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Suppression of *ftsH* Mutant Phenotypes by Overproduction of Molecular Chaperones

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Decreased intracellular levels of FtsH, a membrane-bound ATPase, led to retardation of growth and protein export, as well as to an abnormal translocation of alkaline phosphatase that had been attached to a cytoplasmic domain of a multispanning membrane protein, SecY. The last phenotype is designated Std (stop transfer defective). In this study, we examined the effects of overproduction of some molecular chaperones on the phenotypes of *ftsH* mutants. The growth retardation was partially suppressed by overproduction of GroEL/GroES (Hsp60/Hsp10) or HtpG (Hsp90), although these chaperones could not totally substitute for FtsH. Overproduction of HtpG specifically alleviated the Std phenotype, while that of GroEL/GroES alleviated the protein export defect of *ftsH* mutants. These results suggest that FtsH functions can be somehow compensated for when the cellular concentrations of some molecular chaperones increase.

The FtsH (HflB) protein of *Escherichia coli* (28, 32, 34) consists of an amino-terminal region with two transmembrane segments and a carboxy-terminal cytoplasmic domain that is homologous to members of the AAA ATPase family, which have diverse cellular functions (7, 20).

We previously identified an *E. coli* mutation, *ftsH101* (*std101*), which allowed significant export of the alkaline phosphatase mature domain that had been attached to the normally cytoplasmic C-terminal region of SecY, a membrane protein that spans the membrane 10 times. This phenotype was called Std (stop transfer defective) (3). The *ftsH101* mutation causes an amino acid substitution in the periplasmic region of FtsH (3). Cellular depletion of FtsH retarded cell growth, retarded protein export, and caused a strong Std phenotype (3). These results suggested that FtsH is somehow involved in protein assembly into and through the membrane. Recent studies additionally indicated that the FtsH function is involved in proteolytic degradation of some unstable proteins (11, 12, 18, 31). Although uncomplexed forms of SecY are substrates of the FtsH-dependent proteolysis, evidence suggests that the Std phenotype is not a consequence of stabilization of the SecY-PhoA fusion protein (18). These diverse phenotypes of *ftsH* mutants as well as recent demonstrations of chaperone-like activities for the ClpA and the ClpX ATPase subunits of the Clp protease (9, 10, 36, 37) made us suspect that FtsH might also interact with a set of proteins, thereby affecting their localization and stability.

We were thus prompted to examine the effects of overproduction of various chaperones on the multiple phenotypes of *ftsH* mutants. Cell growth was partially restored when HtpG (Hsp90) or GroEL/GroES (Hsp60/Hsp10) was overproduced in FtsH-depleted cells. Overproduced HtpG partially alleviated the Std phenotype but not the translocation defect. The opposite was the case for GroE overproduction.

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MATERIALS AND METHODS

Bacterial strains and media. *E. coli* strains were all K-12 derivatives. CU141 (3) was a derivative of MC4100 (6) carrying F′ *lacZ*$^+$ *lacI*^{q}. AD315 carried Δ*ftsH3*::*kan* on the chromosome, while functional FtsH was supplied from a plasmid (pSTD40) under the control of the *lac* promoter (3). AD245 (Δ*phoA* Δ*lac ftsH101 zgi-231*::Tn*10*) was constructed by introducing the *ftsH101* mutation into KS474 (30) by joint transduction with *zgi-231*::Tn*10* (3). AD247 was an isogenic *ftsH*$^+$ counterpart of AD245. CU194 was a derivative of MC4100 into which Δ*htpG1*::*lacZ*$^+$ and *zba-315*::*kan* were transduced (5, 35). YJ37 (MC4100 except Δ*groE*::*tet srl-268*::Tn*5* *recA1*/F′*lacZ*$^+$ *lacI*^{q}/pKY326) was constructed by transducing the Δ*groE*::*tet* marker (17) into CU141, which carried pKY236 with the p*lac*-controlled *groES*/*groEL* operon.

L medium (8), peptone medium (14), and M9 medium (26) were used. Ampicillin (50 μg/ml), tetracycline (25 μg/ml), and chloramphenicol (20 μg/ml) were added for growth of plasmid-bearing cells as well as for selection of transformants and transductants.

Plasmids. Plasmids pKY221 and pKY321 (both carrying *secY-phoA* 66-6 under the control of the *lac* promoter), pSTD40 (p*lac-ftsH40*; as described below, a mutation, Thr-199 to Ala, has fortuitously been introduced), pSTD401 (p*lac*–wild-type *ftsH*), and pSTD41 (p*lac-ftsH41*) were described previously (3). Although our original construction of pSTD40 complemented the *ftsH1* (Ts) mutation, which is weakly dominant with respect to some phenotypes (3), the plasmid extracted from a stock of strain AD315 subsequently proved to have lost the complementation activity against *ftsH1* and to contain a Thr-199-to-Ala mutation. This form of FtsH is still functional in that it complements the *ftsH*::*kan* disruption. Since phenotypes we examined under the condition of repression of the complementing *ftsH* function would not have been affected by the fortuitously introduced mutation, we report here results obtained with strain AD315. We confirmed essentially the same results with a reconstructed strain with a *ftsH*$^+$ plasmid (29).

Plasmid pYJ99 carried *htpG*; a 2.6-kbp *Eco*RI-*Eco*RV fragment of pMSY3 that carried *htpG* (35) was cloned into the multicloning site of pTWV229 (a pBR322-based *lac* promoter vector obtained from Takara Shuzo). Plasmid pNRK267 carried *groES*/*groEL* (21), and pAK330 carried *secB* (19). Plasmid pKY326 in strain YJ37 carried *groES*/*groEL* placed under the control of the *lac* promoter and was constructed by PCR such that the promoter of the *groE* operon was replaced by that of the *lac* operon (1). Plasmid pKY327 carried *dnaK*; a 3.6-kbp *Xho*I-*Hin*dIII fragment of pNH1 that carried p*trp-dnaK* (27) was cloned into the *Sal*I-*Hin*dIII site of pBR322. Plasmid pYJ135 also carried *dnaK*; a 3.6-kbp *Xho*I-*Hin*dIII fragment of pNH1 was cloned into the multicloning site of pMW119 (a pSC101-based vector obtained from Nippon Gene).

Trypsin digestion and immunoblotting of the SecY-PhoA C6 fusion protein. Cells were grown in peptone medium supplemented with appropriate antibiotics. The procedures of trypsin digestion for examining folding states of alkaline phosphatase (PhoA) were as described by Akiyama et al. (3). Proteins were precipitated with trichloroacetic acid for subsequent gel electrophoresis (2, 13).

Protein samples were separated by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (23) and electroblotted onto an Immobilon polyvinylidene difluoride membrane (Millipore). The filter was treated with anti-PhoA serum (obtained from 5 Prime 3 Prime, Inc.) and detected by using the ECL detection kit (Amersham).

Other procedures. The procedures for assay of PhoA activity and the unit definition were as described by Manoil and Beckwith (24). Pulse-labeling and

immunoprecipitation for examining the export of β-lactamase (Bla) and OmpA were carried out as described previously (2, 3); proteins were separated by SDS–10% polyacrylamide gel electrophoresis and visualized with a BAS2000 image analyzer (Fuji Film).

RESULTS

Growth defects of FtsH depletion can be suppressed by overproduction of HtpG or GroE. *E. coli* AD315 has its chromosomal *ftsH* disrupted by *kan* and its episomal *ftsH*$^+$ controlled by the *lac* repressor (3). Growth of this strain depends on isopropyl-β-D-thiogalactopyranoside (IPTG), an inducer of the *lac* system. We overproduced various chaperones to examine their effects on the growth of AD315 cells.

A series of plasmids that carried *htpG*, *groES*/*groEL*, *dnaK*, or *secB* were introduced into strain AD315, and the growth of transformants in the presence and absence of IPTG was examined. At about 4 h after removal of IPTG, the growth of cells without a chaperone-overproducing plasmid began to decline (Fig. 1A and B). This growth cessation should have been due to a decreased cellular abundance of FtsH, which is brought about by dilution and possible degradation of the preexisting FtsH molecules. At this time point, the cellular amount of FtsH became undetectable by immunoblotting (Fig. 2, lanes 4 and 8). Plasmid overproducing either HtpG or GroEL/GroES eliminated the early growth cessation in the absence of IPTG (Fig. 1A and B). Immunoblotting experiments showed that even in the presence of overproduced HtpG or GroEL/GroES, the FtsH level remained undetectable (Fig. 2, lanes 2 and 10 for HtpG overproduction and lanes 6 and 14 for GroE overproduction). The SecB plasmid improved the growth only slightly (Fig. 1C). The DnaK plasmids did not improve growth. This was true for both pKY327 (a pBR322-derived plasmid) that actually retarded growth (Fig. 1C) and pYJ135 (a pSC101-derived plasmid) that did not cause such growth retardation (data not shown).

Restoration of cellular growth by HtpG or GroE overproduction was also evident when bacterial growth on agar plates in the presence or absence of IPTG was examined (data not shown). However, none of these chaperone proteins completely substituted for FtsH, since we were unable to transduce the Δ*ftsH*::*kan* marker into cells that carried the HtpG or GroE plasmid unless they carried an *ftsH* plasmid as well. Presumably, the uninduced level of *ftsH* expression in strain AD315 was essential, even in the presence of excess HtpG or GroE.

Suppression of the Std phenotype of the *ftsH101* mutant by overproduction of HtpG. FtsH was previously suggested to be involved in the correct assembly of a SecY-PhoA C6 fusion protein with an attached topology reporter (PhoA). In wild-type cells, the PhoA domain, which has been attached to cytoplasmic domain 6, is localized in the cytoplasm. When the FtsH function is compromised, a substantial fraction of the PhoA moiety of the SecY-PhoA fusion protein is exported to the periplasm, where it is folded into a conformation that is enzymatically active and resistant to trypsin. The efficiency of such abnormal translocation varied from about 20% in the *ftsH101* mutant to almost 100% in cells expressing an ATPase site mutant of FtsH (3, 4). We examined the effect of overproduction of HtpG or GroE on the Std phenotype of the *ftsH101* mutant. Strain AD245 (*ftsH101*)/pKY321 (*secY-phoA* C6) was further transformed with a chaperone-overproducing plasmid. Translocation of the PhoA domain was assessed by colony color, PhoA enzyme activity, and generation of a trypsin-resistant PhoA fragment (3).

On agar plates that contained a chromogenic substrate (XP) of PhoA, cells with the HtpG-overproducing plasmid formed white colonies whereas those with a control (vector) plasmid, as well as those with the GroEL/GroES-overproducing plasmid, remained blue. PhoA enzyme activities were measured. HtpG overproduction decreased the enzyme activity by about fourfold. The GroE plasmid did not significantly affect the enzyme activity.

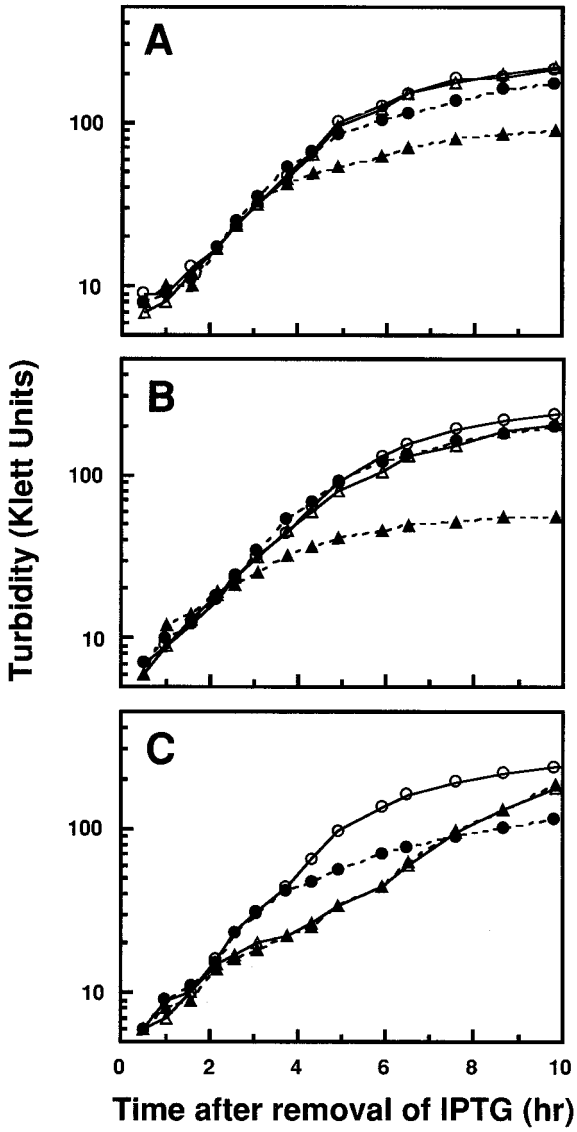

FIG. 1. Effects of chaperone overproduction on growth of cells under FtsH deprivation. Cells of AD315 (Δ*ftsH*::*kan*/pSTD40) that additionally carried the plasmids indicated below were grown to stationary phase in M9 glucose medium containing Casamino Acids (0.2%), thiamine (2 μg/ml), chloramphenicol, ampicillin, and IPTG (1 mM). Cells were centrifuged, washed four times with the same medium without IPTG, and then inoculated into the same medium with (open symbols) or without (closed symbols) IPTG. Cell growth was monitored by measuring the turbidity with a Klett-Summerson colorimeter with filter 54. Plasmids carried were as follows. (A) Circles, pYJ99 (*htpG*); triangles, pTWV229 (vector). (B) Circles, pNRK267 (*groE*); triangles, pBR322 (vector). (C) Circles, pAK330 (*secB*); triangles, pKY327 (*dnaK*).

The results of trypsin digestion assays are shown in Fig. 3. In contrast to the *ftsH*$^+$ cell extract, which gave only a small amount of the trypsin-resistant PhoA fragment (lane 12), the *ftsH101* cell extract gave a much higher proportion of the trypsin-resistant PhoA fragment (lanes 4 and 10). In the presence of the HtpG-overproducing plasmid, this proportion decreased to that in the *ftsH*$^+$ cells (lane 2). The increased trypsin

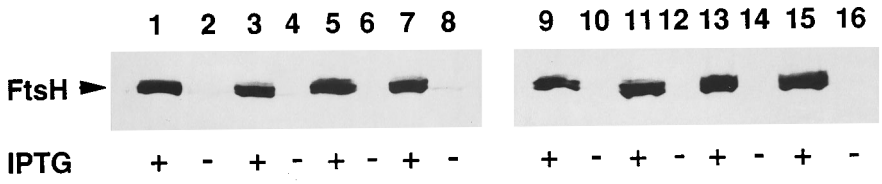

FIG. 2. Cellular abundance of FtsH. Cells of AD315 that additionally carried the plasmids indicated below were grown as described in the legend to Fig. 1 with (lanes with odd numbers) or without (lanes with even numbers) IPTG for 4 h (lanes 1 to 8) or 6 h (lanes 9 to 16). A portion of each culture was treated with trichloroacetic acid, and denatured proteins were dissolved in SDS. Fixed amounts of protein (adjusted by the turbidity of the cultures) were electrophoresed, and FtsH was stained immunologically. Plasmids carried were as follows. Lanes 1, 2, 9, and 10, pYJ99 (*htpG*); lanes 3, 4, 11, and 12, pTWV229 (vector); lanes 5, 6, 13, and 14, pNRK267 (*groE*); lanes 7, 8, 15, and 16, pBR322 (vector).

sensitivity indicates an increased cytoplasmic localization of the PhoA part of the fusion protein. Thus, overproduction of HtpG suppressed the Std phenotype of the *ftsH101* mutant. However, depletion of HtpG did not itself cause an Std phenotype (29). Overproduction of either GroE (lane 8) or SecB (lane 6) was ineffective in suppression.

FtsH is involved in degradation of unstable proteins, such as uncomplexed forms of SecY (18). However, the SecY-PhoA C6 fusion protein was much more stable than overproduced SecY, and HtpG overproduction did not affect the stability of the fusion protein (29). Thus, it is unlikely that suppression of the Std phenotype by overproduced HtpG is caused by decreased stability of the fusion protein.

Suppression by overproduction of GroE of the protein export defect caused by *ftsH* depletion. Depletion of FtsH causes export (translocation) defects of Bla and OmpA (3). We examined whether overproduction of HtpG or GroE had any effects on the export phenotypes under the condition of decreased FtsH content. Pulse-labeling experiments showed that the labeling of precursor forms of Bla and OmpA decreased to nearly wild-type levels when GroE was overproduced in AD315 cells that had been incubated in the absence of IPTG (Fig. 4, lane 3). Thus, overproduction of GroE alleviated the export defect caused by FtsH depletion. In contrast, overproduction of HtpG did not significantly improve the export of these proteins (Fig. 4, lane 1).

Expression of an ATP binding site mutant form of *ftsH*, *ftsH41*, from a plasmid causes a dominant translocation interference (4). We investigated whether overproduction of HtpG or GroE had any effect on this dominant translocation defect. Processing of Bla and OmpA was examined by using cells of CU141 (*ftsH*$^+$) that carried pSTD41 (*ftsH41*) as well as a chaperone-overexpressing plasmid. Overproduction of either chaperone did not suppress this mode of translocation defect (29).

The above results suggest that GroE has positive roles in the export of Bla and OmpA in cells deprived of FtsH. It was shown previously, using temperature-sensitive *groE* mutant strains, that GroE function is required for optimum export of Bla but not of OmpA (22). We addressed the involvement of GroE in optimal export of the OmpA protein, using a simpler system of GroE depletion (17). In strain YJ37 in which *groE* on the chromosome had been disrupted by *tet* while functional GroEL/GroES was supplied by a plasmid under *lac* promoter control, the export of OmpA was slightly (but significantly) retarded upon removal of IPTG (Fig. 5).

DISCUSSION

The pleiotropic phenotypes of *ftsH* mutants suggest that FtsH has multiple functions. Tomoyasu et al. (33) first observed that overproduction of GroE partly suppressed the defect in Bla export in the *ftsH1* mutant. In the present study, we investigated the effects of overproduction of various chaperones on phenotypes of *ftsH* mutants.

Growth of the FtsH-disrupted cells was retarded by shutting off the expression of the complementing *ftsH* plasmid, and such growth inhibition was partially compensated for by overproduction of HtpG or GroEL/GroES, although the proteins could not totally substitute for FtsH.

Overproduction of HtpG and overproduction of GroEL/

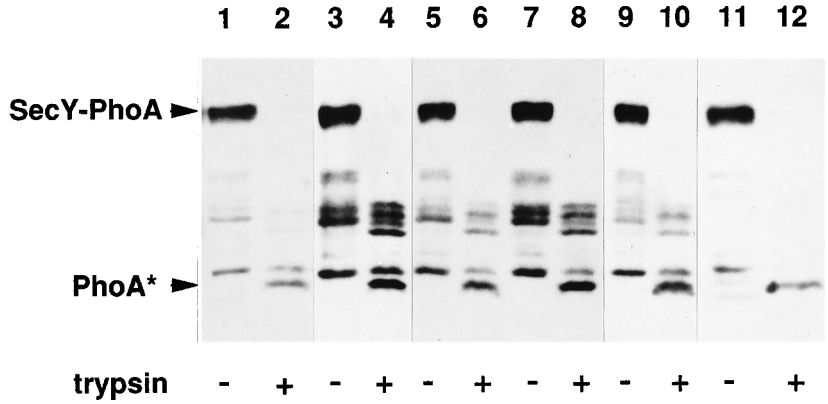

FIG. 3. Effect of overproduction of HtpG or GroE on the Std phenotypes of *ftsH101* mutant cells. Cells of AD245 (Δ*phoA ftsH101*)/pKY321 (*secY-phoA* 66-6)/pYJ99 (*htpG*) (lanes 1 and 2), AD245/pKY321/pTWV229 (vector) (lanes 3 and 4), AD245/pKY321/pAK330 (*secB*) (lanes 5 and 6), AD245/pKY321/pNRK267 (*groE*) (lanes 7 and 8), AD245/pKY321/pBR322 (vector) (lanes 9 and 10), and AD247 (Δ*phoA ftsH*$^+$)/pKY321/pBR322 (lanes 11 and 12) were grown in peptone medium containing chloramphenicol and ampicillin. They were harvested, disrupted, and treated with 50 μg of trypsin per ml (lanes with even numbers) or mock treated (lanes with odd numbers); both treated and mock-treated cells were then subjected to SDS–10% polyacrylamide gel electrophoresis and immunoblotting with anti-PhoA serum. PhoA*, trypsin-resistant PhoA fragment.

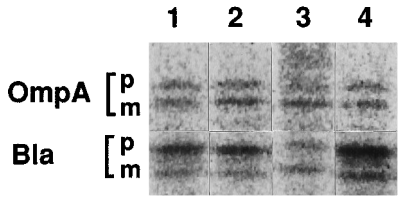

FIG. 4. Effects of overproduction of HtpG or GroE on protein export defects of cells deprived of FtsH. Cells of AD315 (Δ*ftsH*::*kan*/pSTD40) that additionally carried pYJ99 (*htpG* [lane 1]), pTWV229 (vector [lane 2]), pNRK267 (*groE* [lane 3]) or pBR322 (lane 4) were first grown in the presence of 1 mM IPTG, washed, and then grown in the absence of the inducer for 4.5 h as described in the legend to Fig. 1. Cells were pulse-labeled for 30 s with [^{35}S]methionine. Labeled OmpA and Bla were isolated by immunoprecipitation and analyzed by SDS–10% polyacrylamide gel electrophoresis. p, precursor form; m, mature form.

GroES exerted different effects on the phenotypes associated with *ftsH* mutants. The defect in export of Bla and OmpA seen upon depletion of FtsH was suppressed by overproduction of GroE but not by HtpG overproduction. The observation that GroE can affect export of OmpA is in apparent contradiction with the results of Kusukawa et al. (22), who showed that temperature-sensitive mutations in *groES* or in *groEL* impaired export of Bla but not of OmpA. It should be noted, however, that mutational alterations of molecular chaperones could exhibit adverse effects. For instance, a mutation that causes fortuitous substrate binding and impaired substrate release could lead to a kind of defect that does not necessarily indicate the participation of the wild-type gene product in the process. Using GroE-depleted cells, we demonstrated in this study that a simple loss of GroE function caused retardation of protein export. It is noteworthy that the GroE requirement for optimum export was not restricted to Bla but was observed for OmpA as well. Thus, GroE may be more generally required for cells to optimize translocation efficiencies of envelope proteins than considered previously. It should be noted that such a requirement may well be indirect, since the depletion takes several hours of incubation.

The Std phenotype of the *ftsH101* mutant was suppressed partially by overproduction of HtpG but not by overproduction of GroE or SecB. Since the *htpG*-disrupted strain did not show any Std phenotype, HtpG is not intrinsically involved in the stop transfer event, although the existence of another factor with an overlapping function cannot be ruled out. The eukaryotic members of the Hsp90 family are involved in the formation of heteromeric protein complexes, keeping a specific set of proteins competent for activation until they encounter proper environments (15). Chaperone-like activities of HtpG have recently been demonstrated (16). Our results of multicopy suppression are consistent with a chaperone function of HtpG, an increased concentration of which could influence assembly of the SecY-PhoA fusion protein. Recently, we have found that purified FtsH protein can bind to a denatured form of PhoA protein without degrading it (for the proteolytic function of FtsH, see the discussion below) (1a). If this activity is related to the stop transfer function of FtsH, it is speculated that HtpG might bind to the PhoA moiety of the fusion protein as well.

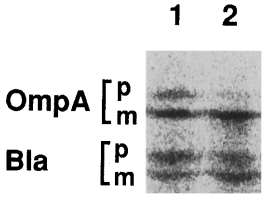

FIG. 5. Effect of depletion of GroE on export of OmpA. Cells of YJ37 (Δ*groE*::*tet*/p*lac-groE*$^+$) were first grown in the presence of 1 mM IPTG, washed, and then grown in the presence (lane 2) or absence (lane 1) of the inducer for 4.5 h as described in the legend to Fig. 1. Cells were pulse-labeled for 30 s with [^{35}S]methionine, and OmpA and Bla immunoprecipitates were analyzed by SDS–10% polyacrylamide gel electrophoresis. p and m represent the precursor and mature forms, respectively.

Recent studies indicate that FtsH is involved in proteolysis (11, 12, 18, 31) and that its targets include uncomplexed forms of SecY protein (1a, 18). The SecY24 mutant protein is degraded at high temperatures by the FtsH system (18). Previously, we reported that a high level of overproduction of HtpG suppressed the temperature sensitivity of the *secY24* mutant (35). However, this suppression required much higher levels of HtpG overproduction than those required for *ftsH* suppression. Moreover, overproduction of HtpG did not stabilize the SecY24 mutant protein (29), and we now suspect that this suppression might have been due to a slight growth inhibition (23a) that proved to accompany high-level HtpG overproduction. Neither the stability of the overproduced SecY protein nor that of the SecY-PhoA fusion protein was affected by HtpG overproduction (29). Therefore, it seems difficult to explain all the SecY-related phenotypes of *ftsH* mutants, as well as their suppression by chaperone overproduction, in terms of proteolysis of the SecY or SecY-PhoA protein. To distinguish between direct and indirect consequences of *ftsH* mutation, it will be essential to define the basic biochemical activities of FtsH, which now include proteolysis of selected substrate proteins (1a, 31) and binding to some denatured proteins (1a). Dual functions with proteolytic and chaperone-like activities have been proposed for the Clp protease complexes (9, 10, 25, 36, 37).

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