance differently (Jung *et al.* 2002; Hung and Masison 2006; Kurahashi and Nakamura 2007). It is possible such alterations affect how different Hsp70s interact with Hsp104 or how Hsp70 and its cochaperones are able to influence Hsp104 activity in one of these two processes.

Earlier work showing that ClpB was unable to remodel amyloid *in vitro* or to support yeast prion propagation raised a view that Hsp104 acquired amyloid remodeling activity through evolution, and that this activity coevolved with yeast prion proteins to promote prion replication (Shorter and Lindquist 2004; Shorter 2008; Tipton *et al.* 2008; Halfmann *et al.* 2010). Since yeast prions propagate as amyloid, our results indicate that ClpB is able to fragment amyloid fibrils *in vivo*. Therefore amyloid remodeling activity of Hsp100 chaperones is conserved through evolution, challenging this view.

Our findings strengthen emerging ideas regarding chaperone interactions, in particular with regard to regulation of various functions. Microorganisms and plants have evolved an Hsp100 disaggregation motor that plays the major role in recovery from stress by solubilizing aggregated proteins. Hsp70 is not only a critical component of this machine, but also a key player in all aspects of protein quality control. Not surprisingly, a large cohort of cochaperones has coevolved to fine tune its functions. Our data support the notion that as cells increase in complexity, an expanded array of J proteins and other cochaperones evolved to help direct the function or targeting of cellular chaperone machinery.

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Prokaryotic Chaperones Support Yeast Prions and Thermotolerance and Define Disaggregation Machinery Interactions

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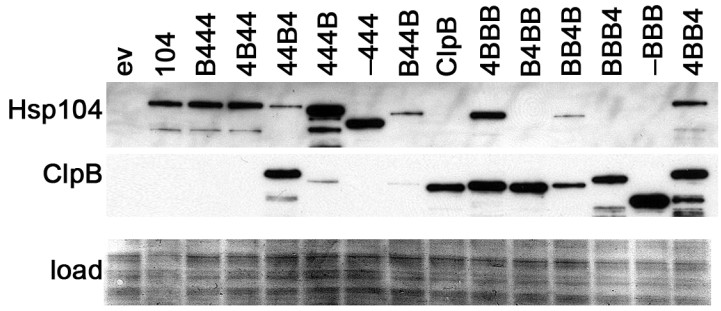


Figure S1 Abundance of Hsp104/ClpB hybrid proteins. Lysates of cells of strain 1408 expressing the indicated wild type or hybrid chaperones were separated on SDS-PAGE gels, blotted and probed with antibodies to Hsp104 or ClpB as indicated. Load panel shows representative portion of the blotted membrane stained by amido-black. There are noticeable variations in ability to detect hybrid proteins due to re-assortment of epitopes. It is evident that the ClpB antibodies recognize a major epitope in the M region, but do not recognize the NTD or NBD1. The Hsp104 antibodies react well with the Hsp104 NTD and an epitope that spans the M-NBD2 region, but do not react with NBD1 and NBD2. Thus, the weaker signals for B44B and BB4B are due at least in part to the lower recognition of these proteins by the antibodies.

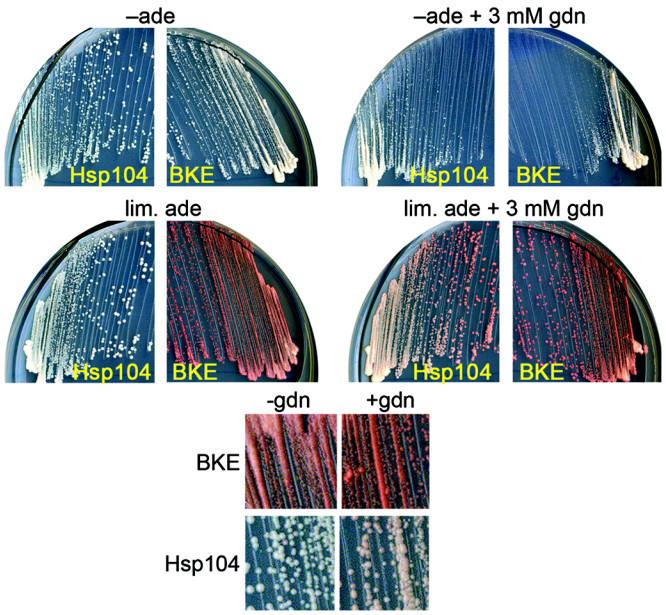
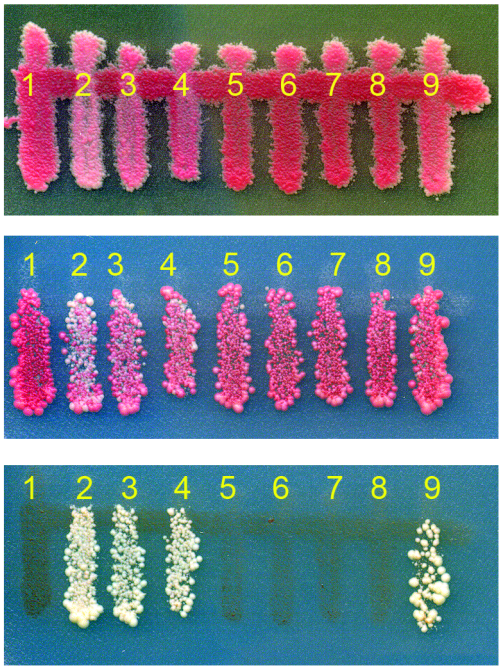


Figure S2 [*PSI*$^{+}$] propagation in BKE cells is cured by guanidine. Cells were grown for six days at 23º without adenine (upper plates) or with limiting adenine (lower plates) and either lacking (left plates) or containing (right plates) 3 mM guanidine-hydrochloride. Guanidine impairment of ClpB and Hsp104 weakens [*PSI*$^{+}$] and therefore reduces growth on medium without adenine (upper right). Prion weakening caused by guanidine is also seen as increased accumulation of pigment on limiting adenine (lower right). Bottom panels show magnification of the limiting adenine plates to show color difference more clearly.



1. B
2. 104
3. BKE
4. BKEJ (+)
5. BKEJ (–)
6. BK*E
7. BK*EJ*
8. BK*EY*
9. BK*ES*

Figure S3 Confirmation of [*PSI*$^{+}$] propagation in BKEJ cells and in BK*E cells expressing J*, Y* and S*. Control cells in lanes 1, 2 and 3 express ClpB (B), Hsp104 (104) or BKE as indicated. Cells in lanes 4 and 5 express BKEJ and are [*PSI*$^{+}$] (+) and [*psi*$^{-}$] (–), respectively. Cells in lanes 6-9 express BK*E and the indicated J-protein (same strains as in Figure 4B). All strains were crossed with [*psi*$^{-}$] strain 621. Growth of diploids on medium lacking adenine (lower panel) indicates presence of [*PSI*$^{+}$] (e.g. samples 2, 3, 4, 9).

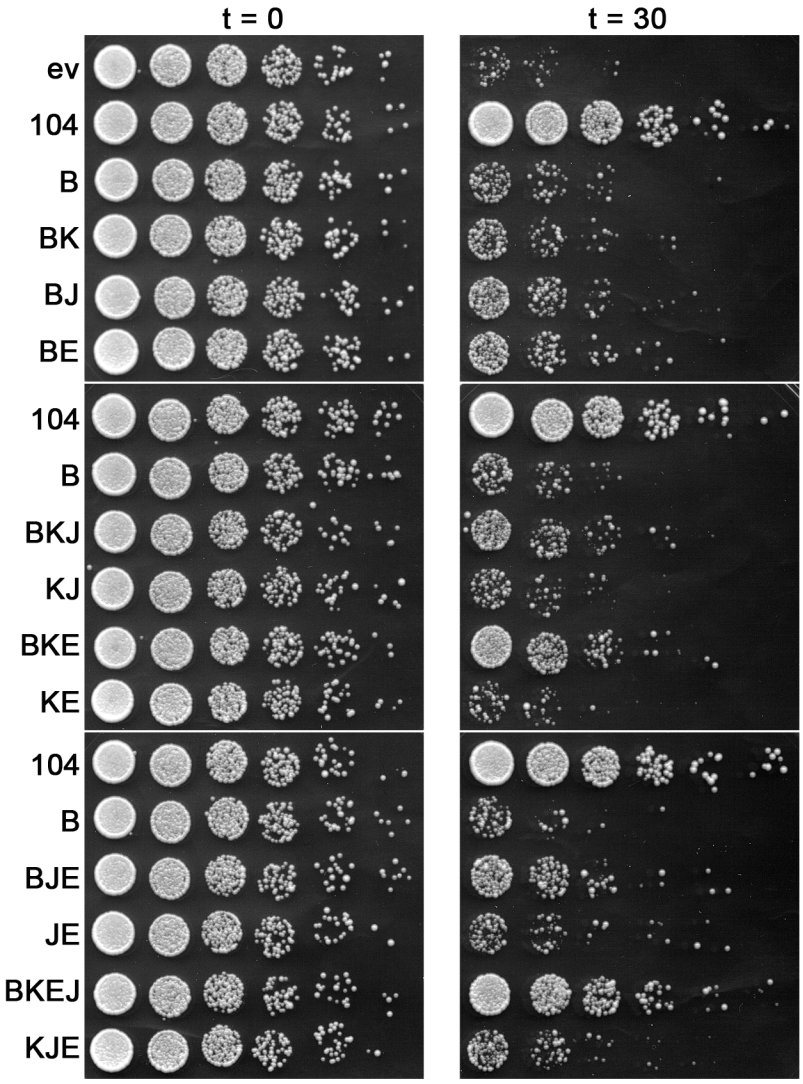


Figure S4 Thermotolerance of strain BY4741 *hsp104Δ* cells expressing Hsp104 (104) or empty vector (ev), or various combinations of *E. coli* chaperones ClpB (B), DnaK (K), DnaJ, (J) and GrpE (E). Cells express indicated chaperones from genes regulated by the constitutively active glyceraldehyde-3-phosphate dehydrogenase promoter on single-copy plasmids. Cells grown at 30º on liquid medium selecting for plasmids to OD_{600} = 0.25 were pretreated at 37º for 30 minutes and then exposed to 50º for 30 min. Five microliters of five-fold serial dilutions of cultures were grown three days at 30º on YPAD plates.