Conditional Proteolysis of the Membrane Protein YfgM by the FtsH Protease Depends on a Novel N-terminal Degron

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Background: YfgM is a membrane protein under regulatory control by FtsH.

Results: Growth phase-dependent degradation of YfgM in Escherichia coli requires a novel N-terminal degron. YfgM interacts with the response regulator RcsB and acts as a negative regulator.

Conclusion: The YfgM degron mediates degradation under stress conditions when the Rcs pathway is needed.

Significance: The study reveals a new degron and provides insights into the function of YfgM.

Regulated proteolysis efficiently and rapidly adapts the bacterial proteome to changing environmental conditions. Many protease substrates contain recognition motifs, so-called degrons, that direct them to the appropriate protease. Here we describe an entirely new degron identified in the cytoplasmic N-terminal end of the membrane-anchored protein YfgM of Escherichia coli. YfgM is stable during exponential growth and degraded in stationary phase by the essential FtsH protease. The alarmone (p)ppGpp, but not the previously described YfgM interactors RcsB and PpiD, influence YfgM degradation. By scanning mutagenesis, we define individual amino acids responsible for turnover of YfgM and find that the degron does not at all comply with the known N-end rule pathway. The YfgM degron is a distinct module that facilitates FtsH-mediated degradation when fused to the N terminus of another monotopic membrane protein but not to that of a cytoplasmic protein. Several lines of evidence suggest that stress-induced degradation of YfgM relies on the response regulator RcsB and thereby permits cellular protection by the Rcs phosphorelay system. On the basis of these and other results in the literature, we propose a model for how the membrane-spanning YfgM protein serves as connector between the stress responses in the periplasm and cytoplasm.

Permanently changing environmental conditions require regulatory processes to adjust the cellular protein pool. Besides transcriptional and translational regulation, proteolysis is a fast and efficient mechanism to adapt the proteome at the post-translational level. In Escherichia coli, five ATPases associated with various cellular activities (AAA⁺ proteases), ClpXP, ClpAP, HslUV, Lon, and FtsH, are responsible for regulated proteolysis (1, 2). Among them, FtsH is the only membrane-anchored and essential protease. It is responsible for an optimal ratio between phospholipids and LPSs in the outer membrane by controlling the level of the LPS biosynthesis enzyme LpxC (3). Given that degradation of intact proteins is irreversible and wasteful, it must be strictly regulated. Often, dedicated sequence motifs, called degrons, direct a substrate to a certain protease (4). These degrons can either be located at the termini of a protein, at internal sides, or can form structured elements for recognition (4, 5). Degron accessibility regulates the stability of protease substrates (reviewed in Refs. 2, 6, 7).

Although FtsH is the most important protease in E. coli, the principles of substrate recognition are poorly understood (8). A common degron does not exist, and it seems that most FtsH substrates follow their unique recognition and degradation pathway (9–18). For instance, proteolysis of LpxC depends on a C-terminal tail (9, 10). Its sequence is very similar to the common ssrA tag recognized by nearly all AAA⁺ proteases in E. coli (19–21). However, the C-terminal degron alone is not sufficient for FtsH-specific degradation of LpxC, and internal sequences or adaptor proteins needed for proteolysis remain to be discovered (10). Degradation of the heat shock sigma factor RpoH (σ32) involves the chaperones DnaK, DnaJ, and GroEL (11, 13, 18, 22). The RpoH degron is a structured motif located in an exposed α helix (15, 23). Because of its localization in the inner membrane, FtsH is predestined to degrade membrane proteins such as YccA, SecY, PspC, and F0a. They are degraded by FtsH in the context of quality control, responsible for eliminating unfolded proteins or those not assembled with their particular interaction partners (24–27). Their recognition by FtsH depends on exposed N or C termini of about 20 residues but does not require a certain sequence context (28, 29).

In a substrate trapping approach, we recently identified the membrane protein YfgM as an FtsH substrate (30). YfgM consists of a short cytoplasmic N-terminal region (19 amino acids), a single transmembrane domain, and a longer C terminus (approximately 160 amino acids) located in the periplasm (31). The cellular function of YfgM is still under investigation. On one hand, it forms a complex with the membrane protein PpiD, a chaperone for outer membrane proteins (31). Both proteins together interact with the SecYEG translocon (32, 33), and it has been suggested that YfgM is part of the periplasmic chap-
erone network. It may mediate the transport of proteins released from the SecYEG translocon to periplasmic chaperones like SurA or Skp (32, 34). On the other hand, YfgM interacts with the cytoplasmic response regulator RcsB of the Rcs phosphorelay system (35). This signal transduction system is important for adaptation to acidic stress (36–38), osmotic stress (39), and stationary phase conditions (40–42). It is also involved in cell division (43). YfgM is rapidly degraded in stationary phase and after osmotic shock and has been postulated to act as a negative regulator of RcsB under optimal growth conditions (30).

In this study, we systematically define the YfgM degtron, which is located in the N-terminal region. It is modular and confers FtsH-mediated degradation to a topologically related membrane protein. Moreover, evidence for a role of YfgM as negative regulator of the Rcs stress response pathway is provided.

**Experimental Procedures**

**Bacterial Strains and Growth Conditions**—The following E. coli strains were used in this study: W3110 (44), ΔrelA/ΔspoT (45), Δppkx (46), ΔrelA251 (45), SpoT_E319Q (R. Harinarayan-an and M. Cashel) ΔproS359 (47), BW25113 (48), ΔppiD (48), ΔrcsB (48), ΔyfgM (48), and ΔftsH (44). Cells were grown in liquid Luria Broth (LB) medium in a water bath shaker (180 rpm) or on LB agar plates at 37 °C (or 30 °C for ΔftsH). When needed, antibiotics were used as follows: ampicillin (Amp, 100 µg/ml), chloramphenicol (25 µg/ml), kanamycin (Kan, 50 µg/ml), and tetracycline (12.5 µg/ml).

**Construction of Plasmids**—A list of all plasmids and oligonucleotides used in this study is available upon request. For N- and C-terminal truncations of yfgM, E. coli K12 genomic DNA was used as template for PCR. The coding regions of C- and N-terminal truncated YfgM variants were inserted into the backbone of pBO2561 (using Bsp1407I/HindIII sites) or pBO2593 (using Nhel/Xhol sites), respectively. Point-mutated N-terminal YfgM variants were generated by QuikChange PCR using pBO2871 as a template. Plasmids coding for N-terminal fusions of the YfgM N terminus to GST were constructed by using pGEX6P-2 DNA as a template for PCR. Coding regions for the first 19 or 23 amino acids of the YfgM N terminus (or the YfgM_E2A N terminus) were fused to GST via a PstI site. Fusion constructs were inserted into the backbone of pBO2871 (using Nhel/Xhol sites). Plasmids coding for PpiD containing the YfgM N terminus (or the YfgM_E2A N terminus) instead of the PpiD N terminus were constructed by using E. coli K12 genomic DNA as a template for PCR. Coding regions for the first 19 or 23 amino acids of YfgM N terminus (or the YfgM_E2A N terminus) were fused to ppiID, which lacked the coding region for the first 12 N-terminal amino acids, via a BglII side. Fusion constructs were inserted into the backbone of pBO2871 (using Nhel/Xhol sites). Cloning results were confirmed by sequencing. All used expression plasmids encode for proteins with a terminal His₆ or His₉ tag under the control of an anhydrotetracycline (AHT)-inducible promoter.

**Separation of Cytosolic and Membrane-inserted Proteins**—To analyze the membrane insertion abilities of YfgM variants, *E. coli* cells containing plasmids encoding for those variants were grown in LB + Amp. Protein expression was induced using 50–200 ng/ml AHT for 30 min, and cells were harvested by centrifugation (10 min, 16,000 × g, 4 °C). The pellet was suspended in 5 ml of lysis buffer A (0.2 mM DTT, 1 mM PMSF, and 0.2 mg/ml DNase I) and disrupted by French press. After sedimentation of cell debris, the membrane and cytosolic fractions were separated by ultracentrifugation (60 min, 200,000 × g, 4 °C). The supernatant, representing the cytosolic fraction, was removed, and the pellet was suspended in 5 ml of lysis buffer A and ultracentrifuged (60 min, 200,000 × g, 4 °C). The pellet, representing the membrane fraction, was suspended in 5 ml of lysis buffer B (0.2 mM DTT, 1 mM PMSF, 0.2 mg/ml DNase I, and 2% (w/v) CHAPS). After centrifugation (2 min, 16,000 × g, 4 °C), membrane proteins were present in the supernatant. Protein samples of the cytosolic and membrane fractions were subjected to SDS-PAGE and immunodetection. The cytosolic chaperone GrpE and the membrane-anchored protease FtsH served as controls for each fraction.

**In Vivo Degradation Experiments in E. coli**—To analyze the stability of different YfgM variants, cells containing plasmids encoding corresponding YfgM proteins were grown in LB + Amp at 37 °C to different growth phases. Protein expression was induced by adding 50–200 ng/ml AHT for 30 min. Translation was blocked by addition of 300 µg/ml spectinomycin (Sp). Samples were taken at different time points, frozen into liquid nitrogen, and subjected to SDS-PAGE and immunodetection.

**Preparation of Protein Extracts and Immunodetection**—Depending on their optical density, cell pellets were resuspended in TE buffer (10 mM Tris/HCl (pH 8), 1 mM EDTA, and 50 µl TE buffer per A₅₈₀ nm of 1.0) and mixed with protein sample buffer (final concentrations of 2% SDS (w/v), 0.1% (w/v) bromophenol blue, 10% glycerol (v/v), 1% (v/v) β-mercaptoethanol, and 50 mM Tris/HCl (pH 6.8)). After incubation for 5 min at 95 °C, samples were centrifuged (1 min, 16,000 × g), and protein samples were subjected to SDS-PAGE and Western transfer using standard protocols (49). His-tagged fusion proteins were detected using a His₆-HRP conjugate (Qiagen). Protein signals were visualized using Luminata Forte Western HRP substrate (Millipore) and Chemilumager Ready (Alpha Innotech). Half-lives of proteins were calculated with AlphaEaseFC software (version 4.0.0, Alpha Innotech).

**Pulldown Experiments**—To validate the interaction between YfgM and RcsB, we performed copurification experiments with C-terminally Strep-tagged YfgM (or E2A or Y4A variants) and C-terminally His-tagged RcsB. After overexpression of YfgM and RcsB, cells were harvested by centrifugation (10 min, 16,000 × g, 4 °C), and cell pellets were suspended in lysis buffer (140 mM NaCl, 2.7 mM KCl, 1.8 mM KH₂HPO₄, 10 mM Na₂HPO₄ (pH 7.3), 1 mM PMSF, and 0.2 mg ml⁻¹ DNase) and disrupted by French press. Unbroken cells were removed by centrifugation (60 min, 16,000 × g, 4 °C), and cell lysates containing cytosolic and membrane proteins were used for the pulldown experiment. Cell lysates were mixed and incubated overnight on ice. Strep-tagged and His-tagged proteins were purified using streptactin-Sepharose (IBA Co.) or Ni-NTA agarose (Qiagen), respectively. His-tag-based purification was performed by washing the column using increasing amounts of imidazole buffer with 50 mM imidazole at maximal concentra-
tion. Elution occurred in three steps using 100, 250, and 1000 mM imidazole. Strep-tagged-based purification was performed using the standard protocol of the purification kit from IBA Co. Elution fractions were analyzed by Western blot analysis using Strep tag-specific (IBA Co.) or His tag-specific (Qiagen) antibodies and by Coomasie-stained gels.

Survival after Acidic Stress—To analyze the survival rate after an acidic shock, E. coli W3110 cells carrying plasmids encoding YfgM variants or the empty vector were grown in LB + Amp to exponential growth phase. Protein expression was induced by adding 50–200 ng/ml AHT for 30 min. Expression of YfgM variants was verified by SDS-PAGE and immunodetection. E. coli strains from the Keio collection (Keio-WT: BW25113, ΔyfgM) were grown in LB (+ Kan when needed) to exponential growth phase. Cells were harvested by centrifugation (10 min, 4,000 × g, room temperature) and resuspended in LB (pH 5.7) and incubated at 37 °C for 70 min. Then cells were harvested (10 min, 4,000 × g, room temperature), resuspended in LB (pH 2.4), and incubated at 37 °C for 2 h. After acidic shock, cells were harvested (10 min, 4,000 × g, room temperature) and resuspended in PBS buffer, and 1.5 μl of serial dilutions (10^6–10^7) were spotted on LB (+ Amp or + Kan when needed) agar plates and incubated overnight at 37 °C. Colonies were counted, and the survival rate was calculated. The survival rate of cells carrying the empty vector preincubated with LB (pH 5.7, 70 min) and stressed with LB (pH 2.4, 2 h) was set to 100%.

Microscopy—E. coli W3110 cells carrying plasmids encoding YfgM variants or the empty vector were grown overnight in LB + Amp + 100–200 ng/ml AHT. Expression of YfgM variants was verified by SDS-PAGE and immunodetection. E. coli strains from the Keio collection (Keio-WT: BW25113, ΔyfgM) were grown overnight in LB (+ Kan when needed). Cultures were diluted 1:1 with LB and subjected to light microscopy (BX41 microscope, Olympus).

Preparation of Total RNA and Northern Blot Analysis—E. coli W3110 cells carrying plasmids encoding YfgM or the empty vector were grown in LB + Amp to exponential growth phase. Protein expression was induced by adding 100 ng/ml AHT for 30 min. 1 ml of culture was harvested by centrifugation (10 min, 16,000 × g, 4 °C). Expression of YfgM was verified by SDS-PAGE and immunodetection. Total RNA of cell pellets was isolated using the hot acid phenol method (50). Northern blot analyses were performed as described previously (51). 8 μg of total RNA was separated on a 1.5% agarose gel, blotted on nylon membranes (Bio-Rad), and hybridized with a specific digoxigenin-labeled (Roche Applied Sciences) RNA probe. Signals were detected by the Hyperfilm ECL system (GE Healthcare).

Results

Growth Phase-dependent Degradation of YfgM Is Influenced by (p)pGpp but Not by Inorganic Polyphosphate (polyP) or RpoS—The membrane protein YfgM is degraded by FtsH in a growth phase-dependent manner, as shown with an N-terminally His-tagged YfgM variant (30). Because certain FtsH substrates are recognized by terminal degrons (9, 10, 52), tags sometimes interfere with proteolysis. To exclude this for YfgM, a C-terminally His-tagged YfgM variant was constructed and analyzed for FtsH-dependent proteolysis in different growth phases (Fig. 1A). It was stable in early growth phases and rapidly degraded by FtsH when cells reached stationary phase (Fig. 1B), showing that the position of a tag did not interfere with the recognition or degradation of YfgM.

Because YfgM degradation is most rapid in stationary phase, we examined the influence of three factors known to contribute to adaptation under this condition: the alarmone (p)pGpp (53–55); inorganic polyphosphate (polyP) (56–58); and the alternative factor of the stationary phase, RpoS (σ^52) (59, 60). YfgM proteolysis was normal in strains lacking RpoS (ΔrpoS) or polyP (ΔpolyP) (data not shown). Also, in strains containing reduced amounts of (p)pGpp caused by a defect in either RelA or SpoT, YfgM degradation was not affected (data not shown). By contrast, accelerated degradation of YfgM already in early growth phases was observed in strain (p)pGpp^Δ completely devoid of the alarmone (45) (Fig. 1C). YfgM Degradation Does Not Depend on the Presence of its Interaction Partners RcsB and PpiD—The faithful delivery of a substrate to a protease often requires the assistance of accessory proteins (2, 6, 7). Therefore, we tested whether the previously reported YfgM interactors PpiD (31–34) and RcsB (35) influence proteolysis. Because the degradation profiles were not altered in the ΔpPpiD (Fig. 2B) or ΔrcsB (Fig. 2C) mutants in

![Figure 1](image-url)

**FIGURE 1.** YfgM degradation is influenced by (p)pGpp. A–C, the stability of plasmid-encoded YfgM_His<sub>6</sub> was analyzed in different growth phases of a typical E. coli growth curve (A) in E. coli W3110 (WT) (B) and ΔppGpp<sup>C</sup> (ΔppGpp<sup>C</sup>, the stabilization phase). After induction of YfgM synthesis for 30 min, translation was blocked by addition of spectinomycin. Samples were taken at different time points, and protein half-lives were determined after SDS-PAGE, Western transfer, and immunodetection. Standard deviations were calculated from at least three independent experiments.

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**Proteolysis of YfgM**

| A | Degradation of YfgM, His6, time [min] after addition of Sp | T<sub>1/2</sub> [min] | growth phase |
|---|---|---|---|
| Keio_Wt | 0 10 20 30 45 60 90 120 | 95 ± 33 | exp |
| | | 63 ± 5 | late exp |
| | | 36 ± 15 | early stat |
| | | 12 ± 3 | stat |

| B | YfgM Degradation Does Not Require the C-terminal End—Condition-dependent degradation of several protease substrates is mediated by terminal degrons (2, 6). YfgM crosses the inner membrane by a single transmembrane domain (Fig. 3A). The first 19 N-terminal amino acids face the cytoplasm, and most of the protein is located in the periplasm (31). Terminally truncated YfgM variants were generated to investigate which part of YfgM is important for degradation by FtsH in E. coli. YfgM variants lacking the C-terminal 35 or 53 residues (YfgM<sub>AC35</sub> and YfgM<sub>AC53</sub>), respectively inserted properly into the membrane (Fig. 3B). They were degraded with the same profile as the wild-type protein (Fig. 3, C and D), indicating that the last 53 periplasmic amino acids of YfgM are not required for growth phase-dependent degradation. The YfgM Degron Is Located in Its Very N Terminus—Next we paid attention to the N terminus of YfgM. Strikingly, many residues in this region are highly conserved, suggesting a common functional or regulatory relevance (Fig. 4). Deletion of the first five residues downstream of the initiator methionine (YfgM<sub>AN2–6</sub>) led to a protein that was able to insert into the inner membrane (data not shown) but was no longer degraded in stationary growth phase (Fig. 5). To refine the analysis, we deleted the second and third residue either in combination (YfgM<sub>AN2–3</sub>) or individually (YfgM<sub>AN2</sub> and YfgM<sub>AN3</sub>). All three proteins were able to properly insert into the inner membrane (shown for YfgM<sub>AN2–3</sub> and YfgM<sub>AN2</sub> in Fig. 5B) and were stable throughout the growth curve (shown for YfgM<sub>AN2–3</sub> and YfgM<sub>AN2</sub> in Fig. 3, E and F, respectively; summarized for all three proteins in stationary phase in Fig. 5A). The results strongly suggest that the YfgM degron is located in its cytoplasmic N terminus and includes the residues Glu-2 and Ile-3.

**Contribution of Individual Residues to the YfgM Degron**—YfgM is conserved in all β and γ proteobacteria, and the aligned N termini of YfgM from various γ proteobacteria show a high sequence similarity (Fig. 4). The glutamate at position 2, which is critical for proteolysis, is strictly conserved, and position 3 is either an isoleucine or valine. According to the N-end rule, typical destabilizing residues in the N terminus are charged positively (61) and not negatively like the glutamate at position 2 in YfgM. To analyze whether the amino acid property is critical for proteolysis, we exchanged Glu-2 with various other residues. YfgM<sub>E2A</sub> (Fig. 6), YfgM<sub>E2K</sub>, and YfgM<sub>E2Q</sub> were stable under all growth conditions (Fig. 5A). Only when the acidic character was retained (YfgM<sub>E2D</sub>) was the protein degraded, although more slowly than the wild-type protein (half-life of 70 min versus 10 min, respectively, in stationary phase; Fig. 5A). Substitution of the isoleucine at the third position against alanine (YfgM<sub>I3A</sub>) resulted in a protein equally as stable as the YfgM<sub>ΔI3</sub> variant (Fig. 6B).

To investigate the contribution of the remaining amino acids in the N-terminal tail of YfgM, we systematically replaced each residue up to position 18 with an alanine (or aspartate when the wild-type residue is an Ala or Val). The half-lives of all these proteins in stationary phase are summarized in Fig. 5A. Degradation profiles of selected candidates in different growth phases are shown in Fig. 6. In contrast to the stabilizing effect of point mutations in positions 2 and 3, the exchange of residues 4 and 5 with alanine resulted in destabilized YfgM proteins that were prone to degradation in early or late exponential phase (YfgM<sub>YA4</sub> and YfgM<sub>EA5</sub>, respectively). Replacement of amino acids between position 6 and 14 resulted in moderately stabilized proteins in all growth phases (Fig. 5A; shown in detail for YfgM<sub>D9A</sub> in Fig. 6, E and Fig. E), indicating that these variants were degraded by FtsH but not as efficiently as the wild-type protein. Substitutions further downstream did not affect the degradation of YfgM (Fig. 5A, shown in detail for YfgM<sub>F17A</sub> in Fig. 6F). To confirm that degradation of the YfgM variants remains FtsH-specific, we performed in vivo degradation experiments in the ftsH mutant. Degradation was due to the FtsH protease because all variants were completely stable in this strain (Fig. 6, C–F). On the basis of this detailed analysis at amino acid resolution, the YfgM degron comprises the first 14 N-terminal residues (Fig. 5B) in which Glu-2 and Ile-3 are most crucial for degradation. Residues Tyr-4 and Glu-5 are important for the stability of YfgM in early growth phases. Residues 6–14 play a minor role in YfgM degradation, and amino acids between positions 15 and 18 close to the membrane are not involved in YfgM turnover.

To address whether shortening of the N-terminal end affects YfgM degradation, we were restricted to the region downstream of Val-14 because all residues upstream of it had an impact on proteolysis. We constructed YfgM variants lacking either the 15th and 16th (YfgM<sub>ΔK15ΔR16</sub>) or 17th and 18th amino acids (YfgM<sub>ΔF17ΔF18</sub>). These variants were still able to insert into the inner membrane (data not shown). In vivo degradation experiments revealed that they were readily degraded in an FtsH-specific manner (Figs. 5A and 6, G and H).

**FIGURE 2. YfgM degradation is not influenced by the lack of its interaction partners RcsB and PpiD.**—A–C, the stability of plasmid-encoded YfgM_His<sub>6</sub>, was analyzed in E. coli BW25113 (Keio_Wt) (A), ΔppiD (B) and ΔrcsB (C) in different growth phases (exp, exponential phase; stat, stationary phase). After induction of YfgM synthesis for 30 min, translation was blocked by addition of spectinomycin. Samples were taken at different time points, and protein half-lives were determined after SDS-PAGE, Western transfer, and immunodetection. Standard deviations were calculated from at least three independent experiments.
Compared with YfgM_WT, degradation was accelerated. Both short variants were already degraded rapidly in exponential and late exponential growth phase and were not detectable in early stationary and stationary phase. On the basis of all of these results, we conclude that the exact amino acid composition of the YfgM degron is the major determinant for appropriate turnover of this FtsH substrate.

The N-terminal YfgM Degron Confers Degradation to a Monotopic Membrane Protein but Not to a Cytosolic Protein—To analyze whether the N-terminal YfgM degron is able to turn normally stable proteins into FtsH substrates, we fused the first 19 or 23 N-terminal residues of YfgM to the N terminus of the cytosolic protein GST from Schistosoma japonicum (Fig. 7A). These GST variants were soluble like the wild-type protein.
Proteolysis of YfgM

The same was true when the YfgM degron was present at the N terminus of GST regardless of whether the wild-type version of YfgM or the E2A variant was used (shown for the 19-residue fusion in Fig. 7B; the results with the 23-residue fusion were identical). Apparently, the N terminus of YfgM cannot confer proteolytic degradation to a cytoplasmic protein.

As a candidate for a membrane protein we chose PpiD, which has the same topology as YfgM (31). The half-life of PpiD in E. coli is longer than 1 h under all growth conditions (Fig. 7B), making it suitable for the fusion approach. The N terminus of PpiD was exchanged against the first 19 or 23 residues of YfgM_WT or YfgM_E2A (Fig. 7A). All YfgM-PpiD fusions localized correctly to the membrane (data not shown). Interestingly, the presence of the YfgM N terminus destabilized PpiD in stationary phase, where it was degraded like YfgM, with a half-life of about 10 min (shown for the fusion with 19 amino acids from YfgM in Fig. 7B). The fusion was stable in a /H9004ftsH strain (Fig. 7C), showing that FtsH is responsible for degradation. The stabilized YfgM terminus carrying the E2A exchange could not confer PpiD unstable (Fig. 7B). In summary, the fusion approach demonstrated that the YfgM degron is sufficient to confer FtsH-dependent proteolysis to a membrane protein with similar topology in a growth phase-dependent manner.

YfgM Overexpression Negatively Impacts the RcsB-mediated Stress Response—A complex between YfgM and the response regulator RcsB of the Rcs phosphorelay system was identified in a global protein-protein interaction screen using two-dimensional blue native/SDS-polyacrylamide gel electrophoresis in combination with LC-MS/MS identification (35). We validated this finding by a pulldown approach using His-tagged RcsB to copurify YfgM variants by Ni-NTA columns (Fig. 8A) or, conversely, streptactin-tagged YfgM variants to copurify RcsB by streptactin columns (Fig. 8B). YfgM_WT, the stabilized E2A variant, and the destabilized Y4A variant were able to interact with the response regulator.

A role of YfgM as inhibitor of RcsB was recently supported by constitutive overexpression of the yfgM gene, which resulted in elongated and acid-sensitive E. coli cells (30). Given a negative effect of YfgM on the Rcs stress response pathway, the deletion of yfgM should have little effect on RcsB targets and RcsB-dependent pathways. This was indeed the case because a /H9004yfgM strain showed normal cell morphology (Fig. 9A) and acid resistance (Fig. 9B). Three lines of evidence suggest that YfgM acts as an RcsB inhibitor. Induced expression of yfgM resulted in a cell division defect (Fig. 9A), severe acid sensitivity (Fig. 9B), and reduced expression of the small RNA RprA (Fig. 10), a direct RcsB target (41).
Cell morphology and acid resistance were also monitored upon expression of several mutated YfgM variants. Production of the stable YfgM_E2A and YfgM_I3A proteins resulted in very acid-sensitive cells (Fig. 9B). Unexpectedly, the cells were normal and rod-shaped (Fig. 9A). Consistent with its rapid degradation under all growth conditions, the presence of YfgM_Y4A did not interfere with cell division and acid resistance. In line with their half-lives (Fig. 6), production of YfgM_E5A (stable during exponential growth), YfgM_D9A (stable under all conditions), and YfgM_F17A (degraded like YfgM_WT) led to filamentous and acid-sensitive cells.

**Discussion**

The Unusual N-terminal Degron of YfgM—Although FtsH is the only essential protease in E. coli, we know little about the substrate recognition logics of this AAA⁺ protease. Each cytoplasmic FtsH substrate degraded for regulatory reasons (RpoH, LpxC, CII) seems to be degraded by its individual pathway (9–18). To the best of our knowledge, YfgM is the first membrane protein degraded by FtsH in a conditional manner. It is stable during rapid growth and degraded in stationary phase and after osmotic shock (30). To the best of our knowledge, stationary phase-specific proteolysis is not very common. Among FtsH substrates, it was only found for the formate dehydrogenase subunit FdoH, whereas IscC and DadA were degraded with the same slow rate throughout the growth curve (30). The Lon substrate CspD, a DNA replication inhibitor, shows a very different degradation profile and is degraded during rapid growth and stable in stationary phase (62). All previously known membrane-bound FtsH substrates are degraded in the quality control pathway when they are unfolded or not assembled with their particular interaction partners (24–27). Exposed and unstructured termini with a length of about 20 amino acids serve as recognition signals, and the sequence composition of these accessible termini is irrelevant for recognition (28, 29). FtsH-mediated quality control of the D1 subunit of photosystem II from Synechocystis sp PCC 6803 is also dependent on an exposed N-terminal tail of D1 (63).

Turnover of YfgM operates by an entirely different mechanism. It requires a dedicated N-terminal tail that reaches into the cytoplasm and in which the exact amino acid composition is crucial for degradation. The only other N-terminal degron of an FtsH substrate has been described for the E. coli transcription activator SoxS (12, 64). However, the sequence has nothing in common with the YfgM degron, probably because SoxS is primarily degraded by the Lon protease, whereas FtsH plays only
an auxiliary role (12). In contrast to FtsH, the ClpP protease utilizes various N-terminal degrons. There are at least three different N-terminal recognition motifs known for ClpXP substrates (65), and substrates of the ClpAP protease are commonly recognized via the so called N-end rule (66–69).

The YfgM degron comprises the first 14 N-terminal amino acids, and the residues between positions 2 and 5 are the most critical ones in this degron (Fig. 5B). Because their mutation led to protease-resistant proteins, the glutamate at position 2 (Glu-2) and the isoleucine at position 3 (Ile-3) seem to play a major role in destabilizing YfgM in stationary phase. Conversely, tyrosine at position 4 (Tyr-4) and glutamate at position 5 (Glu-5) are needed for stabilization of YfgM in early growth phases because mutations at these positions resulted in unstable proteins. The composition of the YfgM degron is unique and does not obey the N-end rule for regulated proteolysis of ClpP substrates with the help of the adaptor protein ClpS (66). Here destabilizing residues are large hydrophobic (Leu, Phe, Trp, and Tyr) or basic residues (Arg and Lys) (61, 68). It remains a mystery how the negatively charged N-terminal end of YfgM, which contains four glutamate residues, enters the pore region of FtsH containing aromatic and acidic residues (70–72). It is important to note, however, that the first interaction of a substrate with the protease does not necessarily occur at the pore region (6). For instance, the contact can take place at auxiliary sites of the protease, as reported for the FtsH substrate CII of bacteriophage λ (73).

FIGURE 7. The YfgM degron is sufficient to confer instability to a membrane protein but not to a cytosolic protein. A, schematic of the fusion strategy. Either the N terminus of wild-type YfgM or the stable E2A variant were recombinantly fused to GST from S. japonicum or PpiD from E. coli. B, the half-lives of the corresponding C-terminally His-tagged fusion proteins in E. coli W3110 in different growth phases (exp, exponential phase; stat, stationary phase). After induction of fusion protein synthesis for 30 min, translation was blocked by addition of spectinomycin. Samples were taken at different time points, and protein half-lives were determined after SDS-PAGE, Western transfer, and immunodetection. C, the stability of PpiD and the PpiD fusion proteins was analyzed in E. coli ΔftsH. Standard deviations were calculated from at least two independent experiments.

FIGURE 8. Protein-protein interaction between YfgM and RcsB. A, His-tagged RcsB was used to copurify YfgM_WT, YfgM_E2A, and YfgM_Y4A by Ni-NTA columns. B, conversely, strep-tagged YfgM variants were utilized to copurify RcsB by streptactin columns. Elution fractions E1–E3 for His tag-based purification and E2–E5 for strep tag-based purification are shown after detection with the corresponding His tag- or strep tag-specific antisera. Coomassie-stained elution fractions E1–E3 after His tag-based purification and E2–E5 for strep tag-based purification are shown. Protein bands highlighted with an asterisk represent both proteins (YfgM and RcsB) because they have nearly the same size, about 22–23 kDa, without the tag. The protein standard is given in kilodaltons. The results are on the basis of two independent experiments.
Alternatively, the interaction between substrate and protease can be mediated via adaptor proteins (2, 6, 7, 74).

A chimera between the N terminus of YfgM and the membrane protein PpiD was routed to FtsH-dependent degradation, suggesting that the YfgM degron is modular and able to confer degradation to topologically related proteins. The soluble protein GST was not destabilized by the YfgM degron. This cannot be due to the generally weak unfoldase activity of FtsH (75) because a chimera between GST and the C-terminal degron of LpxC was directed to FtsH (10). It therefore seems that degrons act in a localization-specific manner; i.e. the degron of a membrane-anchored protein retains functionality when transplanted to another membrane protein, whereas a degron of a cytoplasmic protein confers proteolysis to another soluble protein. This proposal is supported by the finding that the N-terminal degron of the cytoplasmic protein SoxS promotes Lon-mediated degradation when fused to GFP (64).

**Factor(s) Potentially Involved in YfgM Degradation**—The growth phase- and stress-dependent degradation of YfgM suggests the contribution of auxiliary factor(s) to this process. The most obvious candidates, the YfgM-interacting proteins PpiD and RcsB, were excluded because degradation was normal in the respective mutant strains. Moreover, proteolysis of YfgM was neither influenced by the alternative $\sigma$ factor RpoS, needed for expression of genes related to survival in stationary phase (59), nor by inorganic polyphosphate, known to accumulate in stationary phase and to trigger Lon-dependent degradation of ribosomal subunits for amino acid recycling (76–78). YfgM degradation, however, was accelerated by the complete lack of the alarmone (p)ppGpp, which accumulates under starvation conditions and induces a stringent stress response (53–55, 79). The precise mechanism is unknown, but it is noteworthy that growth rate-dependent degradation of the FtsH substrates LpxC and AciI also is influenced by (p)ppGpp (80, 81). The effect seems to be FtsH-specific because growth phase-dependent degradation of the DNA replication inhibitor CspD by the Lon protease was barely affected by the absence or presence of (p)ppGpp (62).
Toward the Biological Function of YfgM—The function of YfgM deserves further studies because it is present in all β and γ proteobacteria (31). To date, two alternative but not mutually exclusive functions of YfgM have been postulated (Fig. 11). The reported interaction between YfgM and PpiD (31, 33) and between YfgM and the SecYEG translocon (32) suggests that it plays a role in the periplasmic chaperone network and transfers proteins released from the SecYEG translocon to the periplasmic chaperones SurA and Skp. On the other hand, the interaction between YfgM and RcsB, reported previously by Lasserre et al. (35) and validated for several YfgM variants in this study, suggests that YfgM is equally involved in intracellular stress signal transduction. The Rcs phosphorelay system plays an important role in stress adaptation and biofilm formation (36, 37, 39, 40, 42, 43, 82). Because constant inhibition of RcsB by YfgM would be deleterious, the rationale for rapid elimination of YfgM by FtsH in stationary phase or under stress conditions is clear. Consistent with an inhibitory role of YfgM on the Rcs system, we found that an yfgM deletion strain was not impaired in cell division and has acid tolerance (pH 2.4). A somewhat conflicting report showed slightly reduced survival of the yfgM mutant at pH 2.0 (34). Acid sensitivity was exacerbated in combination with deletion of the skp or surA genes, which supports a role of YfgM in the periplasmic chaperone network (Fig. 11). In line with a role in RcsB-mediated stress response pathways are our findings that the overexpression of yfgM negatively impacts cell division, acid resistance, and RprA expression.

The collection of YfgM variants with different half-lives provided the opportunity to further investigate its role in the stress response network. Several variants (E5A, D9A, and F17A) behaved like YfgM_WT in eliciting filamentous and acid-sensitive cells. YfgM_Y4A, which is able to interact with RcsB in vitro, had no negative effect in vivo, most likely because it is an extremely unstable protein that is rapidly degraded even in exponential growth. The only counterintuitive results were obtained with the stable E2A and I3A variants. As expected, cells expressing these alleles were extremely acid-sensitive. Unexpectedly, cell division was undisturbed because the YfgM_E2A- and YfgM_I3A-producing cells were normal and rod-shaped. A possible explanation for this observation is that YfgM has several other interaction partners and that binding of these partners may be altered when the N terminus is changed. Most conspicuous in this context is the essential membrane-bound cell division protein FtsL, which was found as the interaction partner of YfgM in a bacterial two-hybrid screen (83).

Because YfgM is a membrane-spanning protein that reaches into two separated compartments of the cell, it is ideally suited to coordinate stress responses across the inner membrane via a dynamic protein-protein interaction network inside and outside of the membrane (Fig. 11). Because of the high conservation of YfgM, RcsB, and PpiD, our findings may have implications for understanding the stress response physiology of many other bacteria, including human pathogens.

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added by the SsrA-tagging system. 

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