FliT Selectively Enhances Proteolysis of FlhC Subunit in FlhD_4C_2 Complex by an ATP-dependent Protease, ClpXP*

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Yoshiharu Sato‡, Akiko Takaya§, Chakib Mouslim∥, Kelly T. Hughes‡, and Tomoko Yamamoto‡,‡,‡
From the ‡Department of Microbiology and Molecular Genetics, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, 260-8675 Japan and the §Department of Biology, University of Utah, Salt Lake City, Utah 84112

Background: The flagellum-related protein FliT and the ATP-dependent protease ClpXP negatively regulate the activity of the flagellar master transcriptional regulator, FlhD_4C_2.

Results: FliT selectively enhances the ClpXP-dependent proteolysis of FlhC subunit of FlhD_4C_2.

Conclusion: FliT and ClpXP work concertedly to repress FlhD_4C_2 activity by enhanced degradation of FlhC subunit.

Significance: Enhancement of ClpXP-dependent proteolysis of FlhC by FliT is a novel example of regulated proteolysis.

We previously reported that the ClpXP ATP-dependent protease specifically recognizes and degrades the flagellar master transcriptional activating complex, FlhD_4C_2, to negatively control flagellar biogenesis. The flagellar-related protein, FliT, is also a negative regulator of flagellar regulon by inhibiting the binding of FlhD_4C_2 to the promoter DNA. We have found a novel pathway of FliT inhibition of FlhD_4C_2 activity connected to ClpXP proteolysis. An in vitro degradation assay using purified proteins shows that FliT selectively increases ClpXP proteolysis of the FlhC subunit in the FlhD_4C_2 complex. FliT behaves specifically to ClpXP-dependent proteolysis of FlhC. An in vitro interaction assay detects the ternary complex of FliT-FlhD_4C_2-ClpX. FliT promotes the affinity of ClpX against FlhD_4C_2 complex, whereas FliT does not directly interact with ClpX. Thus, FliT interacts with the FlhC in FlhD_4C_2 complex and increases the presentation of the FlhC recognition region to ClpX. The DNA-bound form of FlhD_4C_2 complex is resistant to ClpXP proteolysis. We suggest that the role of FliT in negatively controlling the flagellar gene expression involves increasing free molecules of FlhD_4C_2 sensitive to ClpXP proteolysis by inhibiting the binding to the promoter DNA as well as enhancing the selective proteolysis of FlhC subunit by ClpXP.

ClpXP is a member of the AAA^+ proteases (the term AAA comes from ATPases associated with diverse cellular activities) that have important regulatory functions in bacterial cells by adjusting the activity of key metabolic enzymes or limiting the availability of critical regulatory proteins that control gene expression (1, 2). The ClpP component of ClpXP consists of two stacked heptameric rings, which enclose a central chamber containing the 14 active sites of the peptidase (3). The ClpX component is a hexameric ring ATPase that binds substrate proteins, denatures them, and translocates the unfolded polypeptides into the ClpP degradation chamber (4). Substrate recognition is critical for the targeted degradation of regulatory proteins whose level needs to be precisely controlled in cells. In some instances, substrates are recognized directly by ClpXP by a degradation tag (5). In other cases, which are called regulated proteolysis, an accessory protein called an adaptor is required to ensure efficient degradation (1, 6–8). Adaptor proteins enhance or expand the substrate recognition of their cognate proteases. For example, an adaptor protein, SspB, is present in Escherichia coli (7). When E. coli cells encounter amino acid starvation, translation stalls, and the generated truncated nascent peptides are tagged. SspB binds to both SsrA-tagged peptides and ClpX, thereby increasing the effective local concentration of these molecules. This adaptor-mediated degradation would rescue ribosome stalling caused by amino acid starvation.

We have recently reported that a Salmonella protein, YdiV, which was initially identified as a negative regulator of flagellar gene expression (9–11), accelerates ClpXP-dependent degradation of FlhD_4C_2, a master transcriptional regulator that acts at the apex of the transcription hierarchy of flagellar genes organized into three promoter classes. The data show that YdiV acts as an adaptor protein binding the FlhD subunit and delivering the FlhD_4C_2 complex to ClpXP protease for degradation (12). YdiV interacts with FlhD_4C_2 complex bound to the specific promoter DNA to release FlhD_4C_2 from the DNA-protein complex (12). YdiV is the first example of dual function protein that targets the transcriptional regulator for protease-dependent degradation by releasing the previously bound regulator protein from the DNA. Substrates identified for ClpXP proteolysis include many transcriptional activators with DNA binding activity, such as RpoS, CtrA, and FlhD_4C_2 (2, 13, 14). These specifically and strongly bind to the promoter DNA to activate transcription of their corresponding gene. It is therefore assumed that the specific binding of DNA to substrate protein may protect the protein from ClpXP proteolysis. Here we dem-

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1 To whom correspondence may be addressed: Dept. of Biology, University of Utah, Salt Lake City, UT 84112. Tel.: 801-587-3367; Fax: 801-585-9735; E-mail: kelly.hughes@utah.edu.

2 To whom correspondence may be addressed: Dept. of Microbiology and Molecular Genetics, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba 260-8675, Japan. Tel.: 81-43-226-2927; Fax: 81-43-226-2927; E-mail: tomoko-y@faculty.chiba-u.jp.
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Table 1: Bacterial strains used in this study

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Table 2: Plasmids used in this study

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In Vivo Transcription of flh Promoter—In vivo transcription of the FlhD4C2-dependent flil promoter was monitored using a mini-Mu lac operon reporter Mudl (21). Transposition of Mudl into the chromosome of Salmonella Typhimurium strain LT2 was performed as described, selecting for Mudl-encoded kanamycin resistance (22). Selection plates also contained bacteriophage χ (Chi), which kills flagellated cells (23) and provided a positive selection for Mudl insertions that had disrupted flagellar genes. Mudl insertions were then subject to complementation and DNA sequence analysis. Selection for χ resistance and flagellar gene complementation analysis was performed as described (24). Complementation studies followed by DNA sequence analysis of the flh5100::Mudl allele used here determined that the Mudl had inserted after the first base of amino acid 97 in the flil coding region and placed the lac operon under the control of the flil promoter.

Construction of Plasmids—To construct plasmid pHCX1 encoding His6-TEV-ClpX, the clpX gene was amplified from the chromosome of strain χ3306, using the primers clpX-NheTEV-f (GGTGATCCCTCGAGGAAACCTGTACTTCCAAATGACAGATAACGCAAAG) and clpX-Xho-r (GCAATCGAGCTAGCGAAAACCTGTACTTC). The fragment generated was cleaved with NcoI at the 5’ end and XhoI at the 3’ end, and cloned into pTrcHisA. To construct plasmid pHCX1 encoding FlhD-His6-TEV site-FlhC, the flhD gene was amplified from the chromosome with flhD-Neo-f (CTTCCCCTAGGAAACAATGCATACA) and flhD-Xho-r (GCAATCTCGAGTTTATGCAGAAGCCCT). The fragment generated was cleaved with Ncol at the 5’ end and Xhol at the 3’ end and cloned into pTrcHisA. To generate the flhc-containing plasmid, the gene was amplified by pHCN-Ndel-TEV-f (CAGCTGTCATCATGCAACATCATCACCATATGCAATGAGGAAACCTGTACTTCCAAATGACAGATAACGCAAAG) and flhC-Xho-r (ACACCGCTCAGATTAAACAGCCTGTTGAC) from the plasmid of the strain χ3306. The fragment generated was cleaved with Ndel and Xhol and initially cloned into pCold. The region of His6-TEV site-flhc on pCold was amplified by using the primers flhc-Xho-f (CCGATCTCGAGGAAACCTGTACTTCCAAATGACAGATAACGCAAAG) and flhC-Pst-r (GATCCCTCGAGTTTATGCAGAAGCCCT). The fragment generated was cleaved with Xhol at the 5’ end and SstI at the 3’ end and cloned into pTrcHisA-FlhD constructed as described above.

Purification of ClpP, Clpx, Flit, and FlhD₄C₂(His₆FlhC₂) Complex—To purify ClpP, 1 liter of cultured E. coli DH5αZ1 derivative carrying pTKY610 (CS5123) was incubated at 37°C until the cells reached an A₆₀₀ of 0.4. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM for 3 h before the cells were collected by centrifugation. Cells suspended in 50 ml of buffer A (50 mM Tris-HCl, pH 7.5, 150 mM KCl, 10% glycerol) were incubated on ice for 30 min. After washing, the medium was supplemented with chloramphenicol (20 μg ml⁻¹) tetracycline (10 μg ml⁻¹), ampicillin (25 μg ml⁻¹), kanamycin (25 μg ml⁻¹), and spectinomycin (25 μg ml⁻¹).
lysis by sonication, the supernatant after centrifugation was loaded onto a HiTrap Q HP column (1 ml; GE Healthcare) and washed with buffer C (50 mM Tris-HCl, pH 7.5, 1 mM MgSO₄, 1 mM DTT, 40 mM NaCl, 10% glycerol). The proteins were eluted with 20 ml of a 0–1000 mM NaCl linear gradient. The eluent was run on gel chromatography using Superdex 200 10/300GL (24 ml; GE Healthcare) and washed with buffer D (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 20 mM imidazole, 0.02% Triton X-100, 20% glycerol) containing 1 tablet of protease inhibitor (Complete EDTA-free, Roche Applied Science). After lysis by sonication, the supernatant collected by centrifugation was batch-bound to 1 ml of Ni-NTA-agarose (Qiagen) and washed with buffer E (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 10% glycerol). For concentration, the fractions containing ClpX were loaded onto HiTrap Q HP (1 ml; GE Healthcare) and washed with buffer H (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 20% glycerol). The proteins were eluted with 20 ml of a 0–1000 mM NaCl linear gradient. The peak fractions of ClpX were collected and dialyzed against buffer H.

To purify ClpX, 1 liter of a culture of E. coli MC4100, ΔclpX-Δlon::Cm, ΔhslUV-Δgal::Tc carrying pHCX1 (CS6764) was incubated at 37 °C until the cells reached an A₆₀₀ of 0.4. Isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM for 2 h before the cells were collected by centrifugation. They were resuspended in 100 ml of buffer D (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 20 mM imidazole, 0.02% Triton X-100, 20% glycerol) containing 2 tablets of Complete EDTA-free protease inhibitor (Roche Applied Science). After lysis by sonication, the supernatant collected following centrifugation was batch-bound to 1 ml of Ni-NTA-agarose (Qiagen) and washed with buffer D. Bound ClpX proteins were eluted with buffer E (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 500 mM imidazole, 0.02% Triton X-100, 20% glycerol). A one-twentieth molar amount of His-TEV protein was added to the elution fraction to remove the His-TEV cut site tag. The sample was dialyzed against buffer F (50 mM HEPES-KOH, pH 7.5, 300 mM NaCl, 20 mM imidazole, 0.02% Triton X-100, 20% glycerol) in a cold room for 4 h. Fractions containing ClpX were run on gel chromatography using Superdex 200 10/300GL (24 ml; GE Healthcare) with buffer I (50 mM NaHPO₄, pH 8.0, 300 mM NaCl, 20 mM imidazole, 1 mM DTT) containing 5 units of Precision protease overnight at 4 °C. The dialyzed sample was rebound to GST-Sepharose beads, and the flow-through fraction was passed through a HiTrap Q HP 1-ml column (equilibrated with buffer J and eluted with a 0–1000 mM NaCl linear gradient).

**FliT Enhances a ClpXP-catalyzed Proteolysis of FlhC**

In Vitro Degradation Assay—The degradation assay was carried out in Clp assay buffer (25 mM HEPES, pH 7.6, 50 mM KCl, 10 mM MgCl₂, and 1 mM DTT) containing purified proteins. FlhD₄-(His₆FlhC)₂ complex was incubated with ClpX and ClpP at 37 °C in the presence or absence of 3 mM ATP. To see the effect of FliT on the ClpXP proteolysis, a FlhD₄-(His₆FlhC)₂ complex or intact FliT was preincubated with FliT for 5 min and then proteolysed as above. A portion of the reaction mixture was mixed with SDS-sample buffer and processed on 16% SDS-PAGE and Coomassie Brilliant Blue staining.

In Vitro Pull-down Assay—Purified FlhD₄-(His₆FlhC)₂ (1 μM) was batch-bound to Ni-NTA resin in 100 μl of Clp binding buffer with 10 mM imidazole (25 mM HEPES-KOH, pH 7.6, 50 mM KCl, 10 mM MgCl₂, 5% glycerol, 1 mM DTT, 1 mM ATP, or AMP-PNP, 10 mM imidazole). 0.1 μM ClpX₆ and 1 μM FliT₂ were preincubated with FlhD₄-(His₆FlhC)₂ for 5 min. Bound beads were washed twice with 500 μl of Clp binding buffer. ClpX and FliT bound to FlhD₄-(His₆FlhC)₂ were eluted with 500 mM imidazole-containing Clp assay buffer. A pull-down assay with GST-Sepharose beads was used to detect ternary complex of FliT–FlhD₄C₂–ClpX. 1 μM purified (GST–FliT)₂ was batch-bound to GST resin in 100 μl of Clp binding buffer with 1 mM ATPγS. 0.1 μM ClpX₆ and 1 μM FlhD₄-(His₆FlhC)₂ were preincubated with GST–FliT for 5 min. Bound beads were washed twice with 500 μl of Clp assay buffer. ClpX and FlhD₄-(His₆FlhC)₂ bound to GST–FliT were coeluted with 100 μl GSH-containing Clp assay buffer.

**ATPase Assay**—ATPase activity was measured according to the colorimetric method of Lanzetta et al. (25) in 25 μl of Clp assay buffer. Samples contained 0.5 μg of ClpX and 3 mM ATP. 2.0 μg of FlhD₄C₂, 0.5 μg of ClpP, and 0.5 μg of FliT, respectively, were added to the samples when required. The reactions...
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were stopped by the addition of 5 μl of 500 mM EDTA. The reaction mixture was mixed with 200 μl of malachite green mixture (3:1:1 volumes of water, 2 mM malachite green, 50 mM ammonium molybdate, and 2.5% polyvinyl alcohol), and 25 μl of 1 mM sodium citrate. Absorption at 600 nm was measured after 30 min of incubation at room temperature. Results were compared with the calibration curve prepared for the phosphate salt.

Biolayer Interferometry (BLI) Analysis—To measure affinity and kinetics of the interaction of FlhD₄₋₅(His₆FlhC)₂ and DNA, a biolayer interferometry assay was done in an Octet RED96 instrument. Purified FlhD₄₋₅(His₆FlhC)₂ was captured on an Ni-NTA sensor (Fortebra). Purified flhB promoter DNA was serially diluted in Clp assay buffer and bound to captured FlhD₄₋₅(His₆FlhC)₂. One-shot kinetics analysis was done with parallel sensors, and a local R_max model was fitted to estimate K_d and k_on/k_off. The first 10 s of dissociation time is omitted from the curve fitting analysis to minimize the buffer effect. To prepare DNA fragments of FlhD₄₋₅ binding site, the class II promoter region ranging from −226 to +48 of the transcriptional start site of flhB was amplified by PCR (generated fragment size is 274 bp). To prepare unrelated DNA fragment unbound to FlhD₄₋₅ as a negative control, a DNA mixture (3:1:1:1 volumes of water, 2 mM malachite green, 50 mM ammonium molybdate, and 2.5% polyvinyl alcohol), and 25 μl of 1 mM sodium citrate. Absorption at 600 nm was measured after 30 min of incubation at room temperature. Results were compared with the calibration curve prepared for the phosphate salt.

RESULTS

FlhD₄₋₅C₂ Inhibition by FliT Is Partially Suppressed in ClpXP Mutants—The overexpression of FliT inhibits motility and transcription of flagellar genes (26). This effect has been shown to be dependent on the binding of FliT to the FlhD₄₋₅C₂ complex through the direct interaction with the ClpX subunit (17, 26). We recently established that overexpression of YdiV promotes degradation of FlhD₄₋₅C₂ in a ClpXP-dependent manner (12), leading to decreasing flagellar gene expression. This ClpXP-dependent effect of YdiV inhibition of flagellar genes transcription appears to be contingent on the degree of expression of YdiV because the expression of YdiV from a tetracycline-inducible promoter (P_tet) inhibits transcription of FlhD₄₋₅C₂-dependent gene expression in a ClpXP-dependent manner, yet the expression of YdiV from a stronger arabinose promoter (P_arabin) inhibits FlhD₄₋₅C₂-dependent transcription in a ClpXP-independent pathway (12). The ClpXP-independent role of YdiV in suppressing gene expression was due to its interaction with FlhD₄₋₅C₂ bound to the class II promoter DNA to release FlhD₄₋₅C₂ from the DNA-protein complex (12). We thus investigated whether the effect of FliT on FlhD₄₋₅C₂-dependent transcription of flagellar genes also involved the ClpXP pathway. To test this hypothesis, the activity of a transcriptional fusion to a class II flagellar gene, fliL, in a strain overexpressing fliT under the control of P_arabin (in strain TH18233 (P_arabin::fliT−/ flL::MudI) was measured. In the presence of the arabinose inducer, overproduction of FliT resulted in inhibition of transcription of fliL, which appeared to be moderately suppressed in the absence of ClpX because the FliT-dependent inhibition of fliL transcription was partially diminished, but not abolished, in a clpX null mutant background (Table 3). This suggests that the ClpXP pathway is involved in the FliT-dependent inhibition of fliL transcription.

FliT Directly Accelerates the Degradation of FlhC Subunit in FlhD₄₋₅C₂ Complex by ClpXP Protease—The finding that the FliT-dependent inhibition of fliL transcription was suppressed partially by clpXP mutation suggests that FliT might directly enhance the degradation of FlhD₄₋₅C₂ by ClpXP. Thus, the effects of FliT on the degradation of FlhD₄₋₅C₂ by ClpXP were tested in vitro. Purified ClpXP slowly degraded FlhC and FlhD subunits (Fig. 1A). In vitro degradation with purified FliT showed that it enhanced the ClpXP-dependent degradation of FlhC subunit but not the FlhD subunit in FlhD₄₋₅C₂ (Fig. 1A). In the absence of ATP, both FlhC and FlhD subunits remained undegraded, suggesting that the enhancement effect of FliT is linked to ATPase activity of ClpX. When ClpXP was removed from this reaction, degradation of FlhD₄₋₅C₂ did not occur (data not shown), excluding the possibilities that FliT also possesses protease activity and that proteases contaminate the purified FliT fraction. The effect of FliT on degradation of individual FlhC by ClpXP in vitro was not determined because the FlhC subunit could not be purified as a soluble protein, whereas FlhC could be purified when complexed in FlhD₄₋₅C₂. Purification of individual FlhC has so far not been successful (27, 28).

In vitro degradation assays, using purified FlhD₄₋₅C₂ complex whose FlhC subunit was His₆-tagged at the N terminus, were also done to test the effect of N-terminal tagging of FlhC (Fig. 1B). ClpXP-dependent degradation of the FlhC subunit of the His-tagged FlhD₄₋₅C₂ complex was stimulated in the presence of FliT to the same extent as intact FlhC (Fig. 1, A–C). Hence, tagging the N terminus of FlhC subunit does not affect the function of FliT in enhancing ClpXP-dependent FlhC degradation. This His-tagged version of FlhD₄₋₅(His₆FlhC)₂ was therefore used for subsequent analyses. To check the effect of ADP that is generated during the reaction and inhibits the activity of ClpX, an in vitro degradation assay under regeneration condition was also performed by adding creatine kinase and creatine phosphate into the reaction. A consistent result was obtained in the degradation assay by the regeneration system, as expected from the clear difference of FlhC degradation between FliT(+) and FliT(−) at the early time point of reaction (~15 min), where the generated ADP is negligible (data not shown).

N-terminal Domain of ClpX Is Required for Enhancement Effect of FliT on FlhC Degradation—ClpX unfolds proteins and presents them to ClpP for degradation. A zinc-binding domain (ZBD) at the N terminus of ClpX is proposed to play a role in the recognition of substrates destined for degradation (20, 29, 30). We asked whether the ZBD domain of ClpX is required for the enhancing effect of FliT on ClpXP proteolysis of FlhC by an in

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Table 3. Effect of FliT overexpression on transcription of class 2 fliL gene in the clpX⁺ and ΔclpX backgrounds
vitro degradation assay with the ZBD-depleted mutant ClpX. Removal of the N-terminal domain (deletion of amino acids 1–60) of ClpX completely abolished the enhancement of ClpXP-catalyzed proteolysis of FlhC by FliT (Fig. 2, a and b). The ZBD mutant of Salmonella ClpX used in this study could rapidly degrade the model substrate, GFP-SsrA (data not shown), as in E. coli ClpX (20). The requirement of the ZBD domain of ClpX for the inhibitory effect of FliT was also examined in a strain overexpressing the fliT gene under the control of P<sub>araBAD</sub>. Depletion of the ZBD domain of ClpX partially suppressed the inhibitory effect of the overproduction of FliT on flIL transcription to the same extent as complete deletion of ClpX (Table 3). These results suggest that the N-terminal ZBD of ClpX is essential for the enhancement of ClpXP proteolysis of FlhC by FliT.

**FliT Acts Efficiently and Specifically**—Most of enhancement factors for AAA<sup>+</sup> proteases are recycled during enhancement of the degradation of target substrate by protease, whereas some of the enhancement factors are degraded together with the target substrate by protease, such as Vir (31) and MecA (32); thus, a stoichiometric amount is required for an efficient enhancement reaction. To reveal in which way FliT acts, we examined whether FliT is recycled in the enhancement reaction. In vitro degradation assays using four different concentrations of FliTs showed that only 0.02 μM FliT<sub>2</sub> sufficiently enhanced the degradation of a 100-fold molar excess of FlhC relative to FliT (Fig. 2, c and d). At a time of 10 min, about 30% of FlhC degradation was enhanced, corresponding to enhancement of 0.6 μM FlhC degradation by 0.02 μM FliT<sub>2</sub> (30-fold molar excess). Moreover, the effect of FliT<sub>2</sub> was almost saturated around 1 μM. These results suggest that FliT is recycled during enhancement of FlhC degradation, and only a small amount of FliT relative to FlhD<sub>4</sub>C<sub>2</sub> is required for efficient enhancement of FlhC degradation.
To determine whether FliT is a specific enhancement factor for FlhC, *in vitro* degradation was assayed using purified model substrates, GFP-SsrA and LambdaO. Whether in the presence or absence of FliT, His$_6$GFP-SsrA and LambdaO were degraded at similar rates (Fig. 3, *a* and *b*). As shown in the degradation of the FlhD$_4$C$_2$ complex (Fig. 1, *b* and *c*), degradation of the FlhD subunit was unaffected by the presence of FliT. These results suggest that FliT works specifically on FlhC, based on the specific interaction between FlhC and FliT rather than as a general enhancement factor promoting chaperone activity of ATPase subunit, like MecA for ClpC (33), or promoting the protease activity of ClpP (34).

**FliT, FlhD$_4$C$_2$, and ClpX Form Ternary Complex in Vitro—**

To understand the mechanism of action of FliT on FlhD$_4$C$_2$ degradation, interaction between FliT and ClpX was assayed. Previously known adaptors enhancing the degradation of ClpXP substrates, such as SspB and UmuD, act as a tethering mechanism. In this way, an adaptor interacts strongly with both the cognate substrate and ClpX to mediate a tighter interaction between ClpX and the substrate. Because ZBD is involved in the enhancing effect of FliT (Fig. 2a), it was assumed that ZBD directly interacts with FliT. BLI analysis allowed us to investigate the interaction between ClpX and FliT. LambdaO, known as a ZBD-binding protein (35), bound directly with ZBD in a concentration-dependent manner (data not shown). In contrast, there was no interaction between FliT and ZBD under the same condition. To examine the interaction between FliT and ClpX further, we also performed an *in vitro* pull-down assay using GST-tagged FliT. As reported previously, binary interaction between GST-FliT and FlhD$_4$C$_2$ was detected by the pull-down assay (Fig. 4a, lanes 1–3). No direct interaction between FliT and ClpX could be detected (Fig. 4a, lanes 4–6). On the other hand, preincubation of ClpX, GST-FliT, and FlhD$_4$-(His$_6$FlhC)$_2$ generated a ternary complex of the three compo-

**FIGURE 2. Effect of FliT to enhance *in vitro* degradation of FlhC requires N-terminal zinc binding domain of ClpX, and a very small amount of FliT efficiently enhances the degradation of FlhC.** The degradation assay using N-terminal zinc-binding domain-depleted mutant ClpX was performed as described in the legend to Fig. 1. The level of FlhC during degradation assay is shown in *a*, and the quantification of the band intensity of FlhC is shown in *b*. Mean and S.D. values (error bars) of triplicate independent experiments are shown. The *solid lines* and the *dashed lines* show the data of FliT(-) and FliT(+), respectively. *Squares* and *circles* show the data of wild type ClpX and ΔZBD-ClpX, respectively. Degradation of 1.0 μM FlhD$_4$C$_2$ by 0.2 μM ClpXP was examined in the presence of four different concentrations of FliT$_2$ (0, 0.02, 1.0, and 3.0 μM) in the degradation assay, as shown in *c*. The level of FlhC during the degradation assay is shown. Quantification of FlhC relative to the value at time 0 in the degradation assay is shown in *d*. *Diamonds*, *squares*, *triangles*, and *circles* show the data of 0, 0.02, 1.0, and 3.0 μM, respectively. Mean and S.D. values of triplicate independent experiments are given. 0.02 μM FliT$_2$ is sufficient to enhance the degradation of 2.0 μM FlhC by 0.2 μM ClpX$_6$ClpP$_{14}$.

**FIGURE 3. Effect of FliT on ClpXP-catalyzed proteolysis of GFP-SsrA and of LambdaO *in vitro*.** Degradation of 1.5 μM GFP-SsrA (*a*) and LambdaO (*b*) in the presence or absence of 1 μM FliT$_2$ was assayed. The same reaction buffer with the FlhD$_4$C$_2$ degradation assay was used. Mean and S.D. values (error bars) of the results of triplicate independent experiments for GFP-SsrA and LambdaO are given in *c* and *d*, respectively.
nents (Fig. 4, a (lanes 7–9) and b). When purified GST instead of GST-FliT was incubated with ClpX and FlhD4C2, no ClpX or FlhD or FlhC was detected (Fig. 4, a (lanes 10–12) and b), which suggests that FliT mediates the formation of the ternary complex with ClpX and FlhD4C2. Whereas FliT stimulated ATPase activity of ClpX in the presence of FlhD4-(His6FlhC)2 and ClpP (Fig. 5, p < 0.01, Student’s t test), there was no stimulation in the absence of FlhD4-(His6FlhC)2. The data also suggest that FliT does not directly interact with ClpX but does so with FlhC, forming a FliT-FlhD4C2-ClpX ternary complex.

Binding of FlhD4C2 to ClpX Is Promoted by FliT—The formation of a ternary complex of FliT-FlhD4C2-ClpX (Fig. 4) suggests that the enhancing effect of FliT is exerted by a stepwise mechanism rather than a simple tethering mechanism, as follows: (i) binding of FliT to FlhD4C2 complex, (ii) alteration of the accessibility of the recognition region on FlhC, and thereby (iii) increase of the affinity between ClpX and FlhD4C2. To test this hypothesis, the binding between ClpX and FlhD4-(His6FlhC)2 complex was assessed by an in vitro pull-down assay using Ni-NTA-agarose beads. Coeluted ClpX bound to His-tagged FlhD4C2 in the presence or the absence of FliT was examined. In the presence of FliT, binding of ClpX to FlhD4C2 complex increased (Fig. 6, a and b, p < 0.01, Student’s t test), suggesting that affinity between ClpX and FlhD4C2 complex is promoted by FliT. A pull-down assay using a different FlhD4C2 protein with a His tag at the N terminus of FlhD also promoted interaction.
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To distinguish whether FliT enhances the recognition of FlhD4C2 complex by ClpX and/or promotes the unfolding of FlhD4C2 complex requiring ATPase activity of ClpX, a pull-down assay was performed with a non-hydrolyzable ATP analog, AMP-PNP. Increased affinity between ClpX and FlhD4C2 complex by FliT was also obtained with AMP-PNP (Fig. 6b). Thus, the FliT effect works at a very early stage of substrate processing by ClpX that does not require ATPase activity.

*FlhD4C2 Is Protected from Degradation by ClpXP When Bound to Class II Promoter DNA—*FlhD4C2 is a DNA-binding protein that strongly binds to the class II promoter region. To determine whether the FliT-ClpXP system degrades FlhD4C2 bound to the promoter DNA, interaction between FlhB promoter DNA and His-tagged FlhD4C2-(His6FlhC)2 was initially assessed by the BLI method. Sensor responses for FlhD4C2-bound DNA interaction showed concentration dependence, with responses reaching a plateau around maximum concentration (Fig. 7a), indicating a specific interaction between FlhD4C2-(His6FlhC)2 and DNA (Fig. 7a). Control unrelated DNA fragments showed only faint or no interaction with FlhD4C2-(His6FlhC)2 (Fig. 7b). K〈sub〉D of 2.1 ± 1.0 nm and t〈sub〉50 of 12.8 min ± 4.8 (decay of FlhD4C2-DNA complex) were estimated from the sensorgrams of triplicate independent BLI assays. Hence, purified FlhD4C2 strongly binds with flhB promoter DNA and dissociates slowly under the conditions of the degradation assay.

In examining the effect of DNA on the degradation of FlhD4C2 by ClpXP in the presence of DNA (Fig. 7, c and d), it strongly inhibited the degradation of FlhC and FlhD, suggesting that the DNA-bound form of FlhD4C2 is resistant to proteolysis. Control DNA fragment unbound to FlhD4C2 did not affect the degradation of FlhD4C2, suggesting that this inhibition is due to specific interaction between FlhD4C2 and its binding site on class II promoter DNA. On the other hand, when 1 μM FliT2 was given before the addition of DNA, FlhC was rapidly degraded by ClpXP, from which we can infer that inhibition of the FlhD4C2-DNA interaction by FliT has an additional important role in the ClpXP-dependent pathway of FliT inhibition; FliT increases the proteolytically sensitive form of free FlhD4C2, which is preferentially degraded by ClpXP. If FliT was incubated after the addition of DNA, the inhibition of FlhC degradation by DNA was partially suppressed (Fig. 7, c and d). This suggests that once FlhD4C2 binds to DNA, interaction between FliT and FlhD4C2 is decreased due to the hindered access to the interaction site of FliT on FlhD4C2, consistent with the surface plasmon resonance experiments by Aldridge *et al.* (36). Remaining enhancement of FlhC degradation compared with the FliT(−)/flhB promoter DNA(+) reaction suggests that FlhD4C2 released from DNA during the degradation assay is rapidly captured by FliT and delivered to ClpXP-dependent proteolysis.

**DISCUSSION**

**Mechanism of Enhancement of ClpXP-dependent Degradation of FlhC by FliT**—Several mechanisms that enhance substrate degradation by AAA<sup>+</sup> proteases have been proposed. These include the induction of subcellular co-localization of a protease and a substrate (2), promotion of substrate-protease
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ClpX more tightly than the monomeric state (39, 40). Tight recognition and disassembly of only one protomer in the MuA tetramer by ClpXP is also an example of conformation-dependent control of substrate recognition (41). Elucidation of the mechanism of an enhanced interaction between FlhC and ClpX from the structural viewpoint is an on-going project in our laboratory.

Degradation of FlhC Subunit in FlhD$_4$C$_2$ Complex Is Selectively Enhanced by FliT—The FliT effect of enhancing the degradation by ClpXP was found only for the FlhC subunit in the FlhD$_4$C$_2$ complex (Fig. 1), despite FliT promoting the binding of ClpX to FlhD$_4$C$_2$ (Fig. 6). The lack of direct interaction of ClpX with FliT and the fact that FliT interacts only with FlhC, but not with FlhD (17), suggest that FliT binding to FlhD$_4$C$_2$ specifically affects the FlhC subunit and ClpX-FlhC interaction. Considering the previously proposed model that ClpX does not recognize the substrate without degron (5), along with our observation that ClpXP degrades both subunits of the FlhD$_4$C$_2$ complex (Fig. 1), it seems that both subunits of the FlhD$_4$C$_2$ complex have their own individual degrons. Hence, it is of interest that ClpX-FlhD$_4$C$_2$ interaction is promoted by FliT, but only FlhC is enhanced. Degradation rates of FlhD and FlhC in vitro differ substantially (Fig. 1), suggesting that degradation of both subunits in the FlhD$_4$C$_2$ complex does not proceed simultaneously. Therefore, the individual subunits in FlhD$_4$C$_2$ are likely to be independently recognized and processed by ClpXP. Dissociation of the FlhD subunit from FlhC-FliT-ClpXP ternary complex during processing of FlhC subunit is probably the reason that it has no effect on FlhD degradation in the presence of FliT. Interestingly, in contrast to FliT, YdiV interacts with FlhD subunit in FlhD$_4$C$_2$ (42), but it enhanced ClpXP-dependent proteolysis of both subunits, FlhC and FlhD, of FlhD$_4$C$_2$ (12). Rapid degradation of the FlhD subunit in the FlhD$_4$C$_2$ could generate unstable dissociated individual FlhC and cause drastic conformational change to FlhC, which may lead to its enhanced proteolysis.

FlhD and FlhC exist as two forms in the cell, FlhD$_2$ and FlhD$_4$C$_2$ (43). For efficient binding of FlhD$_4$C$_2$ to class II promoter DNA, both subunits of FlhD$_4$C$_2$ are required (44), suggesting that enhanced degradation of only the FlhC subunit is sufficient to control FlhD$_4$C$_2$ activity. The fact that FlhD$_2$ also has a DNA binding ability (43) indicates its distinctive role and suggests that selective enhancement of FlhC degradation has an additional effect other than decreasing FlhD$_4$C$_2$ activity. FlhD$_2$ also has roles in stabilizing FlhC and promoting the DNA binding ability of FlhC by forming stable hetero-oligomer FlhD$_4$C$_2$. Thus, the cellular pool of individual FlhD$_2$ might affect FlhD$_4$C$_2$ activity and support rapid construction of active FlhD$_4$C$_2$ when cells require flagella. Enhancement of FlhC degradation by FliT-ClpXP may be involved in adjusting the cellular pool of FlhD$_2$.

Multiple Degradation Enhancement Factors for FlhD$_4$C$_2$—We found a second factor that enhances FlhD$_4$C$_2$ degradation in addition to YdiV. Intriguingly, the ClpXP-dependent effect of FliT in vivo was stronger than that of YdiV (data not shown), suggesting that both work on the ClpXP-dependent pathway but in dissimilar ways. The two factors interact with different partners; YdiV interacts with FlhD, whereas FliT interacts with
the FlhC subunit in FlhD$_{4}$C$_{2}$. Another notable difference relates to the DNA binding state of FlhD$_{4}$C$_{2}$. We previously showed that YdiV stripped off FlhD$_{4}$C$_{2}$ that was prebound to DNA (12). On the other hand, FliT could not interact with FlhD$_{4}$C$_{2}$ prebound to DNA (36). ClpXP moderately degrades both subunits of FlhD$_{4}$C$_{2}$ even without FliT; however, the DNA-bound form of FlhD$_{4}$C$_{2}$ becomes insensitive to proteolysis by ClpXP (Fig. 7). Furthermore, the FlhD$_{4}$C$_{2}$-DNA complex is very stable, as shown from the slow dissociation of FlhD$_{4}$C$_{2}$ from FlhD$_{4}$C$_{2}$-DNA complex ($t_{1/2} = 12.8$ min from our BLI assay and $>40$ min as reported by Claret and Hughes (43)). These results suggest that control of FlhD$_{4}$C$_{2}$ activity by FliT or ClpXP is mostly restricted by the state of the DNA-unbound free form of FlhD$_{4}$C$_{2}$. The FliT effect, shifting the equilibrium from the DNA-bound to the DNA-unbound form by inhibiting DNA binding of FlhD$_{4}$C$_{2}$, may have an additional role in the ClpXP-dependent pathway (i.e. increasing the amount of recognizable FlhD and FlhC in FlhD$_{4}$C$_{2}$, which leads to the enhanced proteolysis by ClpXP) (Fig. 8).

In E. coli, multiple anti-adaptors for RssB are expressed to control RpoS levels in multiple modes when cells adapt to the different stresses (e.g. anti-adaptors IraM and IraP are up-regulated for protecting RpoS from rapid degradation by RssB in response to magnesium and phosphate deprivation, respectively) (45). Such multiple adaptor-related factors present a rational strategy for the bacteria to respond to different stresses. In the case of the control of FlhD$_{4}$C$_{2}$, FliT and YdiV seem to have distinct roles and have different timings, considering that expression of YdiV only occurs when Salmonella encounter nutrient poor conditions (10). YdiV may have an important role in shutting off flagellar biogenesis inside macrophage and evading the host immune system (46). For such purposes, YdiV ought to completely inhibit FlhD$_{4}$C$_{2}$ activity; to accomplish a complete shut-off, YdiV acts even on the DNA-bound form of FlhD$_{4}$C$_{2}$ (i.e. on all states of FlhD$_{4}$C$_{2}$). In contrast, FliT expression is regulated by flagellar class II and III promoters (i.e. downstream of flhDC). Hence, accelerated FlhC degradation by FliT-ClpXP is assumed to be important in controlling the number of flagella by a negative feedback mechanism. Salmonella depleted of fltT have more flagella irrespective of the nutrient levels in the culture media (36). Aldridge et al. (36) showed that overexpression of FliT could not completely abolish the basal level of flagellar expression. Consistent with these results, our data show that the effect of FliT-ClpXP regulation is maximum on the free DNA-unbound FlhD$_{4}$C$_{2}$ (Fig. 7) (i.e. the DNA-bound state of FlhD$_{4}$C$_{2}$ becomes resistant to FliT and to ClpXP). This moderate negative regulation dependent on the DNA binding state of FlhD$_{4}$C$_{2}$ may contribute to the retention of a basal level of flagellar expression in order to construct a substantial number of flagella or work as a buffer to respond rapidly to external stimuli favoring the flagellated state, as in the model proposed by Aldridge et al. (36). The insensitivity of DNA-bound FlhD$_{4}$C$_{2}$ to FliT inhibition and the long half-life of DNA-bound FlhD$_{4}$C$_{2}$ indicate the importance of the stringent control of the cellular level of the free form of FlhD$_{4}$C$_{2}$ in the regulation of flagellar biogenesis. The fact that only a very small amount of FliT efficiently enhanced ClpXP-dependent degradation of FlhC (Fig. 2, c and d) suggests the relative impact and efficacy of the ClpXP pathway compared with the stoichiometric action of FliT alone inhibiting DNA binding of FlhD$_{4}$C$_{2}$. From these results, the ClpXP pathway of the FliT-dependent anti-FlhD$_{4}$C$_{2}$ effect can be considered as having a substantial role in the negative feedback loop that controls flagellar biogenesis.

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