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Filamentous Morphology in GroE-Depleted *Escherichia coli* Induced by Impaired Folding of FtsE[▽]

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The chaperonin GroE (GroEL and the cochaperonin GroES) is the only chaperone system that is essential for the viability of *Escherichia coli*. It is known that GroE-depleted cells exhibit a filamentous morphology, suggesting that GroE is required for the folding of proteins involved in cell division. Although previous studies, including proteome-wide analyses of GroE substrates, have suggested several targets of GroE in cell division, there is no direct in vivo evidence to identify which substrates exhibit obligate dependence on GroE for folding. Among the candidate substrates, we found that prior excess production of FtsE, a protein engaged in cell division, completely suppressed the filamentation of GroE-depleted *E. coli*. The GroE depletion led to a drastic decrease in FtsE, and the cells exhibited a known phenotype associated with impaired FtsE function. In the GroE-depleted filamentous cells, the localizations of FtsA and ZipA, both of which assemble with the FtsZ septal ring before FtsE, were normal, whereas FtsX, the interaction partner of FtsE, and FtsQ, which is recruited after FtsE, did not localize to the ring, suggesting that the decrease in FtsE is a cause of the filamentous morphology. Finally, a reconstituted cell-free translation system revealed that the folding of newly translated FtsE was stringently dependent on GroEL/GroES. Based on these findings, we concluded that FtsE is a target substrate of the GroE system in *E. coli* cell division.

The chaperonin GroE (i.e., GroEL and the cochaperonin GroES) is a highly conserved molecular chaperone that assists protein folding in the cell (2, 12). Although extensive in vitro studies have clarified the sophisticated mechanism of GroEL as a protein-based molecular machine, the in vivo roles of GroE are still poorly understood. GroE is essential for the viability of *Escherichia coli* at all temperatures (7). One prevailing explanation for the requirement of GroE for cell viability is that the folding of proteins involved in essential cellular processes is strictly dependent on GroE. Therefore, the proteins that are obligate substrates of GroE should be identified.

One approach to answer this question is the use of a proteome-wide analysis. Hundreds of GroE substrates have been identified using mass spectrometry, and they provide a valuable resource to elucidate the role of GroE in the cells (3, 14, 18, 25). In particular, Kerner et al. have identified ~250 substrates that interact with *E. coli* GroEL, and they observed enrichment of ~85 obligate substrates (referred to as "class III" substrates), including 13 essential proteins (18). In addition, Chapman et al. have identified ~300 substrates from an inclusion body fraction in an *E. coli* strain that expresses a lethal GroEL temperature-sensitive mutant (3).

Another approach to investigate the in vivo substrates of GroE is detailed analysis of the phenotype associated with GroE-depleted *E. coli*. In a pioneering study, McLennan and Masters demonstrated that GroE is vital for cell wall synthesis (20). They found that *E. coli* cells deprived of GroE tend to lyse because the folding of DapA, which is an essential enzyme for synthesis of the cell wall precursor diaminopimelic acid (DAP), is stringently GroE dependent (20). Such a detailed phenotypic analysis provides a precise physiological role for GroE in a specific cellular process and is considered to be complementary to the global proteomic analysis.

In addition to the cell lysis phenotype in GroE-depleted *E. coli*, it has been reported that cells with impairment of GroE (e.g., severely temperature-sensitive *groE* mutant [13]) exhibit a filamentous cell morphology (3, 8, 13). This filamentous phenotype is not restricted to *E. coli*, since GroE-depleted *Caulobacter crescentus* and *Streptococcus mutans*, both of which are phylogenetically distinct from *E. coli*, also have a defect in cell division (19, 27), suggesting that GroE plays a universal role in cell division in eubacteria. There are several candidates for GroEL substrates among the dozens of proteins involved in cell division. The candidate proteins based on proteome-wide analyses include FtsE and ParC, both of which have been designated obligate class III GroE substrates (18), FtsZ, FtsA, and FtsI (3). In addition, the FtsZ-dependent localization of GroEL at possible division sites has suggested a possible role of GroEL in cell division (21). However, the GroEL substrate that is directly responsible for the filamentous morphology has not been identified.

In this study, we provide in vivo and in vitro evidence that the cell division defect in GroE-depleted *E. coli* is induced by the impaired folding of FtsE. The general utility of the approach for investigating the physiological roles of GroE is also discussed.

MATERIALS AND METHODS

Plasmids. pMCS, in which the T7 promoter region of the pET vector was replaced with *tac*, was constructed from pET15b(+) (Novagen). To replace the T7 promoter with the *tac* promoter, the *tac* promoter was amplified from

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pMD137 (4) and cloned into pET15b(+) digested with BglII/XhoI. To construct pWARA3(*ftsA-gfp*), pWARA7(*zipA-gfp*), pWARA8(*gfp-ftsQ*), and pWARA9(*ftsX-gfp*), fragments encoding full-length *ftsA*, *zipA*, *ftsQ*, and *ftsX*, respectively, were amplified by PCR from *E. coli* K-12 strain MG1655 chromosomal DNA, and *gfp* was amplified from plasmid YCp-GAL1p-SUP35 (NM)–GFP (17). The amplified *ftsA*, *zipA*, *ftsX*, and *ftsQ* fragments were digested with NdeI/BamHI (*ftsA*, *zipA*, and *ftsX*) or XhoI/BglII (*ftsQ*). The amplified *gfp* fragment was digested with BglII/XhoI (*ftsA*, *zipA*, and *ftsX*) or KpnI/XhoI (*ftsQ*). The digested fragment of *gfp* and that of either *ftsA*, *zipA*, *ftsX*, or *ftsQ* were ligated into pMCS. To construct pWARA1, pWARA2, pWARA5, and pWARA6, the full-length *metK*, *ftsZ*, *ftsE*, and *parC* genes, respectively, were amplified from *E. coli* K-12 strain MG1655 chromosomal DNA by PCR. The amplified fragments were digested with NdeI/XhoI and cloned into pMCS. Due to the presence of an NdeI site in *ftsE*, a partially digested fragment was used to construct pWARA5.

Complementation of cell filamentation. *E. coli* MGM100 cells [MG1655 *groE*::*araC*-P_{BAD}-*groE*(Kan^r)] (20) harboring pMCS, pWARA1, pWARA2, pWARA5, and pWARA6 were grown in LB medium containing 200 μg/ml ampicillin and 0.2% arabinose at 37°C to an optical density at 660 nm (OD_{660}) of 0.5, and then the cells were washed twice with LB medium. The washed cells were diluted 1:1,000 into LB medium with 1 mM DAP containing either 0.2% arabinose or 0.2% glucose. The cell morphology was monitored after 5 h of cultivation with a differential interference microscope (IX71; Olympus).

Western blotting. To prepare the polyclonal antibody against FtsE, recombinant FtsE with an N-terminal six-His tag, which was expressed from pET15b(+), was purified from inclusion bodies by urea extraction, followed by affinity chromatography using Ni-nitrilotriacetic acid. The purified protein was used to raise the antibody. MGM100 cells harboring either pMCS or pWARA5(*ftsE*) were grown using the procedure described above. Cells were harvested periodically, suspended in lysis buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA) so that the preparations contained equivalent OD_{660} units, and sonicated (Branson Sonifier). The insoluble fraction was separated from the soluble fraction by centrifugation (20,000 × *g*, 30 min) and was resuspended in 8 M urea. Total, soluble, and insoluble extracts were compared by fractionation by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and were detected by immunoblotting using anti-FtsE antiserum or anti-FtsZ antibodies (a gift from Masaaki Wachi).

Overexpression of MetK or ParC in GroE-depleted cells. MGM100 cells harboring either pWARA1(*metK*) or pWARA6(*parC*) were grown in LB medium with 200 μg/ml ampicillin and 0.2% arabinose at 37°C to an OD_{660} of 0.5, and then the cells were washed twice with LB medium. The washed cells were diluted 1:100 into LB medium with 1 mM DAP containing either 0.2% arabinose or 0.2% glucose. After 2 h of cultivation, each protein was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 h. The cells were then harvested, suspended in lysis buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA) so that the preparations contained equivalent OD_{660} units, and sonicated. The insoluble fraction was separated from the soluble fraction by centrifugation (20,000 × *g*, 30 min) and was resuspended in 8 M urea. Total, soluble, and insoluble extracts were analyzed by SDS-PAGE, and the gel was stained with Coomassie brilliant blue.

Localization of GFP fusion proteins. MGM100 cells harboring pWARA3(*ftsA-gfp*), pWARA7(*zipA-gfp*), or pWARA8(*gfp-ftsQ*) were grown in LB medium containing 200 μg/ml ampicillin and 0.2% arabinose at 37°C to an OD_{660} of 0.5. To express all of the green fluorescent protein (GFP) fusion proteins except FtsX-GFP, 100 μM (FtsA-GFP and GFP-FtsQ) or 10 μM (ZipA-GFP) IPTG was added to the culture. After 30 min of induction, the cells were washed twice and suspended in LB medium. To express FtsX-GFP, 1 μM IPTG was added to the preculture and no additional induction was performed due to the toxicity of overexpressed FtsX-GFP. The washed cells were diluted 1:250 into LB medium containing 1 mM DAP and either 0.2% arabinose or 0.2% glucose. After 5 h of cultivation at 37°C, the cells were observed by fluorescence microscopy (IX71; Olympus).

NaCl requirement. MGM100 cells harboring either pMCS or pWARA5(*ftsE*) were grown in LB medium containing 200 μg/ml ampicillin and 0.2% arabinose at 37°C to an OD_{660} of 0.5, and then the cells were washed. The washed cells were diluted 1:500 into LB medium containing 1% NaCl or no NaCl supplemented with 1 mM DAP and either 0.2% arabinose or 0.2% glucose. Growth was monitored periodically by determining the OD_{660}.

In vitro translation of FtsE using the reconstituted cell-free translation system (PURE system). Transcription-translation-coupled cell-free translation of FtsE was performed for 2 h. Then the productivity and solubility of the synthesized protein were evaluated by an autoradiographic analysis as previously described (31), except that the insoluble fractions were isolated by centrifugation at 20,000 × *g*. Ultrafiltration assays were performed using Microcon Ultracel YM-100 (Millipore). The reaction mixtures were filtered by centrifugation at 1,500 × *g* for 30 min. The concentrations of the chaperones were as follows: 1 μM GroEL, 1 μM GroES, 4 μM DnaK, 2 μM DnaJ, 2 μM GrpE, and 2.5 μM trigger factor. The products were radiolabeled with 0.1 MBq of [^{35}S]methionine and then were analyzed by SDS-PAGE. Bands were detected and quantitated with a BAS5000 imager (FUJIFILM). Purified DnaK, DnaJ, GrpE, GroEL, and GroES were obtained commercially. Purified trigger factor was prepared as described previously (31).

RESULTS

Excess FtsE suppresses the filamentous morphology of GroE-depleted *E. coli*. In this study, we used a conditional GroE expression strain, MGM100, in which the native *groE* chromosomal promoter region has been replaced with the *araC* gene and the *araBAD* promoter (20). When the sugar in the growth medium is changed from arabinose to glucose, the GroE levels decrease by ~90% within 2 h in this strain (20). To suppress the cell lysis phenotype due to the loss of DAP in the GroE-depleted cells, the growth medium was supplemented with 1 mM DAP throughout this study. Following arabinose removal in the presence of DAP, we observed the typical filamentous morphology of the cells (Fig. 1A), confirming again that cell division is prevented in the GroE-depleted cells (3, 13).

Since the overproduction of DapA suppresses the cell lysis phenotype in the GroE-depleted cells (20), we hypothesized that the filamentous phenotype derived from the GroE depletion would be delayed when critical substrates of GroE were synthesized in excess, before the GroE depletion. We first selected two essential proteins, FtsE and ParC, which are required for cell division (16, 28) and have been proposed to be obligate class III substrates of GroEL (18). *E. coli* strain MGM100 was transformed with plasmids bearing *ftsE* or *parC* in the presence of arabinose. We then cultured the transformed cells in the absence of arabinose to deplete the GroE and observed the cell morphology after 5 h of growth in the glucose-containing medium. Strikingly, we observed obvious suppression of filamentation in the GroE-depleted cells bearing the *ftsE* plasmid (Fig. 1C). Note that leaky expression of FtsE protein with the *tac* promoter, even without IPTG, was sufficient to induce the suppression (Fig. 2B and 2C, compare 0-h lanes). A statistical analysis revealed that the average and the distribution of the cell lengths for the cells with the *ftsE* plasmid grown in glucose medium and for the cells with the empty vector control grown in arabinose medium were almost indistinguishable (Fig. 1B, C, and E).

On the other hand, we observed the typical filamentous morphology in the GroE-depleted cells bearing the *parC* plasmid (Fig. 1D). The failure to suppress the filamentous phenotype in the ParC-expressing *E. coli* strain was not due to the lower expression of the ParC protein, since the overproduction of ParC by induction with 100 μM IPTG for 30 min before the GroE depletion also did not complement the filamentous morphology (data not shown).

Next we examined the fate of the FtsE protein by Western blotting. The soluble fraction of the endogenous FtsE was constant in the presence of arabinose (Fig. 2A). When the cells were shifted to glucose medium, the amounts of total and soluble FtsE drastically decreased with the GroE depletion, and the FtsE eventually disappeared (Fig. 2B). The absence of

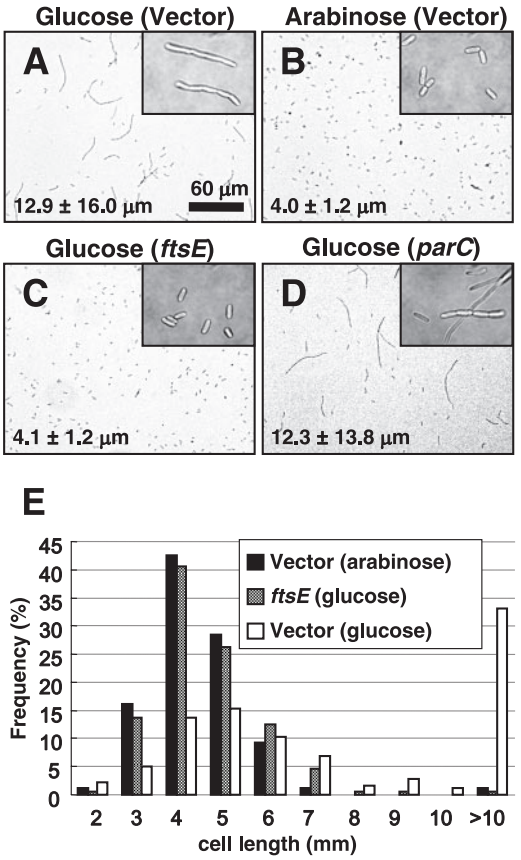

FIG. 1. FtsE overexpression before GroE depletion suppresses filamentous growth. (A to D) Cell morphology of GroE conditional mutants (MGM100) with various expression plasmids observed 5 h after a shift to LB medium containing 0.2% arabinose or 0.2% glucose. The insets show 10-fold magnifications. (A and B) MGM100/pMCS (control); (C) MGM100/pWARA5(*ftsE*); (D) MGM100/pWARA6 (*parC*); (A, C, and D) cells grown in LB medium with glucose; (B) cells grown in LB medium with arabinose. The average lengths ± standard deviations of the cells are indicated. The unusually large standard deviations in panels A and D are due to the presence of both filamentous and normal-length cells. (E) Histograms of the lengths of the cells shown in panels A (pMCS, growth in glucose), B (pMCS, growth in arabinose), and C [pWARA5(*ftsE*), growth in glucose].

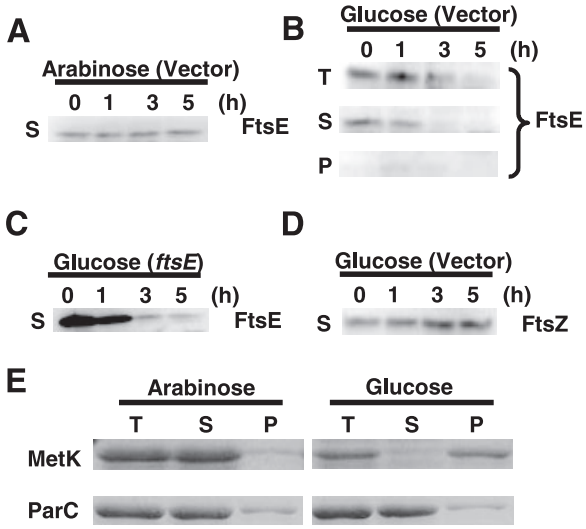

FIG. 2. Decrease in FtsE during GroE depletion. The times after the shift to fresh LB medium containing 1 mM DAP with either glucose or arabinose are indicated above the lanes. Equal numbers of cells were used in all lanes. (A to C) Levels of endogenous (A and B) or preoverexpressed (C) FtsE in the presence of arabinose (A) or glucose (B and C). Total (T), soluble (S), and pellet (P) fractions of MGM100/pMCS (A and B) and MGM100/pWARA5(*ftsE*) (C) lysates were analyzed by immunoblotting using anti-FtsE antiserum. (D) Level of endogenous FtsZ in glucose medium. Soluble fractions of MGM100/pMCS lysates were immunoblotted using an anti-FtsZ antibody. (E) ParC and MetK overexpression in GroE-depleted cells (MGM100). Cells harboring either pWARA6(*parC*) or pWARA1(*metK*) were grown in arabinose medium and were shifted to arabinose- or glucose-containing medium. After 2 h of cultivation, each protein was induced by IPTG for 1 h. Equivalent amounts of cells were withdrawn, lysed, and then centrifuged for preparation of total (T), soluble supernatant (S), and insoluble pellet (P) fractions. These fractions were analyzed by SDS-PAGE, and the gel was stained with Coomassie brilliant blue. Only the bands of overexpressed MetK and ParC are shown.

FtsE from the insoluble fraction during the reduction in FtsE (Fig. 2B) suggests that the newly translated FtsE was rapidly degraded under the conditions in which GroE was depleted, as suggested previously in the case of DapA (18, 20). In FtsE-overexpressing cells grown in glucose medium, in which the filamentous phenotype was suppressed, the FtsE levels also decreased. However, significant amounts of soluble FtsE (Fig. 2C) remained even after 5 h. Quantification of three independent experiments revealed that the FtsE levels of FtsE-overexpressing cells in glucose medium after 5 h were 103% ± 22% of the endogenous levels of FtsE in arabinose medium (Fig. 2A). This retention of FtsE in the GroE-depleted cells led to the suppression of filamentation in the cells. The level of FtsZ, the other candidate protein, was almost constant under the GroE-depleted conditions (Fig. 2D), showing that the solubility of FtsZ was not affected by the GroE depletion. In addition, almost all of the ParC was soluble under the GroE-depleted conditions, even when it was overexpressed (Fig. 2E). These results suggest that the filamentous morphology is due to the loss of FtsE function in GroE-depleted cells, although we cannot rule out the possibility that unknown pleiotropic effects in the GroE-depleted cells might cause the filamentation.

Another phenotype associated with FtsE dysfunction in GroE-depleted cells. It has been reported that *E. coli* with a FtsE dysfunction requires at least 0.5% NaCl for viability (5, 22, 24). Even the FtsE null mutant is viable in the presence of salts or osmolytes, although global knockout analyses of *E. coli* have shown that FtsE is essential (9). To further confirm the loss of FtsE function in the GroE-depleted cells, we tested whether the GroE depletion exhibits the salt requirement. Cells in LB medium containing arabinose grew normally with or without 1% NaCl (Fig. 3A). Upon a shift to LB medium containing glucose, the lack of NaCl almost arrested the cell growth after ~3 h, whereas 1% NaCl maintained the growth even after 6 h, albeit at reduced rates compared to those in the presence of arabinose. The growth arrest in the glucose medium lacking NaCl was substantially restored by prior overexpression of FtsE, indicating that the growth arrest is caused by impairment of FtsE in the GroE-depleted cells. In the salt-free glucose medium, about 80% of the GroE-depleted cells showed filamentous morphology. The filaments were smooth,

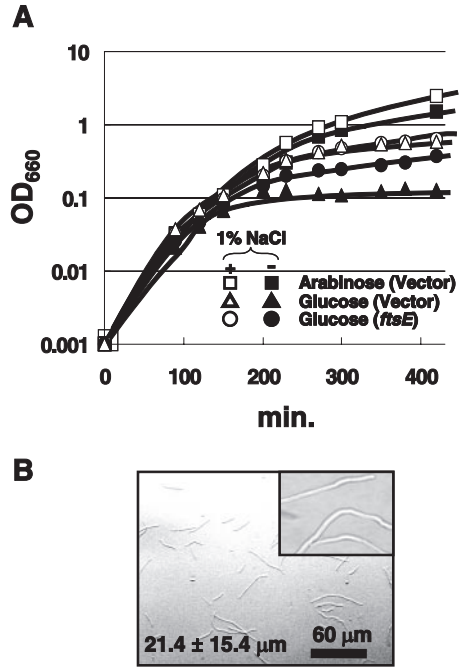

FIG. 3. (A) Growth defect of GroE-depleted cells in NaCl-free medium. Cells were grown in LB medium without NaCl (filled symbols) or in LB medium containing 1% NaCl (open symbols). Growth was measured periodically by determining the OD_{660}. Squares, MGM100/pMCS in arabinose-supplemented medium; triangles, MGM100/pMCS in glucose-supplemented medium; circles, MGM/pWARA5(*ftsE*) in glucose-supplemented medium. (B) Smooth filamentous morphology of GroE-depleted cells in NaCl-free medium. MGM100 cells harboring pMCS were observed 5 h after a shift to LB medium containing 0.2% glucose (the conditions indicated by filled triangles in panel A). (Inset) Tenfold magnification.

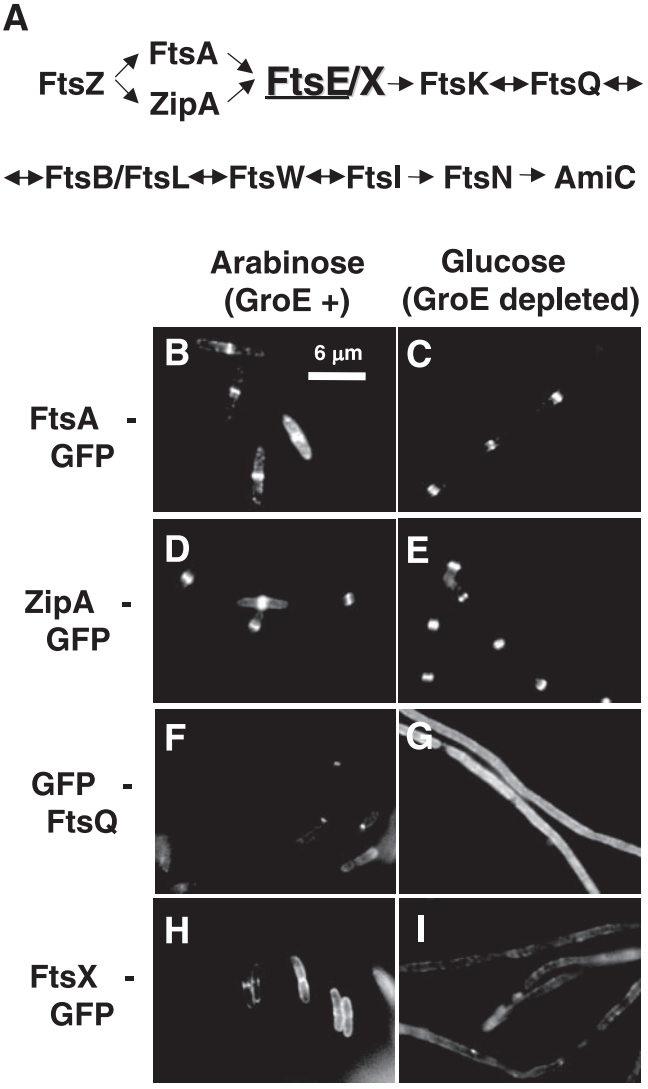

FIG. 4. Localization of cell division proteins fused with GFP. (A) Model for the assembly of proteins into the septal ring of *E. coli* (10, 29). (B to I) GroE conditional mutant cells (MGM100) expressing various GFP fusions were grown in LB medium containing arabinose (B, D, F, and H) or glucose (C, E, G, and I). The cells expressed FtsA-GFP (B and C), ZipA-GFP (D and E), GFP-FtsQ (F and G), or FtsX-GFP (H and I). Typical fluorescence micrographs are shown.

in contrast to the constriction-containing filaments (so-called chain morphology) in medium containing 1% NaCl (compare Fig. 1A and D insets and Fig. 3B inset). It has been shown previously that an FtsE-deficient mutant in the salt-free medium also shows the smooth filamentous morphology (24), further supporting our conclusion that FtsE is dysfunctional in the GroE-depleted cells.

In addition, we noted that the growth rates in the glucose medium were reduced, even in the presence of 1% NaCl (Fig. 3A). This finding, combined with the finding that further addition of 1 mM DAP to the glucose medium in the presence of 1% NaCl did not allow colony formation (data not shown), implies that the GroE depletion inhibits cell growth by affecting the folding of other substrates besides FtsE.

FtsA, but not FtsX and FtsQ, becomes localized to the septal site in GroE-depleted cells. In *E. coli*, the various cell division proteins become localized to the septal ring in a defined order (Fig. 4A) (for a review, see reference 29). The process starts with the polymerization of FtsZ at the inner face of the cytoplasmic membrane (Z ring). The Z ring is stabilized by two other essential division proteins, FtsA and ZipA. Once it is established, FtsE/X, FtsK, FtsQ, and other division proteins are recruited in a more or less linear fashion to the septal ring (Fig. 4A) (29), although some deviation from this strict linearity, such as the concerted interactions among FtsQ, FtsL, and FtsB, has recently been reported (1, 10). In any case, the localization of a cell division protein to the septal ring is dependent on the local presence of the preceding protein. Therefore, provided that FtsE is the only substrate of GroE in cell division, we expected that the impairment of FtsE in the GroE-depleted cells should affect the localization of the protein following FtsE (e.g., FtsQ) or the interacting partner of FtsE (FtsX) but not that of the preceding protein (e.g., FtsA).

To investigate whether the GroE depletion affects the ordered localization of the division proteins to the septal ring, we visualized the proteins by fusing them with GFP and expressing them in the MGM100 strain. After the GFP fusions were expressed in the arabinose medium, the cells were washed and diluted into media supplemented with arabinose or glucose.

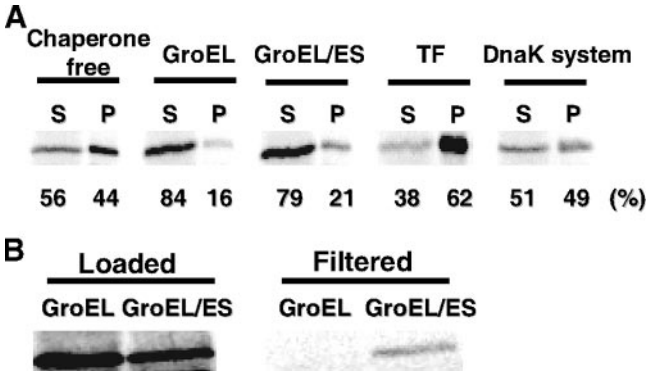

FIG. 5. Folding of newly translated FtsE is stringently GroE dependent. (A) Effects of chaperones on the solubility of nascent FtsE translated by the reconstituted cell-free translation system (PURE system). After centrifugation of the translation mixtures, the soluble (S) and insoluble (P) fractions were subjected to SDS-PAGE, followed by autoradiography. Only the region of the FtsE band is shown. The DnaK system was a mixture of DnaK, DnaJ, and GrpE. The chaperone concentrations were as follows: 1 μM GroEL, 1 μM GroES, 4 μM DnaK, 2 μM DnaJ, 2 μM GrpE, and 2.5 μM trigger factor (TF). (B) Ultrafiltration assay. Translation mixtures containing GroEL or GroEL plus GroES were filtered using a 100-kDa-cutoff membrane. The total mixtures (Loaded) and filtrates (Filtered) were analyzed as described above for panel A.

Under the growth conditions with arabinose, in which GroE was expressed, all of the GFP fusions tested (FtsA, ZipA, FtsX, and FtsQ) were localized at the division sites (Fig. 4B, D, F, and H). Under the growth conditions with glucose, in which the cells exhibited the filamentous morphology (Fig. 4C, E, G, and I), FtsA-GFP and ZipA-GFP were properly localized (Fig. 4C and E) in the filamentous cells, and the lengths between the two locations were almost identical to those in the FtsE-depleted cells reported previously (24) (data not shown), indicating that the localizations of FtsA and ZipA were not affected by the GroE depletion. In contrast, the localizations of FtsX-GFP and GFP-FtsQ were greatly reduced by growth in glucose medium, and the proteins were dispersed (Fig. 4G and I). Collectively, these data indicate that impairment of FtsE affects the localizations of FtsX, the interaction partner of FtsE, and FtsQ, a protein localizing after FtsE in the septal ring assembly, but not the localizations of FtsA and ZipA, the proteins preceding FtsE. These results imply that FtsE is the only substrate of GroE involved in the assembly of the septal ring.

Folding of newly translated FtsE is stringently dependent on the GroE system, as revealed by a reconstituted cell-free translation system. To test whether the folding of newly translated FtsE is strictly dependent on GroE, as Kerner et al. have proposed (18), the solubility of the translated FtsE was examined using a reconstituted cell-free translation system (PURE system) (26). Since the PURE system consists of only proteins essential for translation and does not contain any chaperones, the effect of added chaperones can be clearly evaluated (30, 31). Under the in vitro translation conditions, approximately one-half of the FtsE protein translated in the PURE system was soluble in the absence of chaperones (Fig. 5A). The addition of trigger factor or the DnaK system (DnaK, DnaJ, and GrpE), both of which are general chaperones in *E. coli*, failed to increase the soluble fraction, and the insolubility became even worse in the case of trigger factor (Fig. 5A). In contrast, addition of GroEL or GroEL plus GroES (GroEL/ES) considerably increased the solubility of FtsE to ~80% (Fig. 5A), indicating that the GroE system assists in the folding of newly translated FtsE.

Since GroEL alone (i.e., without GroES) improved the solubility of the synthesized FtsE, we questioned whether the folding of FtsE might not stringently depend on the complete GroE system (i.e., GroEL, GroES, and ATP) under the in vitro conditions. Since some substrates, such as ribulose-1,5-bisphosphate carboxylase/oxygenase, are known to remain bound to GroEL in the absence of GroES even in the presence of ATP (11), we filtered the reaction mixtures using a 100-kDa-cutoff membrane to separate the monomers or putative dimers of FtsE (24 and 47 kDa, respectively [5]) from the GroEL or the GroEL/ES complex (>800 kDa). The translated FtsE passed through the filter only when both GroEL and GroES were present (Fig. 5B), demonstrating the requirement of GroES for release of the soluble FtsE. We concluded that the folding of newly translated FtsE is stringently dependent on the complete GroE system.

DISCUSSION

In this work, we showed that impaired folding of FtsE, a 24-kDa protein involved in cell division, induced the filamentous morphology in GroE-depleted *E. coli*. Prior overexpression of FtsE suppressed the filamentous morphology, indicating that the supply of functional FtsE in the GroE-depleted cells was sufficient for the complementation. In addition to in vivo analyses, an in vitro analysis using a reconstituted cell-free translation system clearly revealed that the folding of newly synthesized FtsE is stringently GroE dependent. Thus, we concluded that GroE is essential for cell division because it assists in the folding of FtsE. The impaired folding of FtsE proteins in GroE-deficient cells might be conserved in eubacteria, since filamentous phenotypes in GroE-deficient cells have also been reported in *Caulobactor* and *Streptococcus* (19, 27), both of which encode an FtsE homolog in their genomes.

Our detailed analyses of FtsE confirmed the previous assignment of FtsE as one of the obligate substrates of GroE (class III substrates), based on a proteomic analysis of the GroE interactants (18). The gradual disappearance of FtsE during GroE depletion (Fig. 2) is similar to the disappearance of DapA and GatY reported by Kerner et al. (18), suggesting that the impaired folding of FtsE in the GroE-depleted cells results in degradation of the protein.

In addition to FtsE, however, other candidates involved in the cell division process (ParC, FtsZ, FtsA, and FtsI) have been assigned as GroE substrates (3, 18). Our conclusion that the impaired folding of FtsE in the GroE-depleted cells induces the filamentous morphology does not necessarily mean that other candidates are not GroE substrates in normal cells. There is a possibility that other chaperones, such as trigger factor or DnaK, might assist in the folding of GroE substrates in the GroE-depleted cells. This is feasible, because enhanced expression of heat shock proteins, including DnaK, in cells with reduced levels of GroE has been reported (15).

Whatever the case, the following observations strongly sug-

gest that the folding of FtsZ and ParC is not dependent on GroE. First, FtsZ remained almost completely soluble in the GroE-depleted cells (Fig. 2D), even under the conditions where FtsZ was overexpressed (data not shown). In addition, the FtsZ ring has been observed even after GroE depletion in *Caulobacter* (27). Although it has been reported that GroEL colocalizes to FtsZ rings (21), we suggest that the folding of FtsZ is independent of the GroE system, but a posttranslational interaction of FtsZ with GroEL might play a role in the function of FtsZ. Second, almost all of the ParC was soluble under the GroE-depleted conditions even when ParC was overexpressed (Fig. 2E).

A previous analysis of GroE interactants predicted that ParC (84-kDa subunit) is one of the class III substrates of GroEL (18), for which folding is supposed to be stringently GroE dependent. However, our observations (Fig. 2E) did not support this finding, suggesting that the predicted class III substrates are not always obligate substrates of GroE. This is possible because the class III substrates have been primarily defined as a subset of the GroEL substrates that are enriched among GroEL interactants (18) and not by an obligate requirement for GroE upon folding. However, why is ParC enriched in the GroE-substrate complex? One possibility is preferential posttranslational complex formation between ParC and GroEL. In such a situation, GroE might play a maintenance role for ParC after it folds. Alternatively, since the molecular mass of ParC is 84 kDa, which is larger than the limit size of the GroEL-GroES cavity (about 60 kDa [6, 18, 23, 25]), ParC cannot be accommodated within the cavity, implying that encapsulation of the substrate into the GroE cavity is necessary for the obligate GroE requirement for folding.

Although hundreds of GroEL interactants in the cell have been identified, how GroE assists in the folding of these proteins in vivo remains to be elucidated. Of particular interest is the folding property of the class III substrates in vivo. One strategy for clarifying the essential role of GroE in vivo is overexpression of candidate substrates before GroE depletion, a method originally developed by McLennan and Masters in the case of DapA (20). We further showed the advantage of this strategy for examining the filamentous phenotype in GroE-depleted cells. Therefore, we can apply this strategy if the cells exhibit some sort of detectable phenotype with GroE depletion or mutation. In this context, we noticed that the growth rates of the GroE-depleted cells were reduced even in the presence of 1% NaCl (Fig. 3A), which suppresses the impairment of FtsE. The reduction in the growth rate implies the requirement of GroE by affecting the folding of other substrates besides FtsE. The use of this growth defect as a detectable phenotype to search for the targets of GroE might provide novel insights into the in vivo role of GroE in the folding of essential substrates.

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