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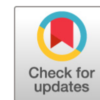
RESEARCH ARTICLE



A Small Periplasmic Protein with a Hydrophobic C-Terminal Residue Enhances DegP Proteolysis as a Suicide Activator

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ABSTRACT DegP is a highly conserved protease that performs regulated proteolysis to selectively remove misfolded proteins in the periplasm of *Escherichia coli*. Binding of misfolded proteins is known to be the main mechanism of DegP activation, but it is unknown whether any native proteins can alter DegP activity. Here, we show that a small periplasmic protein, YjfN, which is highly upregulated by the Cpx envelope stress response, functions as a "suicide activator" for DegP and promotes efficient degradation of misfolded proteins. YjfN readily binds to and is degraded by DegP, for which a hydrophobic C-terminal residue and transient unfolding of YjfN are critical. YjfN also activates DegP in *trans* while it is being degraded and accelerates degradation of a denatured outer membrane protein, OmpA, that is not easily recognized by DegP. Although YjfN also prevents OmpA aggregation, the *trans*-activation effect is mainly responsible for efficient OmpA degradation. Overexpression of YjfN enhances the viability of cells in misfolded protein stress that is induced by the presence of a less-active variant of DegP at high temperature. Collectively, we suggest that YjfN can enhance DegP proteolysis for relieving envelope stresses that may generate toxic misfolded proteins.

IMPORTANCE Proper degradation of toxic misfolded proteins is essential for bacterial survival. This function is mainly performed by a highly conserved protease, DegP, in the periplasm of *Escherichia coli*. It is known that binding of misfolded proteins is the main mechanism for activating the DegP protease. Here, we find that a small periplasmic protein, YjfN, can be a substrate and an activator of DegP. It is the first example of a native protein showing an ability to directly alter DegP activity. The YjfN-mediated *trans* activation of DegP promotes efficient degradation of misfolded proteins. Our results suggest that YjfN is a novel "suicide activator" for DegP that enhances DegP proteolysis under misfolded protein stress.

KEYWORDS protein quality control, proteolysis, suicide activator, YjfN, DegP, HtrA

Proteases in protein quality control (PQC) play a critical role in degrading potentially toxic misfolded proteins (1, 2). Because protein degradation irreversibly destroys proteins, their proteolytic activities should be carefully regulated not to remove functional proteins. Cytoplasmic PQC proteases employ a mechanism in which additional regulatory proteins perform ATP-dependent unfolding and translocation of substrate proteins into a self-compartmentalized proteolytic chamber in a barrel-shape structure that inherently blocks access of normal proteins (3, 4). In contrast, periplasmic proteases should implement a different mechanism for activity regulation, due to the lack of ATP in the bacterial periplasm.

PQC proteolysis in the periplasm of *Escherichia coli* is performed mainly by a member of the highly conserved HtrA family of proteases, DegP (5). Because misfolded proteins in the bacterial periplasm are toxic to cells, DegP proteolysis is essential for cell survival at high temperature or under conditions that generate misfolded proteins (6–8). Two major envelope stress responses, the σ^E and Cpx responses, recognize

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misfolded protein stress as a signal and increase the production of DegP (1, 9–11). These transcriptional upregulations greatly enhance the capacity of DegP proteolysis for removing misfolded proteins.

The activity level of DegP is regulated by balancing two distinct molecular transformations, the conformational change between inactive and active states and the assembly of large polyhedral cages containing 12, 18, or 24 subunits (12–16). Introduction of two mutations that disrupt the balance of the two molecular transformations can generate a hyperactive DegP variant that can kill bacteria by excessive protein degradation (17). Both transformations are allosterically induced by substrate binding, and thus the DegP cage is proteolytically activated only in the presence of proper substrates. DegP preferentially binds to two distinct sequence motifs in substrates: a hydrophobic C-terminal motif for binding to the PDZ1 domain and a cleavage site motif for binding to the active site of DegP (13, 16, 18). These interactions with substrates may help discriminate proper substrates and maintain the activity level during protein degradation.

It is believed that misfolded proteins are the major substrates and activators for DegP, and no other protein factor has been known to modulate the level of DegP activity. In this study, we identify a new native substrate and activator of DegP, YjfN, which is a small periplasmic protein that is highly upregulated by the Cpx envelope stress response (19). YjfN is readily degraded by DegP, activates DegP in *trans*, and greatly accelerates the degradation of an abundant outer membrane protein (OMP), OmpA. We suggest that YjfN functions as a suicide activator for DegP and complements DegP proteolysis by transiently activating DegP.

RESULTS

The C-terminal ends of periplasmic proteins display a much smaller percentage of hydrophobic residues. We initially intended to identify a periplasmic protein that can be a native substrate of the DegP protease. We reasoned that periplasmic proteins with hydrophobic C-terminal residues have a higher chance to bind to and be degraded by DegP. Because the majority of proteins are translocated across the cytoplasmic membrane in unfolded states via the Sec translocation machinery (20), periplasmic proteins with hydrophobic C-terminal residues may undergo wasteful degradation by DegP or other proteases that are known to recognize hydrophobic C-terminal residues, such as Tsp and DegQ (21–23). Periplasmic proteins, therefore, might have evolved to avoid hydrophobic residues at C termini, and those with hydrophobic C-terminal residues may escape wasteful degradation by different mechanisms or function as native substrates of DegP.

To test this idea, we initially analyzed the C-terminal sequences as well as the whole sequences of proteins located in the *Escherichia coli* periplasm or cytoplasm. The subcellular location of *E. coli* proteins was obtained from the *Echo*LOCATION database (24). We included lipoproteins in periplasmic proteins but excluded those expected to be exported by the twin-arginine translocation (Tat) pathway because they are exported in folded states (25). Although the total amino acid compositions of cytoplasmic proteins ($n = 2{,}708$) are not much different from those of periplasmic proteins ($n = 406$), several amino acids showed significantly different compositions at the C-terminal ends (Fig. 1; see Table S1 in the supplemental material). For example, while hydrophobic residues such as Ala and Val are found at much lower rates (17.2% versus 2.0%), hydrophilic residues such as Lys and Gln are found at much higher rates (15.4% versus 34.0%) at the C termini of periplasmic proteins. Six hydrophobic residues (Ala, Val, Leu, Met, Ile, and Phe) compose only 14% of all C-terminal residues in periplasmic proteins, compared to 34.9% in cytoplasmic proteins. This result indicates that C-terminal residues of periplasmic proteins present much lower levels of hydrophobic residues, which may help avoid wasteful protein degradation.

YjfN is degraded by DegP. Among 57 periplasmic proteins with hydrophobic C-terminal residues (Table S2), we chose YjfN, a periplasmic protein of unknown function, as a candidate for DegP substrate for the following reasons. First, the Cpx

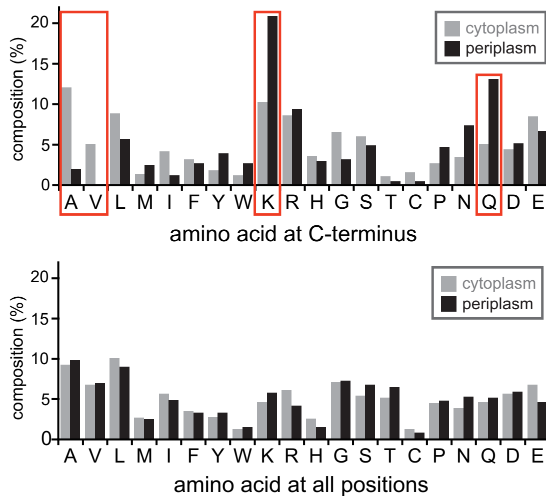

FIG 1 Amino acid compositions of cytoplasmic and periplasmic proteins at all positions or the C-terminal position (the final residue). The correlation between subcellular locations and amino acid positions indicates that the compositions of A, V, K, and Q are significantly different (red rectangles, $P < 0.001$ by Fisher's exact test).

envelope stress response is known to upregulate both *yjfN* and *degP* (10, 19), indicating a potential connection of their functions under the misfolded protein stress. Second, YjfN is a small protein with only 70 residues after cleavage of the signal peptide (26, 27). A small protein may exist in an equilibrium state between folded and unfolded forms, and thus its hydrophobic C terminus may be sufficiently exposed for DegP binding (22).

YjfN is a member of the YhcN family proteins, which include 10 small proteins in the *E. coli* periplasm, and is expected to have a small fold with two α-helices and three β-strands (Fig. 2A and B; Fig. S1) (26, 28, 29). C termini of YhcN family proteins end with one or more lysines or arginines except for YjfN. We incubated purified YjfN with DegP and found that YjfN is efficiently degraded by DegP (Fig. 2C, top band). To determine the importance of the hydrophobic C-terminal residue, we tested the degradation by DegP of two YjfN variants, $YjfN_{A70K}$ and $YjfN_{A \rightarrow KL}$, in which the C-terminal alanine is changed to either lysine or lysine-leucine, and found that only the variant with a hydrophobic C-terminal residue, $YjfN_{A \rightarrow KL}$, showed efficient degradation (Fig. 2C). Experiments with a YjfN homologue and its variant, $YjfO_{WT}$ (where WT means wild type) and $YjfO_{RK \rightarrow A}$, also revealed a more rapid degradation of the variant with a hydrophobic C-terminal residue (Fig. 2C). Of note, the tryptophan fluorescence experiments with different amounts of guanidine suggest that $YjfN_{WT}$, $YjfN_{A70K}$, $YjfO_{WT}$, and $YjfO_{RK \rightarrow A}$ largely exist in a folded state (Fig. S2A and B), and the C-terminal hydrophobicity, not the stability of the fold, mainly determines the degradation efficiency of the YjfN variants (Fig. S2B and C).

Some evidence suggests that DegP mainly binds to and degrades the unfolded state of YjfN. First, to test the direct interaction between YjfN and DegP, we monitored the change of tryptophan fluorescence anisotropy of YjfN with increasing amounts of a catalytically inactive DegP variant, $DegP_{S210A}$. $DegP_{S210A}$ bound to $YjfN_{WT}$ but not to $YjfN_{A70K}$, suggesting that the hydrophobic C terminus of YjfN is critical for direct interaction with DegP (Fig. 2D). However, the hydrophobic C terminus of YjfN is expected to be a part of the central β-strand in the main β-sheet and hidden in the fold (Fig. 2A and B). Second, we determined the cleavage sites by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (Fig. 2E). When the cleavage sites were mapped to the predicted secondary structure, the first cleavage site, I51-T52, was located in the middle of the second β-sheet (Fig. 2A, B, and F). This β-sheet may have to be fully dissociated for proper interaction with the active-site region of DegP. Finally, we isolated a relatively unstable YjfN mutant, $YjfN_{W62A}$, and found that it was degraded faster than the wild-type YjfN (Fig. 2G and H). W62 is

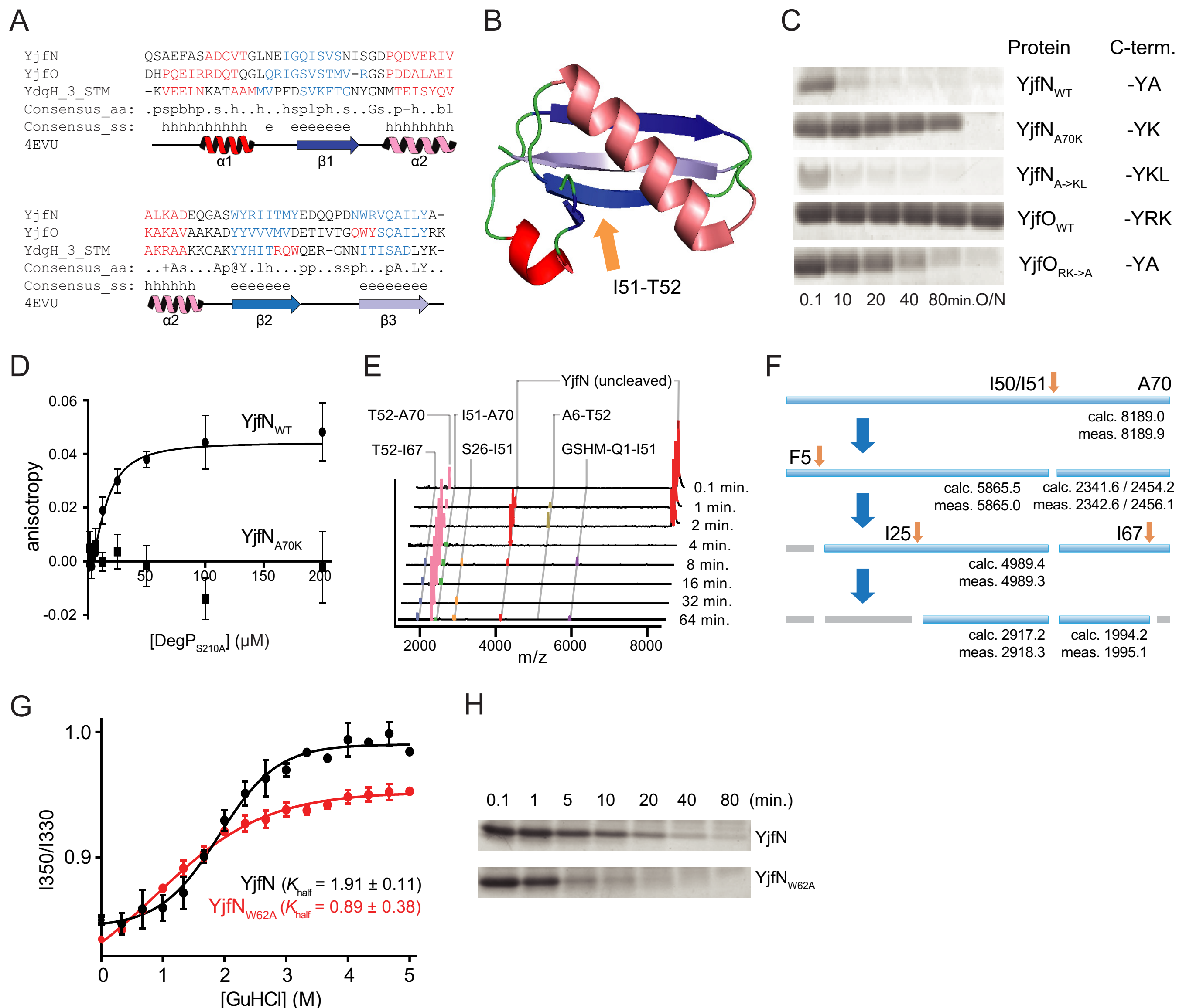

FIG 2 YjfN is degraded by DegP *in vitro*. (A) Multiple-sequence alignment and secondary-structure prediction of YjfN, YjfO (*E. coli*), and the third DUF1471 domain of YdgH (*Salmonella* serovar Typhimurium) using PROMALS3D (29). Residues for predicted helices and β-sheets are colored red and blue, respectively. Consensus amino acids are indicated as follows: p, polar; s, small; b, bulky; h, hydrophobic; l, aliphatic; @, aromatic; −, negatively charged; +, positively charged; capital letters, highly conserved residues. Consensus secondary structures are indicated below the alignment: e, extended (β-sheet) structure; h, helix. The secondary structure of the YdgH third domain based on the crystal structure (PDB ID 4EVU) was aligned for comparison. The N-terminal sequences (C25 to T39) of YjfO were omitted for simplicity. (B) Crystal structure of the third DUF1471 domain of YdgH retrieved from the protein data bank (PDB ID 4EVU) (28). The first cleavage sites of YjfN by DegP (I51-T52) are indicated with an arrow. (C) Cleavage of YjfN or YjfO variants (50 μM) by DegP (10 μM) was monitored by SDS-PAGE. The C-terminal sequence of each variant is shown on the right. O/N, overnight. (D) Tryptophan fluorescence anisotropy of $YjfN_{WT}$ or $YjfN_{A70K}$ (10 μM) was monitored with different amounts of an inactive variant of DegP ($DegP_{S210A}$). Error bars show averages ± 1 SD ($n = 3$). (E) Analysis of the cleavage products of YjfN using MALDI-TOF mass spectrometry. YjfN (10 μM) was mixed with DegP (1 μM), and aliquots of the reaction solution at various time points were taken and quenched by addition of 1% TFA (vol/vol). (F) Schematic diagram of the YjfN degradation by DegP. Cleavage sites are indicated by arrows. Gray fragments were not detected in the MALDI-TOF mass spectrometry spectra. calc., calculated; meas., measured. (G) Tryptophan fluorescence was measured in the presence of different amounts of guanidine HCl. The ratios of the intensities at 350 and 330 nm are shown, and the K_{half} values were determined by fitting the data to a sigmoidal curve. Error bars show averages ± 1 SD ($n = 3$). (H) Degradation of YjfN and $YjfN_{W62A}$ (25 μM) by DegP (2 μM) was monitored by SDS-PAGE.

expected to be located in the hydrophobic core, and thus the W62A mutation may destabilize the folded state. Consistent with this, the W62A mutant was more readily unfolded with a lower concentration of guanidine, as shown in the tryptophan fluorescence experiments (Fig. 2G).

YjfN activates DegP in *trans* and promotes OmpA degradation. DegP is allosterically activated by substrate binding (14, 16, 18, 30). To monitor DegP activation by

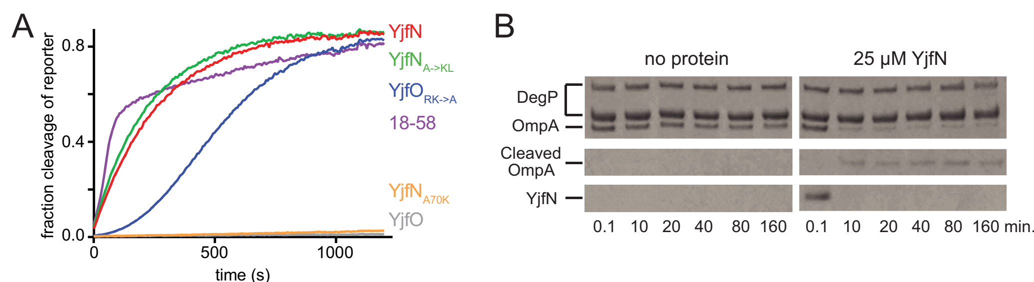

FIG 3 YjfN activates DegP in *trans* and promotes OmpA degradation. (A) DegP (10 μM) activation by YjfN or YjfO variants (50 μM) or 18-58 (50 μM) was monitored by cleavage of the reporter peptide (100 μM). (B) OmpA (2 μM) cleavage by DegP (5 μM) was monitored by SDS-PAGE in the absence or presence of YjfN (25 μM). OmpA was added to the solutions containing DegP and, if any, YjfN.

YjfN, we used a model reporter substrate, which is efficiently cleaved by activated DegP but not by inactive DegP (16). DegP cleavage of reporter separates a fluorophore from a quencher and therefore increases the fluorescence. As previously reported, 18-58, which is a lysozyme-derived peptide that tightly binds to and is quickly cleaved by DegP, efficiently activates DegP, as shown in the high level of reporter cleavage (Fig. 3A) (16). $YjfN_{WT}$, $YjfN_{A\rightarrow KL}$, and $YjfO_{RK\rightarrow A}$, all of which contain a hydrophobic C-terminal residue, also showed robust activation of DegP, whereas $YjfN_{A70K}$ and $YjfO_{WT}$, which have a hydrophilic C-terminal residue, revealed little activation effect (Fig. 3A). This result indicates that YjfN and other variants with hydrophobic C termini can tightly bind to and activate DegP.

To test how YjfN affects protein degradation, we monitored the degradation of denatured OmpA by DegP in the absence or presence of YjfN (Fig. 3B; Fig. S3). When we added urea-denatured OmpA to a solution containing DegP, there was little degradation of OmpA. The simultaneous addition of YjfN (25 μM) (Fig. 3B) to the same solution, however, triggered significant OmpA degradation, as shown in the appearance of a distinct OmpA fragment and the disappearance of full-length OmpA, as well as YjfN degradation. A lower concentration of YjfN (e.g., 5 μM) that would shorten the duration time for activated DegP lowered the level of OmpA degradation (Fig. S3). This result suggests that YjfN promotes the degradation of proteins that are not easily recognized and degraded by DegP.

YjfN-mediated *trans* activation of DegP, not the prevention of aggregation, promotes degradation of denatured OmpA. YjfN-mediated degradation of denatured OmpA may result from the *trans* activation of DegP by YjfN or, alternatively, from the prevention of OmpA aggregation that maintains OmpA in a degradation-competent state. To distinguish between these two models, we initially tested OmpA aggregation in the presence of YjfN variants or another substrate by light scattering experiments. Urea-denatured OmpA was aggregated when it was diluted in a buffer as well as in a solution containing a short peptide substrate, p23, which is a shorter version of the 18-58 peptide that also tightly binds to and activates DegP (Fig. 4A; Fig. S4A) (31). However, OmpA did not show any significant aggregation when it was diluted in solutions containing any YjfN variant ($YjfN_{WT}$, $YjfN_{A70K}$, or $YjfN_{A70K}$ plus p23), indicating that YjfN prevents OmpA aggregation and the hydrophobic C terminus is not required for this property (Fig. 4A). YjfO could also prevent the aggregation of OmpA (Fig. S4B). Aggregation assays with truncated variants of OmpA revealed that YjfN interacts mainly with the periplasmic domain, and not with the β-barrel domain, to prevent OmpA aggregation (Fig. S4C). Consistent with this, YjfN did not prevent aggregation of OmpF, which is composed of only a single β-barrel domain (Fig. S4C). YjfN did not prevent the aggregation of other model proteins, lysozyme and luciferase, suggesting that this function may not be extensive for YjfN (Fig. S4D).

We then added DegP in the above-mentioned solutions after the 15-min incubation for the aggregation assay. The aggregated OmpA in a buffer or the p23 solution was

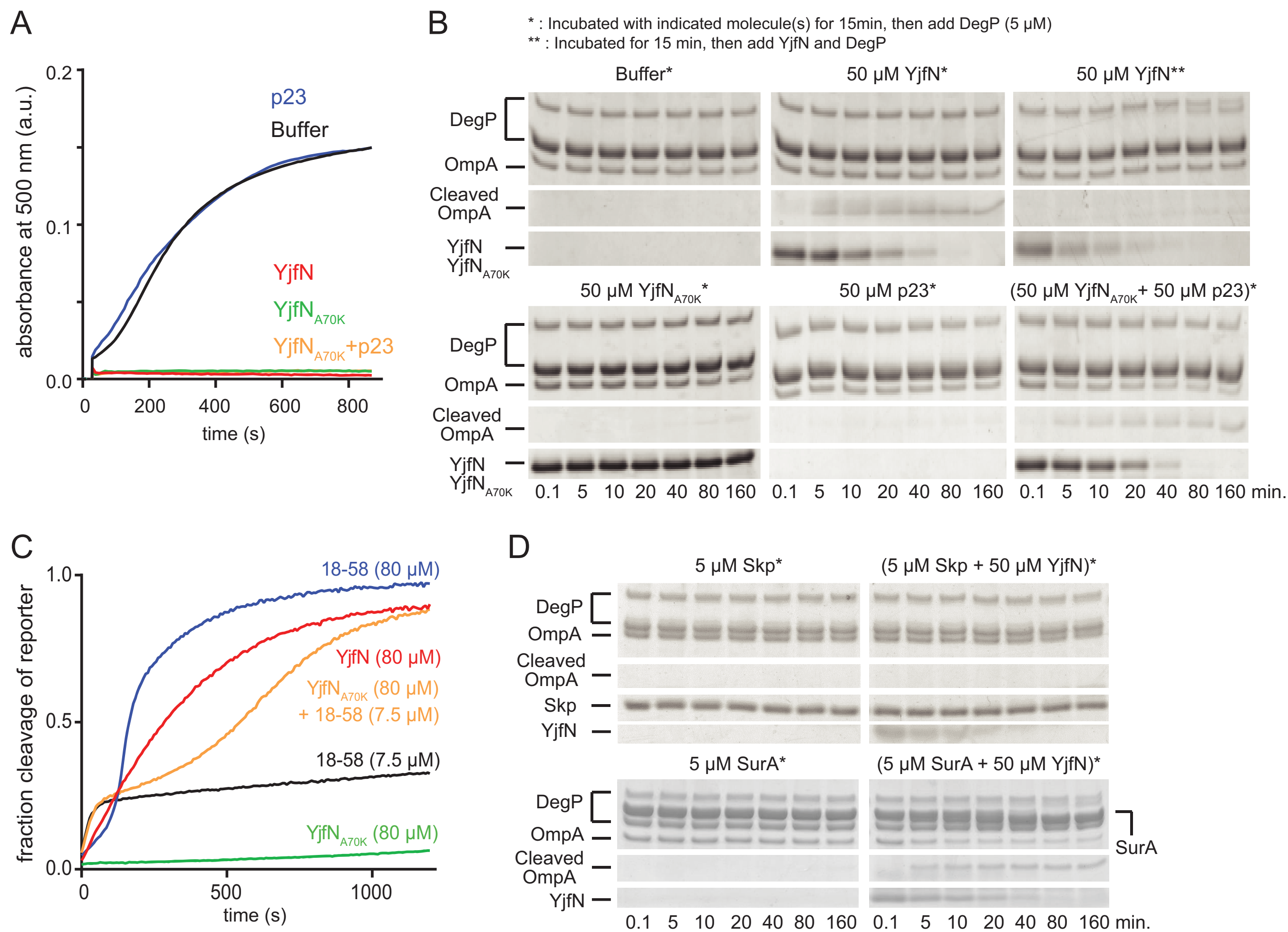

FIG 4 The *trans* activation of DegP by YjfN stimulates efficient degradation of a misfolded protein. (A) Aggregation of OmpA (2 μM) in the absence or presence of YjfN variants or the p23 peptide (50 μM) was monitored by light scattering at 500 nm. (B) DegP (final concentration, 5 μM) was added to the solutions prepared for panel A, and the degradation of OmpA was monitored by SDS-PAGE. For the late addition of YjfN, YjfN was added to the reaction together with DegP after the light scattering assay. (C) DegP (10 μM) activation by YjfN variants (80 μM), 18-58 (7.5 or 80 μM), or both was monitored by cleavage of the reporter peptide (100 μM). (D) Degradation of Skp-bound or SurA-bound OmpA (2 μM; 5 μM for Skp and SurA) by DegP (5 μM) was monitored in the presence or absence of YjfN (50 μM).

barely degraded upon DegP addition (Fig. 4B, top left and bottom center). The late addition of YjfN into the OmpA-only solution also did not show significant degradation (Fig. 4B, top right), indicating that activated DegP cannot degrade the aggregated OmpA. However, a part of the YjfN-stabilized OmpA was degraded by DegP, as shown in the presence of the distinct cleaved OmpA fragment (Fig. 4B, top center), whereas the YjfN$_{A70K}$-stabilized OmpA was largely uncleaved (Fig. 4B, bottom left). These results indicate that a part of the YjfN-stabilized OmpA is in a degradation-competent state but requires DegP activation for degradation. Interestingly, both the YjfN$_{A70K}$-stabilized OmpA and YjfN$_{A70K}$ showed degradation upon the addition of another DegP activator, p23 (Fig. 4B, bottom right), suggesting that the p23-activated DegP can degrade OmpA and YjfN$_{A70K}$, the fragments of which, in turn, keep DegP activated for further OmpA degradation. Consistent with this model, the reporter cleavage assay showed a two-phase activation curve for the solution containing YjfN$_{A70K}$ (80 μM) and a much smaller amount of 18-58 (7.5 μM), in which the endpoint is similar to that of YjfN$_{WT}$ (80 μM) but much higher than that of 18-58 alone (7.5 μM) (Fig. 4C). Data from additional degradation assays under different conditions or with different combination of reagents were also consistent with the above results (Fig. S5). Collectively, our results support a model showing that the YjfN-mediated *trans* activation of DegP, not the prevention of

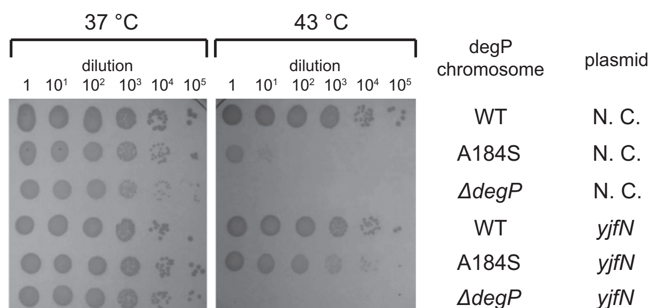

FIG 5 YjfN alleviates misfolded protein stress induced by a less-active DegP variant at high temperature. Strains producing $DegP_{WT}$, $DegP_{A184S}$, or no DegP were transformed with an empty plasmid or a YjfN expression plasmid. They were serially diluted, spotted on LB agar plates with IPTG, and incubated at 37°C or 43°C. The result shown is representative of three independent assays. N.C., negative control (no *yjfN* gene).

aggregation, is responsible for the degradation of denatured OmpA, while YjfN can also keep some OmpA molecules in a degradation-competent state.

Activated DegP can degrade the SurA-bound OmpA but not the Skp-bound OmpA. Two major chaperones for OMPs are Skp and SurA, and, in particular, SurA plays a central role in OMP biogenesis (32–35). They interact with unfolded OMPs and prevent their aggregation *in vitro* (Fig. S4E). We wondered whether Skp- or SurA-stabilized OmpAs are susceptible to degradation by activated DegP. We incubated denatured OmpA with Skp or SurA and subsequently added DegP in the absence or presence of YjfN. Only the SurA-stabilized OmpA showed significant OmpA degradation in the presence of YjfN (Fig. 4D), indicating that activated DegP can trap and degrade the SurA-bound OmpA but that the Skp-bound OmpA is mainly protected from DegP degradation.

YjfN alleviates misfolded protein stress at high temperature in the presence of a less-active variant of DegP. DegP is overexpressed at high temperature and suppresses envelope stress by degrading misfolded proteins (1, 5). The DegP function is essential for cell survival at high temperatures. To test the effect of YjfN overexpression on DegP function, we inserted either an empty plasmid or a YjfN expression plasmid into three strains containing $degP_{WT}$, $degP_{A184S}$, or no *degP* gene. $DegP_{A184S}$ is a less-active variant of DegP and has less ability to combat misfolded protein stress (17). Under heat shock stress (43°C), cells producing wild-type DegP, but not those producing no DegP, were viable, regardless of whether YjfN was overexpressed (Fig. 5). However, cells expressing $DegP_{A184S}$ showed much higher viability (about 1,000-fold) when YjfN was overexpressed (Fig. 5). The deletion of the chromosomal *yjfN* gene did not change this result (Fig. S6A). This result suggests that YjfN alone cannot suppress misfolded protein stress but that YjfN may enhance cell viability by *trans* activation of $DegP_{A184S}$, which greatly facilitates degradation of toxic misfolded proteins.

It has been reported that the toxicity of PapE and the LamB-LacZX90 fusion protein that are misfolded in the periplasm is relieved by activation of the Cpx stress response and DegP proteolysis (36, 37). We tested whether YjfN is required for these processes by using Δ*yjfN* strains (38) and found that the suppression of their toxicity is not dependent on YjfN (Fig. S6B and C). This result suggests that YjfN may be involved in the degradation of a specific group of proteins or that YjfN is merely one of several redundant periplasmic proteins that can help DegP proteolysis in the Cpx stress response.

DISCUSSION

The current model of DegP proteolysis is that binding of misfolded proteins to DegP, as the substrates, activates the protease, which subsequently degrades them. In this study, we show that YjfN can function as a “suicide activator” of DegP by maintaining DegP in an active state while it is being degraded and promote the degradation of

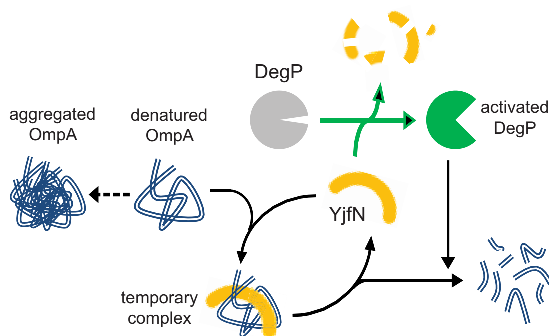

FIG 6 Model for YjfN-mediated DegP activation and OmpA degradation. YjfN prevents the aggregation of denatured OmpA and activates DegP while it is degraded by DegP. Only the activated DegP efficiently degrades denatured OmpA.

other misfolded proteins in *trans* (Fig. 6). It was previously reported that CpxP helps DegP proteolysis as an adaptor that delivers misfolded proteins to DegP for degradation (37). To the best of our knowledge, however, YjfN is the first example that shows that a native protein can activate DegP by direct interaction.

What is the advantage of the new protein factor that can directly modulate DegP activity? Any YjfN expressed in the periplasm may readily bind to and be degraded by DegP. The seemingly wasteful expression of YjfN, however, may complement DegP-mediated PQC proteolysis in several aspects. First, YjfN may allow more prompt activation of DegP in the presence of misfolded proteins that DegP cannot readily recognize. Various misfolded proteins have been shown to activate DegP at very different levels (16), and this study also shows that denatured OmpA is not efficiently recognized and degraded by inactive DegP. YjfN may help initiate the activation of DegP under various stress conditions. Second, YjfN may maintain a high level of DegP activity during misfolded protein stress. YjfN is highly overproduced in the Cpx envelope stress response and other stress conditions (19, 39, 40). The constant expression of YjfN may keep DegP in an active state and thus allow efficient PQC proteolysis under stress conditions. Finally, the activation effect of YjfN is transient enough to avoid excessive proteolysis. Constant DegP activation without any noticeable envelope stress may lead to a lot of wasteful degradation of normal proteins and is therefore undesirable. Because DegP is allosterically activated by binding to an intact substrate, it returns to an inactive state when it finishes degradation of YjfN. It is also reported that mRNA of *yjfN* is most unstable in *E. coli* (41).

Several features make YjfN a good DegP activator. The hydrophobic C terminus of YjfN is essential for binding to and activating DegP. Interestingly, all members of the YhcN family of proteins have a lysine or an arginine at their C termini except for YjfN, and the introduction of a hydrophobic residue at the C terminus of YjfO converts it into a substrate and activator of DegP, suggesting that the fold in the YhcN family may be generally vulnerable to DegP proteolysis but that only YjfN is evolved to tightly bind to DegP in a native state. The spontaneous unfolding of YjfN may also be important for interaction with DegP, because the two motifs for DegP binding, a hydrophobic C-terminal motif and a cleavage site motif, appear to be hidden in the fold of the YhcN family. The small size of YjfN may make it exist in an equilibrium of the folded and unfolded states.

Although YjfN keeps denatured OmpA from aggregation, YjfN cannot prevent aggregation of the β-barrel domains of OmpA and OmpF or other proteins such as lysozyme and luciferase. Therefore, the function of aggregation prevention may not be extensive for YjfN. We suggest that the main function of YjfN is to directly activate DegP for efficient degradation of misfolded proteins. In contrast, the two major chaperones for OMPs, Skp and SurA, can prevent aggregation of OMPs (32, 35) but cannot activate DegP. Interestingly, our data show that the YjfN-activated DegP can degrade the

SurA-bound OmpA but not the Skp-bound OmpA. The inner cavity of Skp may protect the denatured OmpA from DegP, but there is no apparent structure in SurA that may block DegP access (42, 43). It was also reported that Skp binds to OMPs more tightly than SurA (44, 45). Since SurA is more important for OMP biogenesis (35), the efficient degradation of OmpA by activated DegP may indicate that the lower levels of OMPs in the envelope stress is the result of not only the transcriptional downregulation of OMPs but also the direct DegP proteolysis of OMPs en route to the outer membrane.

We demonstrate that the composition of the periplasmic proteins at the C-terminal residues is significantly different from that of the cytoplasmic proteins, and, in particular, hydrophobic residues are found at much lower rates. It is not clear what causes this disproportional composition, but one interesting hypothesis is that proteases preferentially recognizing substrates with hydrophobic C-terminal residues such as DegP, DegQ, and Tsp might have imposed a selective pressure to avoid wasteful degradation of proteins that are newly translocated across the cytoplasmic membrane in unfolded states. It is well known that the N-terminal residues of cytoplasmic proteins are regulated by the N-end rule that is associated with specific protein degradation pathways (46, 47). The unusual composition of C-terminal residues and the presence of the three proteases in the periplasm may indicate that the "C-end rule" is loosely applied to periplasmic proteins and associated with distinct protein degradation pathways. However, it should not be as strict as the N-end rule, because there is no ATP-dependent protein degradation pathway. Periplasmic proteins with hydrophobic C-terminal residues may escape proteolysis through fast folding, through sequences near C termini that inherently avoid interaction with proteases, or through translocation in folded states via the Tat system.

It is yet to be determined to what extent the envelope stress response relies on the YjfN-mediated DegP activation. Because the deletion of the *yjfN* gene does not significantly reduce cell viability at high temperature or under other stress conditions (Fig. S6), we believe that the YjfN function is not essential but supplementary for DegP proteolysis. Its effect on cells may not be significant for cell viability but still sufficient for providing a selective advantage under stress conditions. Indeed, the small increase or decrease in the level of DegP activity was shown to be accumulated during multiple replication cycles and meaningfully affects the growth fitness of cells at high temperature (17). Therefore, we suggest that the YjfN-mediated fine-tuning of DegP activity can be an additional mechanism for the regulation of PQC proteolysis in the bacterial periplasm.

MATERIALS AND METHODS

Analysis of amino acid compositions. The lists of proteins in various compartments in *E. coli* were obtained from the *Echo*LOCATION database (https://www.york.ac.uk/res/thomas/echolocadv.cfm) (24). Cytoplasmic proteins also include membrane-associated proteins on the cytoplasmic side of the inner membrane. Periplasmic proteins also include lipoproteins that are anchored to either the outer membrane or the inner membrane. Periplasmic proteins were further analyzed with PRED-TAT to determine whether they use the general secretory pathway (Sec) or the twin-arginine translocase (Tat) and excluded those using the Tat pathway (48). Full sequences of listed proteins were obtained from the annotated information of the *E. coli* K-12 MG1655 complete genome (49). Amino acid compositions of cytoplasmic and periplasmic proteins were analyzed separately at all positions or the C-terminal position.

Construction of plasmids and strains. Bacterial strains, primers, and plasmids used in this study are listed in Table S3 to S5 in the supplemental material. Plasmids expressing Skp (pSK578), YjfN (pSK599), and YjfO (pSK789) were constructed by insertion of the PCR-amplified genes between the NdeI and NotI sites of pET28b. Plasmids expressing variants of YjfN or YjfO (pSK787, pSJ019, pSJ025, and pIN026), C-terminally His-tagged OmpA (pSJ017), or its truncation variants (pSJ052-056) were constructed using an inverse PCR method (50). An IPTG (isopropyl-β-D-1-thiogalactopyranoside)-inducible YjfN overexpression plasmid (pSJ026) was constructed with pACYCDuet-1 and pTrcAgBIS(CO) (51, 52). First, the *trc* promoter and the open reading frame (ORF) region of pTrcAgBIS(CO) were cut and inserted into pACYCDuet-1 (between NdeI and BspHI) to construct pSJ023. The *yjfN* gene was moved from pSK706 (constructed from pSK633 [17] by restriction-free cloning) into pSJ023 by restriction-free cloning (53). pSJ047 (used as a negative control of pSJ026) was constructed by an inverse PCR method.

For the construction of a strain expressing $DegP_{A184S}$ (HYO038), the $degP_{A184S}$ gene was amplified from plasmid pSK735 and used to replace the *degP*::*kan* gene in SK324 by λ-Red-mediated recombineering with the pSIM5 plasmid (17, 54). The mutation was confirmed by DNA sequencing (Macrogen) and a viability assay at 43°C.

Preparation of proteins and peptides. DegP variants and various substrate peptides (18-58, p23, and reporter) were prepared as previously described (16, 31). Wild-type DegP, $DegP_{S210A}$, and SurA were prepared as previously described (16, 55). Newly constructed proteins were expressed using the BL21(DE3) strain. Overnight cultures were diluted 100-fold and grown to an optical density at 600 nm (OD_{600}) of ~0.4, and 0.1 mM IPTG was introduced into the cultures. Induction was conducted for 5 h at 37°C. Cells were lysed by sonication and centrifuged at 20,000 × *g* for 15 min, and the supernatants were collected. The proteins were purified using Ni-Sepharose 6 Fast Flow (GE Healthcare). Wash and elution buffers contained 50 mM sodium phosphate (pH 8), 300 mM NaCl, and 40 mM (wash) or 500 mM (elution) imidazole. The His tags of SurA, Skp, YjfN variants, and YjfO variants were removed by enzymatic cleavage using human thrombin (Millipore) at room temperature. The cleaved proteins were concentrated and exchanged with buffers (10 mM Tris [pH 8], 20 mM sodium acetate [pH 4.75], or 20 mM MES [pH 6], depending on the pI value of each protein) using an Amicon centrifugal filter (Millipore) and purified by ion-exchange chromatography (MonoQ or MonoS 5/50 GL; GE Healthcare). Some of the proteins were further purified by gel filtration chromatography (Superose 6; GE Healthcare).

His-tagged OmpA variants were expressed as described above, and the cells containing OmpA variants were suspended in a 0.1% (vol/vol) Triton X-100 solution. After purification by repetition of resuspension, sonication, and centrifugation at 4,500 × *g*, the inclusion bodies were dissolved in 8 M urea and centrifuged at 20,000 × *g* to remove the undesired pellet. Denatured proteins were purified using Ni-Sepharose 6 Fast Flow (50 mM sodium phosphate; pH 6.3 for wash and pH 4.5 for elution). Eluted proteins were concentrated using an Amicon centrifugal filter (Millipore).

***In vitro* protein degradation assay.** All *in vitro* assays were conducted in a buffer containing 50 mM sodium phosphate (pH 8) and 100 mM NaCl at 26°C. Various YjfN variants or other proteins (50 μM) were mixed with DegP (10 μM) for reactions. For the $YjfN_{W62A}$ degradation assay, YjfN or $YjfN_{W62A}$ (25 μM) was mixed with DegP (2 μM) for reactions. For the OmpA degradation assay, denatured OmpA (200 μM, dissolved in 8 M urea) was diluted 100-fold into the solution containing DegP (5 μM) and protein substrates. Each reaction was quenched with 4× SDS sample buffer at the time points specified in Fig. 3B and 4B and D. For analysis of the YjfN cleavage products, 10-μl volumes of the reaction solution (1 μM DegP and 10 μM YjfN) were taken at the specified time points, quenched by the addition of 1% (vol/vol) trifluoroacetic acid (TFA;1 μl), desalted using a C_{18} ZipTip (Millipore), and analyzed using a Microflex MALDI-TOF mass spectrometer (Bruker).

DegP activation assay. Activation of DegP (10 μM) by various protein/peptide substrates (50 μM) was monitored by the fluorescence change of the reporter peptide (Abz-KASPVSLGY^{NO2}D, 100 μM; Abz is 2-aminobenzoic acid, and Y^{NO2} is 3-nitrotyrosine). Fluorescence was measured using an Infinite M200 microplate reader (Tecan) with excitation at 320 nm and emission at 430 nm. Assay results were normalized with the maximal fluorescence of each reaction mixture, which was measured after the addition of elastase (Promega).

Tryptophan fluorescence. Tryptophan fluorescence was measured to determine the stability of YjfN and $YjfN_{W62A}$. YjfN and $YjfN_{W62A}$ (20 μM) were incubated for 5 min in solutions with various concentrations of guanidinium chloride (0 to 5 M). Fluorescence spectra of YjfN and $YjfN_{W62A}$ (20 μM) were measured using an Infinite M200 microplate reader (Tecan) with excitation at 295 nm (bandwidth, 5 nm) and emission in the range of 320 nm to 360 nm (bandwidth, 20 nm). For YjfN and YjfO variants, proteins (10 μM) were incubated for 5 min in solutions with various guanidinium chloride concentrations (0 to 6 M). Then, fluorescence spectra were recorded with a QuantaMaster 400 steady-state spectrofluorometer (Photon Technology International). The excitation wavelength was 295 nm (bandwidth, 5 nm), and the increment of 0.5 nm was used to scan spectra in the range of 315 nm to 400 nm (bandwidth, 5 nm). All experiments were done at 25°C. K_{half} (the concentration of guanidinium chloride at the half maximal change of the signal) of the ratio of intensities at 350 nm and 330 nm (I_{350}/I_{330}) was determined by fitting the data to the following sigmoidal curve.

$$Y = Y_{\min} + \frac{\left(Y_{\max} - Y_{\min}\right)}{1 + (10^{K\text{half} - x})^{h}}$$

where Y is the intensity ratio (I_{350}/I_{330}), Y_{min} is the minimum value of the intensity ratio, Y_{max} is the maximum value of the intensity ratio, and h is the Hill coefficient.

Tryptophan fluorescence anisotropy. $YjfN_{WT}$ or $YjfN_{A70K}$ was incubated for 5 min with various amounts of $DegP_{S210A}$. Then, fluorescence anisotropy of the solution was measured using a QuantaMaster 400 steady-state spectrofluorometer (Photon Technology International) with excitation at 298 nm and emission at 340 nm.

Aggregation (light scattering) assay. OmpA (200 μM, dissolved in 8 M urea) was diluted 100-fold in buffer containing proteins or peptides. The solution was mixed by pipetting (20 s), and then absorbance (500 nm) was measured every 10 s for 15 min using an Ultrospec 7000 UV-Vis spectrometer (GE Healthcare). After 15 min, the reaction solutions were transferred to 1.5-ml tubes, and then DegP (final concentration, 5 μM) was added to perform degradation assays. Protein aggregation of lysozyme or luciferase was monitored by measuring absorbance (500 nm) using an Infinite M200 microplate reader (Tecan). Dithiothreitol was added (final concentration, 10 mM) to trigger the aggregation of lysozyme (10 μM), and the temperature was elevated to 42°C to trigger the aggregation of luciferase (0.2 μM). Fifty micromolar YjfN was used to determine whether it can prevent protein aggregation.

Viability assay. Overnight cultures grown at 34°C were diluted 100-fold into 4 ml LB with appropriate antibiotics and grown to an OD_{600} of ~0.2 at 34°C. Then, 4-μl aliquots from a set of 10-fold serial dilutions of the cultures were dotted on the plates with appropriate antibiotics and inducer (0.4 mM IPTG). Cell viability was observed after 18 h of incubation at 37°C or 43°C.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00519-17.

SUPPLEMENTAL FILE 1, PDF file, 1.2 MB.

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We declare no conflicts of interest.

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