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# Synergistic Roles of HslVU and Other ATP-Dependent Proteases in Controlling In Vivo Turnover of $\sigma^{32}$ and Abnormal Proteins in *Escherichia coli*

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**Production of abnormal proteins during steady-state growth induces the heat shock response by stabilizing normally unstable $\sigma^{32}$ (encoded by the *rpoH* gene) specifically required for transcription of heat shock genes. We report here that a multicopy plasmid carrying the *hslVU* operon encoding a novel ATP-dependent protease inhibits the heat shock response induced by production of human prourokinase (proUK) in *Escherichia coli*. The overproduction of HslVU (ClpQY) protease markedly reduced the stability and accumulation of proUK and thus reduced the induction of heat shock proteins. In agreement with this finding, deletion of the chromosomal *hslVU* genes significantly enhanced levels of proUK and $\sigma^{32}$ without appreciably affecting cell growth. When the Δ*hslVU* deletion was combined with another protease mutation (*lon*, *clpP*, or *ftsH*/*hflB*), the resulting multiple mutations caused higher stabilization of proUK and $\sigma^{32}$, enhanced synthesis of heat shock proteins, and temperature-sensitive growth. Furthermore, overproduction of HslVU protease reduced $\sigma^{32}$ levels in strains that were otherwise expected to produce enhanced levels of $\sigma^{32}$ due either to the absence of Lon-ClpXP proteases or to the limiting levels of FtsH protease. Thus, a set of ATP-dependent proteases appear to play synergistic roles in the negative control of the heat shock response by modulating in vivo turnover of $\sigma^{32}$ as well as through degradation of abnormal proteins.**

When *Escherichia coli* cells are exposed to high temperature, synthesis of a set of heat shock proteins (HSPs) is rapidly induced. The induction occurs by transient increase in $\sigma^{32}$ (the *rpoH* gene product), which directs RNA polymerase to transcribe the heat shock genes (11, 44). The increase in $\sigma^{32}$ level depends on both increased synthesis and stabilization of $\sigma^{32}$, which is very unstable (half-life of about 1 min) (33). Whereas induction of $\sigma^{32}$ synthesis occurs at the translation level mediated by the *rpoH* mRNA secondary structure (25, 45), stabilization occurs presumably by sequestering $\sigma^{32}$ from DnaK and DnaJ chaperones (34, 35) or proteases such as FtsH/HflB (13, 37). This homeostatic response appears to be regulated by a complex feedback circuit involving chaperones, proteases, and other components of signalling pathways that have yet to be identified (2, 6, 11, 43, 44).

Many HSPs are molecular chaperones or ATP-dependent proteases. The DnaK-DnaJ-GrpE and GroEL-GroES chaperone teams play major roles in protein folding and assembly in many cellular processes, perhaps including protein turnover (6, 11, 12). The HSPs Lon/La, ClpP, ClpX, and FtsH are all ATP-dependent proteases or their subunits: Lon is a single polypeptide that forms homotetramers, whereas Clp is a large complex that consists of a catalytic subunit (ClpP) and a regulatory subunit (ClpA or ClpX) which confers substrate specificity (8, 9, 10, 22). FtsH is a membrane-bound metalloprotease essential for growth and can degrade $\sigma^{32}$ in vivo and in vitro (13, 37). Degradation of cellular proteins is mostly energy dependent, since it is inhibited by 90% upon depletion of ATP. Lon and ClpAP/Ti proteases play major roles in proteolysis: disruption of both *lon* and *clpA* (or *clpP*) inhibits degradation of cellular proteins by 70 to 80% (10, 22).

Production of abnormal proteins by addition of amino acid analogs or puromycin, or accumulation of mutant proteins, membrane precursor proteins, or foreign proteins, can induce HSPs and mimic the heat shock response (7, 15). The functional *rpoH* gene is required for such induction, and the extent of induction depends on the amount of unfolded proteins and not on protein degradation (27). Moreover, the increased $\sigma^{32}$ level under these conditions results primarily from stabilization and not from increased synthesis of $\sigma^{32}$ (17, 39). These and other data suggested that stabilization and enhanced synthesis of $\sigma^{32}$ that occur upon exposure to high temperature involve two distinct pathways or mechanisms (11, 17, 43).

Because degradation of $\sigma^{32}$ and abnormal proteins apparently requires the same set of chaperones (DnaK, DnaJ, and GrpE), it has been suggested that unfolded or misfolded proteins generated under heat shock and other stress rapidly bind and titrate the chaperones away from $\sigma^{32}$ and thus stabilize $\sigma^{32}$ and induce the heat shock response (2, 4). The facts that $\sigma^{32}$ directly binds to DnaK and DnaJ (5, 20) and that overproduction of DnaK reduces the heat shock response at high temperature (35) or upon production of abnormal proteins (17) are consistent with such suggestions. Although little is known about the mechanisms involved, it is quite reasonable that enhanced levels of abnormal proteins trigger a chain of events leading to increased synthesis of HSPs.

To further understand the nature of heat shock response induced by abnormal proteins, we looked for additional genes and factors that may affect the response following production of human prourokinase (proUK), which is known to form partially unfolded structures (17). We report here that overproduction of a pair of HSPs, encoded by the *hslVU* operon (3), markedly inhibits proUK-induced HSP synthesis primarily by promoting degradation of proUK. Analysis of mutants lacking HslVU proteins, with or without simultaneous loss (or reduction) of other ATP-dependent protease(s), led us to propose that HslVU and other proteases jointly participate in the negative control of heat shock response by modulating turnover of $\sigma^{32}$ as well as of unfolded or misfolded proteins in vivo.

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While this work was in progress, we learned that HslVU (also called ClpQY) indeed represents a novel ATP-dependent protease in *E. coli* (24, 29, 42).

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and phages.** *E. coli* K-12 strain MC4100 [F$^-$ *araD* Δ(*argF-lac*)*U169 rpsL relA flbB deoC ptsF rbsR*] and its derivatives were used in most experiments. Strain SG22094 (MC4100 *rcsA166*::Δ*kan clpP1*::*cat* Δ*lon-510*) was kindly provided by S. Gottesman. Strain FS1576 (C600 *thy recD1009*) (32) was used for construction of Δ*hslVU* and Δ(*clpPX-lon*) mutants. Strain YJ415 carrying Δ*ftsH3*::*kan* on the chromosome and *ftsH*$^+$ on a pSC101-based plasmid (pSTD401), as well as the isogenic parental strain (CU141), was donated by Y. Akiyama (1, 31). pUK752 is a pACYC184-based plasmid carrying the human proUK gene (under controls of the *tac* promoter [$tac_p$] and *lacI*$^q$), derived from plasmid pUK-02pm0 described previously (17). An *E. coli* DNA library was constructed by ligating *Sau*3AI-digested chromosomal DNA with Charomid 9-36 (Nippon Gene, Tokyo, Japan) cut with *Bam*HI. pKV1004 is a pBR322-based plasmid carrying the *Esp*3I-*Eco*47III fragment (2.3 kb) which was excised from a plasmid (reported here) containing the entire *hslVU* operon. pKV1007 is a pBR322-based plasmid carrying the *hslVU* operon under the control of $trp_p$ and *trpR* repressor. pKV1174 is a derivative of pKV1007 lacking the *Nsi*I-*Bgl*II fragment (1.7 kb) of the *hslVU* operon. Phage P1*vir* was used for transduction experiments. λ2137, a derivative of Charon 25, was used to construct λ2137-*hslVU*$^+$ carrying the *hslVU* operon by in vivo recombination. λpF13-(*groEp-lacZ*) carried *lacZ* under the control of *groE* heat shock promoter (40).

**Media and chemicals.** L broth has been described elsewhere (36); ampicillin (50 μg/ml), chloramphenicol (10 or 20 μg/ml), or tetracycline (5 μg/ml) was added when necessary. The synthetic medium used was medium E supplemented with 0.5% glucose and 2 μg of thiamine per ml (36); ampicillin was used at 10 μg/ml. L-[$^{35}$S]methionine (29.6 TBq/mmol) was obtained from American Radiochemicals; other chemicals were obtained from Nacalai Tesque, Kyoto, or Wako Pure Chemicals, Osaka, Japan.

**Construction of Δ*hslVU* and Δ(*clpPX-lon*) mutants.** A derivative of pKV1004 carrying the *hslVU* operon with extended flanking regions (1 kb for each side) was first constructed, and the internal portion of the *hslVU* genes (0.6-kb *Nsi*I-*Nru*I fragment) was replaced by 1.4-kb fragment containing the tetracycline resistance (*tet*) gene of pBR322. The resulting plasmid, pKV1172, retained the 5′ portion of *hslV* and the 3′ portion of *hslU*, but these segments were shown to be devoid of activities. This plasmid was treated with an appropriate restriction enzyme and then introduced into strain FS1576 (*recD*) by selecting for tetracycline resistance. One such transformant that carried the Δ*hslVU1172*::*tet* deletion on the chromosome was used to transduce the deletion into MC4100 by phage P1, and the resulting deletion mutant was designated KY2039. Strain KY2263 [MC4100 Δ(*clpPX-lon*)*1196*::*cat*] was similarly constructed by replacing the entire 3.7-kb *Mlu*I-*Sal*I segment (*′clpP-clpX-lon′*) with the *cat* gene derived from pACYC184.

**Radioactive labeling of proteins.** Cells were grown in synthetic medium at 30°C; samples were taken and pulse-labeled with [$^{35}$S]methionine (100 μCi/ml) to determine synthesis rates of specific proteins by immunoprecipitation followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). To determine protein stability, pulse-labeled cells were incubated with excess unlabeled methionine (200 μg/ml), and samples taken at intervals were examined for radioactivities associated with the specific proteins by immunoprecipitation and SDS-PAGE. For determining synthesis rate and stability of proUK, isopropyl-β-D-thiogalactopyranoside (IPTG; final concentration, 5 or 10 μM) was used to induce proUK from $tac_p$.

**Immunoprecipitation and immunoblotting of proteins.** The assays were carried out essentially as described elsewhere (41), using specific antisera. Antisera against proUK and FtsH were kindly supplied by M. Yamada (Toso Co.) and Y. Akiyama (Kyoto University), respectively. A Hybond-ECL nitrocellulose membrane filter (Amersham) or polyvinylidene difluoride membrane (Nihon Millipore Kogyo Co., Osaka, Japan) was used for immunoblotting. Quantitation of protein bands following immunoprecipitation or immunoblotting was done with a BAS2000 BioImaging Analyzer (Fuji Film, Tokyo, Japan) or a BioImage Intelligent Quantifier (BioImage Systems Co., Tokyo, Japan), respectively.

**Other procedures.** Nucleic acid manipulation (30), β-galactosidase assay (23), and SDS-PAGE (36) were performed as described previously.

## RESULTS

**Screening for multicopy genes that can inhibit proUK-induced heat shock response.** To search for novel genes and factors that might inhibit the proUK-induced heat shock response, we used strain MC4100 carrying the λpF13-(*groEp-lacZ*) prophage and a pACYC184-based pUK752 plasmid expressing proUK from $tac_p$. In this strain, IPTG induces synthesis of proUK (partially unfolded form) which stabilizes $\sigma^{32}$ (17) and induces β-galactosidase from the *groE* heat shock promoter. This permits formation of dark blue colonies on L-agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) at 30°C. An *E. coli* gene library constructed with Charomid 9-36 was introduced into this strain, and genes that inhibit proUK-induced heat shock response were screened by looking for light blue colonies. The gene library was constructed from chromosomal DNA of a Δ*lon*-Δ*clpP* double mutant (SG22094) to avoid cloning the known protease genes.

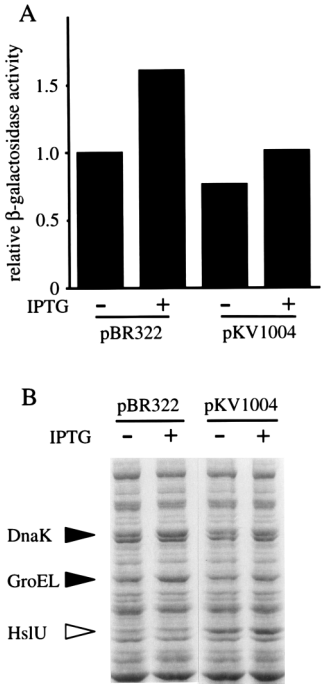


FIG. 1. Inhibition of proUK-induced heat shock response by overexpression of the *hslVU* operon. (A) Strain MC4100 lysogenic for λpF13-(*groEp-lacZ*) and harboring a pACYC184-based proUK expression plasmid (pUK752) and a compatible pBR322 or its derivative pKV1004 carrying *hslVU* was grown to mid-log phase in L broth with or without 5 μM IPTG at 30°C to determine β-galactosidase activity. The data from a typical experiment are shown after normalization to values for the control cells carrying pBR322 grown without IPTG. (B) The same set of cultures was used to extract whole-cell proteins, which were analyzed by SDS-PAGE (10% gel) followed by staining with Coomassie blue.

The initial screening of over 10,000 transformants on L-agar plates containing X-Gal and IPTG gave 42 candidates, and analysis of whole-cell proteins by SDS-PAGE (10% gel) revealed that the levels of DnaK and GroEL proteins were significantly reduced in 19 of them. Subcloning and sequencing of the cloned DNA showed that at least two clones contain the entire *hslVU* operon encoding a pair of HSPs, HslV and HslU (3), whose functions appeared to be related to proteolysis. The putative ATP-binding region of HslU exhibited high sequence similarity (ca. 50% identity) to that of ClpX, a regulatory subunit of ClpXP protease, whereas HslV showed ca. 20% identity to eukaryotic proteasome β-type subunit (3, 21).

**Overproduction of HslVU inhibits the heat shock response by promoting degradation of proUK.** To confirm the effect of overexpression of *hslVU* on the proUK-induced heat shock response, plasmid pKV1004 containing the intact *hslVU* operon or vector alone (pBR322) was introduced into MC4100 [λpF13-(*groEp-lacZ*)] harboring a compatible plasmid (pUK752) expressing proUK from $tac_p$. Addition of IPTG to these cells induced proUK, which in turn induced the heat shock response as judged by *lacZ* expression from $groE_p$ (Fig. 1A). As expect-

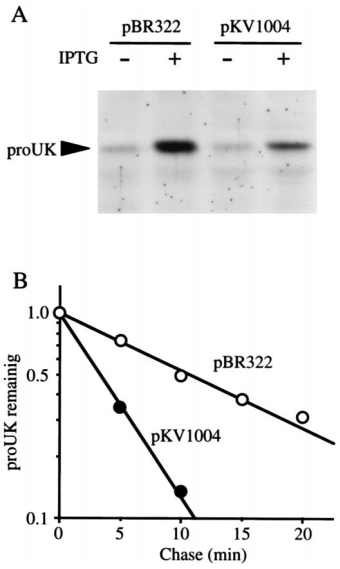


FIG. 2. Destabilization of proUK in cells overexpressing the *hslVU* operon. (A) Samples taken from the cultures used for Fig. 1 were examined for proUK by SDS-PAGE (10% gel) followed by immunoblotting. (B) The stability of proUK was determined with the strains used for panel A grown in synthetic medium. Cells were grown to early log phase at 30°C, IPTG (5 μM) was added, and after 2 h, the cells were pulse-labeled with [$^{35}$S]methionine (100 μCi/ml) for 1 min and chased with excess unlabeled methionine (200 μg/ml). Samples were taken at intervals starting at 1 min (set as time zero), and radiolabeled proUK was analyzed by immunoprecipitation followed by SDS-PAGE. Average values from two experiments were plotted. Symbols: ●, MC4100 [λpF13-(*groE$_p$-lacZ*)]/pUK752 harboring pKV1004; ○, MC4100 [λpF13-(*groE$_p$-lacZ*)]/pUK752 harboring pBR322.

ed, cells carrying pKV1004 elicited lower β-galactosidase activity than those carrying pBR322 when grown with IPTG. The slightly lower β-galactosidase found with pKV1004-carrying cells without IPTG probably means that *hslVU* overexpression affected the basal level as well as the induced level of proUK. Under these conditions, syntheses of DnaK and GroEL encoded by the chromosomal genes were significantly reduced in the strain carrying pKV1004 when grown with IPTG (Fig. 1B); quantitation of these proteins revealed a good correlation with the β-galactosidase levels shown in Fig. 1A.

The effect of HslVU overproduction on proUK was then examined by immunoblotting using an antiserum against proUK. Cells carrying pKV1004 produced significantly lower levels of proUK than those carrying pBR322, both in the presence and in the absence of IPTG (Fig. 2A). Pulse-chase experiments revealed that overproduction of HslVU renders proUK much less stable, the half-life being about one-third of that of the control (Fig. 2B). The synthesis rates of proUK as determined by pulse-labeling followed by immunoprecipitation were virtually unaffected (data not shown). These results indicate that overproduction of HslVU inhibits proUK-induced heat shock response by destabilizing proUK, and perhaps by destabilizing $\sigma^{32}$ as well (see below).

**Deletion of *hslVU* genes enhances HSP synthesis and affects growth at high temperature.** To assess the physiological role of HslVU proteins, we constructed an *E. coli* mutant lacking the chromosomal *hslVU* by homologous recombination using a linearized pKV1172 in which an appreciable portion of *hslVU* had been replaced by *tet* (see Materials and Methods). The resulting deletion mutant carrying Δ*hslVU1172*::*tet* on the chromosome was confirmed by PCR and shown to lack HslVU proteins by immunoblotting (data not shown).

The Δ*hslVU* mutant (KY2039) grew normally on L-agar plates up to 42°C but poorly at 44 to 45°C. When the mutant cells carrying pUK752 were grown in L broth, the level of proUK produced was significantly higher than in the wild type at 30 or 37°C (Fig. 3A). The levels of $\sigma^{32}$ as well as HSPs such as DnaK and GroEL were also elevated (about twofold) in the mutant (Fig. 3B; compare lanes 1 and 2 or 5 and 6); these mutant phenotypes were complemented by λ2137-*hslVU*$^+$ (compare lanes 3 and 4 or 7 and 8). The effects of Δ*hslVU* deletion were clearly seen with cells grown in L broth but were less striking with cells grown in synthetic medium, particularly at 30°C. The induction of HSPs following temperature upshift (30 to 42°C) or addition of azetidine to the medium at 30°C was little affected (data not shown). These results taken together indicated that HslVU proteins are involved in modulating the cellular levels of $\sigma^{32}$ and HSPs as well as of unfolded or misfolded proteins at physiological temperatures. However, HslVU seemed to be required for normal growth only at very high temperatures (>44°C) in rich medium.

**The absence of HslVU further stabilizes proUK and $\sigma^{32}$ in the deletion mutant lacking Lon and Clp proteases.** Assuming that the function of HslVU is related to proteolysis, we transduced the Δ*hslVU* deletion to a Δ(*clpPX-lon*) mutant (KY2263) lacking Lon and Clp proteases and examined the effects on protein stability. The resulting triple-protease-deficient mutant (KY2266) exhibited a more severe temperature-sensitive growth (little or no growth at 42°C on L agar) than the Δ(*clpPX-lon*) double mutant (KY2263) or the Δ*hslVU* mutant (KY2039). The slower growth was observed even at 30°C in synthetic medium, the doubling time being 180 min for the triple mutant, compared to 90 min (double mutant) or 70 min (wild type). The amounts of proUK produced under these conditions were 2.5 or 5-fold higher than for the wild type in the double or triple mutant, respectively (Fig. 4A). In agreement with these results, the stability of proUK increased threefold or five- to sixfold in the respective mutants (Fig. 4B), while the

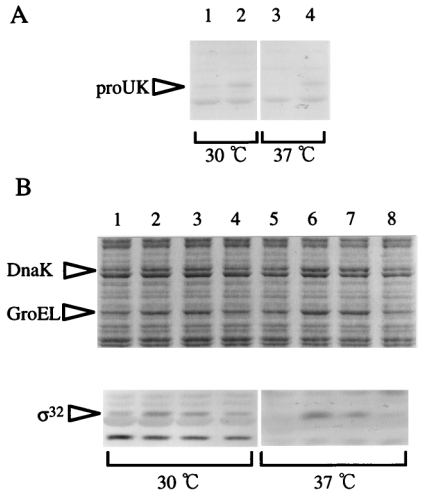


FIG. 3. Elevated levels of proUK, HSPs (DnaK and GroEL) and $\sigma^{32}$ in the Δ*hslVU* mutant. Cells were grown in L broth at 30 or 37°C to mid-log phase, and whole-cell proteins were prepared and analyzed by SDS-PAGE (10% gel) followed by staining or immunoblotting. (A) The proUK levels in the absence of IPTG were determined by immunoblotting. Lanes: 1 and 3, MC4100/pUK752; 2 and 4, KY2039 (Δ*hslVU*)/pUK752. (B) The levels of DnaK and GroEL are shown on a gel stained with Coomassie blue (upper panel), and the $\sigma^{32}$ levels are shown by immunoblotting (lower panel). Lanes: 1 and 5, MC4100 (*hslVU*$^+$); 2 and 6, KY2039 (Δ*hslVU*); 3 and 7, KY2039 (λ2137); 4 and 8, KY2039 (λ2137-*hslVU*$^+$).

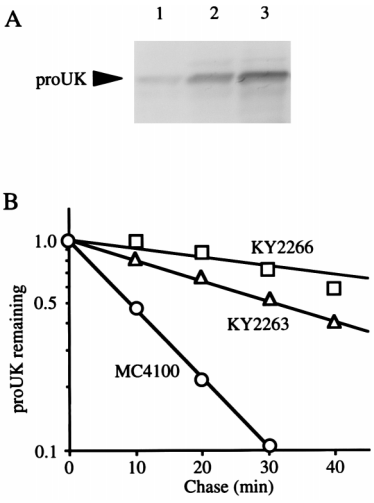


FIG. 4. Stabilization of proUK in the Δ(*clpPX-lon*) double mutant (KY2263) or the Δ*hslVU*-Δ(*clpPX-lon*) triple mutant (KY2266). (A) Cells carrying pUK-02pm0 were grown in synthetic medium at 30°C, IPTG (final concentration, 10 μM) was added at early log phase, and cells were harvested after 1 h. The amount of proUK was determined by immunoblotting. Lanes: 1, MC4100; 2, KY2263 [Δ(*clpPX-lon*)]; 3, KY2266 [Δ*hslVU*-Δ(*clpPX-lon*)]. (B) Stability of proUK was determined by pulse-chase experiments essentially as for Fig. 2B, and averages from two experiments were plotted. Symbols: ○, MC4100; Δ, KY2263; □, KY2266.

synthesis rate of proUK remained virtually unaffected (data not shown).

Striking increases in the levels of $\sigma^{32}$, DnaK, and GroEL due to multiple protease deficiencies were also observed by comparing the same set of mutants (Fig. 5A). The half-life of $\sigma^{32}$ increased from 1.5 min in the wild type to 8 min in the double mutant [Δ(*clpPX-lon*)] and to 40 min in the triple mutant [Δ*hslVU*-Δ(*clpPX-lon*)] (Fig. 5B), whereas the synthesis rate increased only slightly (ca. 1.5-fold) with either mutant, suggesting that stabilization primarily accounted for the observed increase in $\sigma^{32}$ levels. These results confirmed and extended the previous findings that $\sigma^{32}$ is markedly stabilized in the Δ*lon* Δ*clpP* double mutant but not in the Δ*lon* or Δ*clpP* single mutant (19). Thus, proteases Lon, Clp, and HslVU appeared to play synergistic roles in degradation of both proUK and $\sigma^{32}$ in vivo.

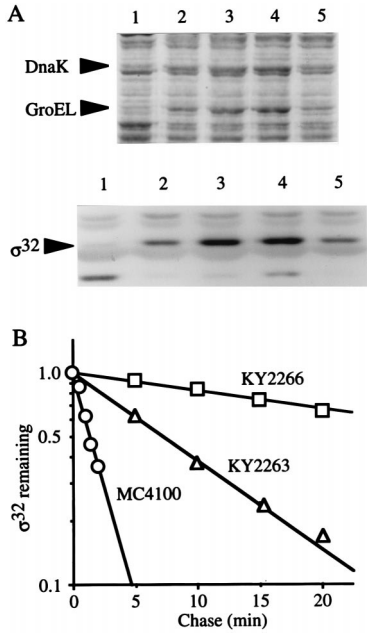


FIG. 5. Stabilization of $\sigma^{32}$ and increased HSP levels in the same set of strains (but lacking pUK-02pm0) used for Fig. 4. (A) Cells were grown in synthetic medium at 30°C to mid-log phase, and whole-cell proteins were analyzed by SDS-PAGE (10% gel) followed by staining with Coomassie blue (upper panel) or immunoblotting (lower panel). Lanes: 1, MC4100; 2, KY2263 [Δ(*clpPX-lon*)]; 3, KY2266 [Δ*hslVU*-Δ(*clpPX-lon*)]; 4, KY2266 (λ2137); 5, KY2266 (λ2137-*hslVU*$^+$). (B) The stability of $\sigma^{32}$ was determined by pulse-labeling cells with [$^{35}$S]methionine for 30 s (MC4100) or 1 min (KY2263 and KY2266) and chasing them for the same period of time (which was set as time zero). Averages from two experiments were plotted. Symbols: ○, MC4100; Δ, KY2263; □, KY2266.

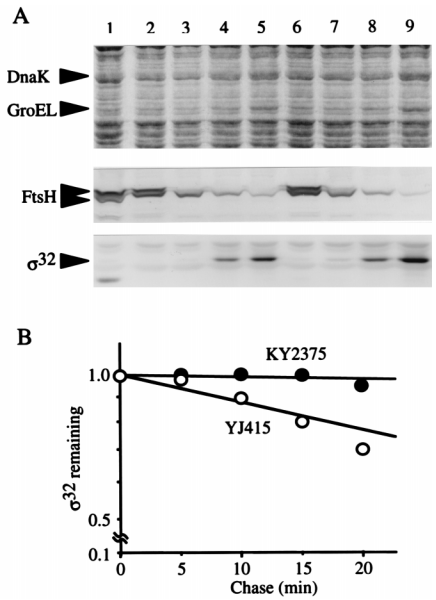


FIG. 6. Stabilization of $\sigma^{32}$ and increase in HSP levels upon depletion of FtsH in the Δ*ftsH*/pSTD401 and Δ*hslVU*-Δ*ftsH*/pSTD401 mutants. (A) Cells were grown in synthetic medium with 1 mM IPTG at 30°C, harvested, washed four times with, and resuspended in, IPTG-free medium, and incubated for 1 (lanes 3 and 7), 2 (lanes 4 and 8), or 3 (lanes 1, 5, and 9) doublings at 30°C. In addition, portions of cells were incubated with 1 mM IPTG as a control and harvested after three doublings (lanes 2 and 6). Whole-cell proteins were analyzed by SDS-PAGE as for Fig. 5A (upper panel). The amounts of FtsH and $\sigma^{32}$ were determined by immunoblotting (lower panel). Lanes: 1, CU141 (*ftsH*$^+$); 2 to 5, YJ415 (Δ*ftsH*)/pSTD401; 6 to 9, KY2375 (Δ*hslVU*-ΔftsH)/pSTD401. (B) The stability of $\sigma^{32}$ was determined by using cells grown in IPTG-free medium for three doublings as for panel A, and pulse-chase experiments were carried out essentially as for Fig. 5B. Averages of two experiments are plotted. Symbols: ○, YJ415; ●, KY2375.

**Synergistic roles of HslVU and FtsH proteases in modulating stability of $\sigma^{32}$.** We then examined the effect of *hslVU* deletion on the stability of $\sigma^{32}$ under conditions of limiting FtsH, an essential membrane-bound ATP-dependent metalloprotease known to degrade $\sigma^{32}$ in vivo and in vitro (13, 37). The *hslVU* deletion was transduced into the Δ*ftsH* strain carrying an *ftsH*$^+$ gene under the *lac* promoter on a low-copy-number plasmid (pSTD401). The resulting mutant (KY2375), when grown with 1 mM IPTG at 30°C, produced FtsH in an amount comparable to that of the *ftsH*$^+$ strain (CU141). Upon depletion of IPTG, the cellular level of FtsH decreased in both the Δ*hslVU* and *hslVU*$^+$ strains. Concomitantly, the levels of $\sigma^{32}$ and HSP gradually increased and were very high after periods of two to three doublings (Fig. 6A). The increase in $\sigma^{32}$ level was more striking in the Δ*hslVU* mutant (30-fold after three doublings) than in the *hslVU*$^+$ strain (15-fold), presumably reflecting differential stabilities. Indeed, $\sigma^{32}$ was quite

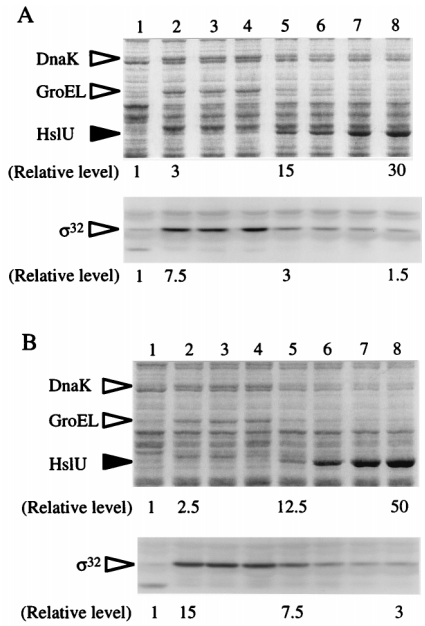


FIG. 7. Effects of overproduction of HslVU on $\sigma^{32}$ levels in the mutants deficient in ClpP-Lon or FtsH protease(s). (A) Cells were grown at 30°C in synthetic medium without (lanes 1 to 3 and 5) or with indoleacrylate at 1 μg/ml (lane 6), 5 μg/ml (lane 7), or 10 μg/ml (lanes 4 and 8). Analyses of whole-cell proteins (upper panel) or immunoblotting (lower panel) were done essentially as for Fig. 5A. The values shown below the gels indicate the levels of HslVU (upper panel) or $\sigma^{32}$ (lower panel) relative to the wild type and were determined by immunoblotting of serial dilutions of samples on separate gels (averages of two experiments). Lanes: 1, MC4100; 2, KY2263 [Δ(*clpPX-lon*)]; 3 and 4, KY2263 carrying pKV1174 (pKV1007 lacking *hslVU*); 5 to 8, KY2263 carrying pKV1007 ($trp_p$-*hslVU*). (B) Cells were grown with IPTG, washed, and depleted for IPTG, and whole-cell proteins were analyzed as for Fig. 6A except that indoleacrylate was added to 1 μg/ml (lane 6), 5 μg/ml (lane 7), or 10 μg/ml (lanes 4, 8) during depletion for IPTG for three doublings at 30°C. Lanes: 1, CU141 (*ftsH*$^+$); 2, YJ415 (Δ*ftsH*/pSTD401); 3 and 4, YJ415/pKV1174; 5 to 8, YJ415/pKV1007. The relative levels of HslVU or $\sigma^{32}$ were determined as for panel A.

stable in both strains, and the absence of HslVU protease resulted in two- to threefold-higher stabilization than in the *hslVU*$^+$ control after three doublings (Fig. 6B). No appreciable differences were seen in the synthesis rates of $\sigma^{32}$ (data not shown). These results demonstrated additive effects of the reduced FtsH level and the absence of HslVU on the stability of $\sigma^{32}$, implying that the two proteases play synergistic roles in turnover of $\sigma^{32}$ in vivo.

**Overproduction of HslVU markedly reduces the $\sigma^{32}$ level in mutants deficient in other proteases.** To further examine the role of HslVU in degradation of $\sigma^{32}$, effects of excess HslVU on the $\sigma^{32}$ level were analyzed in the absence of Lon-Clp proteases or under limited presence of FtsH protease. If $\sigma^{32}$ directly serves as a substrate for HslVU protease, overproduction of HslVU may effectively reduce the $\sigma^{32}$ level under these conditions. When a multicopy plasmid expressing *hslVU* from its own promoter (pKV1004) was introduced into KY2263 [Δ(*clpPX-lon*)], the $\sigma^{32}$ level was twofold lower than in the control carrying pBR322 (data not shown). Similarly, the same plasmid (pKV1004) caused threefold reduction of $\sigma^{32}$ when introduced into strain YJ415 (Δ*ftsH*/pSTD401) and grown under limiting FtsH (data not shown). Consistent with the reduction in $\sigma^{32}$ level, the DnaK and GroEL levels were significantly reduced by excess HslVU.

The quantitative relationship between the level of HslVU and that of $\sigma^{32}$ was further examined by using another plasmid (pKV1007) expressing *hslVU* under the *trp* promoter. The levels of HslVU produced were varied by changing concentration of inducer (indoleacrylate) in the range that had little effect on cell growth. Thus, production of 15- to 30-fold excess HslVU in the Δ(*clpPX-lon*) strain reduced the $\sigma^{32}$ level 2.5- to 5-fold, the effect being proportional to the amount of HslVU within the range tested (Fig. 7A, lanes 5 to 8). Similar results were obtained for the Δ*ftsH*/pSTD401 strain grown under limiting FtsH: the $\sigma^{32}$ level was reduced 2- to 5-fold when HslVU was overproduced 12.5- to 50-fold (Fig. 7B, lanes 5 to 8). The DnaK and GroEL levels were reduced closely in parallel with the reduced $\sigma^{32}$ level. These results taken together strongly suggested that HslVU and other ATP-dependent proteases jointly participate in modulating the stability and levels of $\sigma^{32}$, a key regulatory factor for the maintenance of HSP levels under normal as well as stress conditions. A summary of results on the stability of $\sigma^{32}$ (and proUK) reported above is presented in Table 1. Although the data presented above suggested that HslVU may directly degrade $\sigma^{32}$ in vivo, possible indirect effects of excess HslVU were not excluded.

## DISCUSSION

We showed that the *hslVU* genes on a multicopy plasmid markedly inhibit the heat shock response induced by production of human proUK: overproduction of HslVU reduced the stability and accumulation of proUK appreciably. Consistent with this finding, disruption of the chromosomal *hslVU* genes resulted in a significant increase in the amount of proUK produced. The Δ*hslVU* mutant also produced higher levels of HSPs (e.g., DnaK and GroEL) and $\sigma^{32}$ (Fig. 3) and failed to grow at very high temperatures (44 to 45°C). These results as well as the sequence similarities suggested that *hslVU* encodes a two-component ATP-dependent protease involved in degradation of abnormal proteins.

Recent reports from several laboratories revealed that HslV exhibits a peptidase activity whereas HslU catalyzes ATP hydrolysis, and the HslVU complex acts as an ATP-dependent peptidase or protease (18, 24, 29, 42). Moreover, overproduction of HslVU conferred on the cell with increased resistance to puromycin, whereas the *hslVU* null mutant degraded puromycyl fragments at reduced rates and was hypersensitive to puromycin (24). In agreement with these results, the level of

TABLE 1. Effects of protease mutation(s) or overproduction of HslVU protease on the stability and level of $\sigma^{32}$ and proUK[a]

| Strain | Stabilization (increase in level) | | Destabilization (decrease in level) by excess HslVU (fold) | |
|---|---|---|---|---|
| | $\sigma^{32}$ | ProUK | $\sigma^{32}$ | ProUK |
| Wild type | 1 | 1 | (1)[b] | 3 (3) |
| Δ*hslVU* | (2) | (2) | ND[c] | ND |
| Δ(*clpPV-lon*) (KY2263) | 5.5 (7.5) | 3 (2.5) | (2.5–5)[d] | ND |
| Δ*hslVU*-Δ(*clpPX-lon*) (KY2266) | 25 (22.5) | 5.5 (5) | ND | ND |
| Δ*ftsH*//pSTD401 (YJ415) | 35[e] (15) | ND | (2–5)[d] | ND |
| Δ*hslVU*-Δ*ftsH*/pSTD401 (KY2375) | 80[e] (30) | ND | ND | ND |

[a] Data were taken from Fig. 2 to 7. See figure legends and text for details.
[b] No detectable effect was found, possibly due to the low level of $\sigma^{32}$ produced.
[c] ND, not determined.
[d] The extent of decrease in level varied in parallel with that of HslVU overproduction.
[e] Probably represents overestimation due to much slower growth under the conditions used.

proUK was elevated in the Δ*hslVU* mutant, presumably due to stabilization. Indeed, proUK was stabilized in the Δ*hslVU*-Δ(*clpPX-lon*) mutant to significantly greater extents than in the isogenic Δ(*clpPX-lon*) mutant (Fig. 4B). It thus seemed evident that HslVU, along with Lon and Clp, participates in ATP-dependent turnover of abnormal proteins in *E. coli.*

Similar additive effects of multiple protease deficiencies were observed in stabilization of $\sigma^{32}$, which is normally quite unstable and present in very low amounts. Striking stabilization was observed in the two independently constructed Δ*clpP*-Δ*lon* double mutants (Fig. 5; reference 19), whereas very little stabilization was observed in either of the single mutants (Δ*clpP* or Δ*lon*) (13, 19). Still greater stabilization was found in the Δ*hslVU*-Δ(*clpPX-lon*) triple mutant (Fig. 5B). Furthermore, the increase in $\sigma^{32}$ level in the Δ(*clpPX-lon*) mutant was strongly counteracted by overproduction of HslVU (Fig. 7A). Thus, these proteases (Lon, Clp, and HslVU) can synergistically affect in vivo turnover of $\sigma^{32}$ as well as of abnormal proteins such as proUK. On the other hand, the defective growth of Δ(*clpPX-lon*) mutant at 43.5°C was complemented by a multicopy plasmid (pKV1004) carrying *hslVU* (16), suggesting that the function of HslVU overlaps that of Lon or Clp protease.

FtsH is another important protease involved in degradation of $\sigma^{32}$: $\sigma^{32}$ is highly stabilized in the *ftsH* mutants (13, 37), and histidine-tagged $\sigma^{32}$ is degraded in vitro by purified FtsH in an ATP-dependent manner (37). However, the results reported here make it unlikely that FtsH is solely responsible for in vivo degradation of $\sigma^{32}$. When the cellular amount of FtsH was reduced to 10 to 20% of the wild-type level, the $\sigma^{32}$ level was markedly elevated (Fig. 6); the level obtained was comparable to or higher than that observed with the Δ*ftsH* mutant containing an extragenic suppressor that enhances FabZ activity and fatty acid biosynthesis (26, 28). However, a significantly greater increase in $\sigma^{32}$ level was observed in the Δ*hslVU* mutant than in the *hslVU*$^+$ background under limiting FtsH (Fig. 6B). More importantly, the increase in $\sigma^{32}$ level expected under these conditions was largely canceled out by overproduction of HslVU in a dose-dependent fashion (Fig. 7B), suggesting that HslVU can degrade $\sigma^{32}$ directly. In the related studies, the expected increase in $\sigma^{32}$ level in the Δ*ftsH* mutant (containing the *fabZ* suppressor) was suppressed by a multicopy plasmid expressing Lon (19). This result, together with the finding that $\sigma^{32}$ is markedly stabilized in the Δ*clpP*-Δ*lon* double mutants (Fig. 5; reference 19), suggests that Lon can also directly degrade $\sigma^{32}$. In fact, $\sigma^{32}$ has been found to be degraded in vitro by purified Lon (14) or ClpAP protease (14, 38). Thus, $\sigma^{32}$ and much of bulk abnormal proteins appear to utilize similar degradation pathways involving several ATP-dependent proteases as well as a set of major chaperones, DnaK, DnaJ, and GrpE.

The extents of $\sigma^{32}$ stabilization in the Δ*hslVU*-Δ(*clpPX-lon*) triple mutant and the Δ*ftsH*/pSTD401 strain were comparable (Fig. 5 and 6; Table 1), suggesting that the sum of cytoplasmic proteases (Lon, Clp, and HslVU) and the membrane protease (FtsH) may equally contribute to turnover of $\sigma^{32}$ in vivo. Since the stabilization observed could represent a direct effect of the mutation(s) on $\sigma^{32}$ or an indirect effect through accumulation of unfolded proteins that could compete with $\sigma^{32}$ as the substrate for the remaining protease(s), the actual contribution of the cytoplasmic proteases might be overestimated. Conversely, if FtsH could degrade unfolded proteins that are also substrates for cytoplasmic proteases, the contribution of the cytoplasmic proteases might be underestimated. Thus, the relative contribution of these proteases remains obscure. It seems most probable, however, that several ATP-dependent proteases can all degrade $\sigma^{32}$ and abnormal proteins as well as certain specific substrates characteristic of each enzyme. Such synergistic roles of the proteases with overlapping specificities may provide an effective and versatile means of ensuring a rapid response to increased demands for proteolysis caused by accumulation of unfolded or misfolded proteins. In any events, multiple ATP-dependent proteases appear to play important roles in the heat shock response, and in protein quality control, by jointly modulating the chaperone-mediated and/or chaperone-independent degradation of $\sigma^{32}$, thereby maintaining proper levels of HSP to meet dynamic cellular requirements for HSP under normal as well as a variety of stress conditions.

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