FliT Selectively Enhances Proteolysis of FlhC Subunit in FlhD₄C₂ Complex by an ATP-dependent Protease, ClpXP*

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Background: The flagellum-related protein FliT and the ATP-dependent protease ClpXP negatively regulate the activity of the flagellar master transcriptional regulator, FlhD₄C₂.

Results: FliT selectively enhances the ClpXP-dependent proteolysis of FlhC subunit of FlhD₄C₂.

Conclusion: FliT and ClpXP work concertedly to repress FlhD₄C₂ 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 activator complex, FlhD₄C₂, to negatively control flagellar biogenesis. The flagellum-related protein, FliT, is also a negative regulator of flagellar regulon by inhibiting the binding of FlhD₄C₂ to the promoter DNA. We have found a novel pathway of FliT inhibition of FlhD₄C₂ 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 FlhD₄C₂ complex. FliT behaves specifically to ClpXP-dependent proteolysis of FlhC. An in vitro interaction assay detects the ternary complex of FliT-FlhD₄C₂-ClpX. FliT promotes the affinity of ClpX against FlhD₄C₂ complex, whereas FliT does not directly interact with ClpX. Thus, FliT interacts with the FlhC in FlhD₄C₂ complex and increases the presentation of the FlhC recognition region to ClpX. The DNA-bound form of FlhD₄C₂ 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 FlhD₄C₂ 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 ClpXP, 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 FlhD₄C₂, 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 FlhD₄C₂ complex to ClpXP protease for degradation (12). YdiV interacts with FlhD₄C₂ complex bound to the specific promoter DNA to release FlhD₄C₂ 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 FlhD₄C₂ (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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**TABLE 1**

Bacterial strains used in this study

| Strain | Relevant properties | Reference or source |
|--------|---------------------|---------------------|
| S. enterica serovar Typhimurium | | |
| TH18233 | ΔflhBΔ975;flhT (P\_flhBΔ975-flhT) | | |
| TH18236 | ΔflhBΔ975;flhT· flhL5100::MudJ ΔflhC | | |
| TH18237 | ΔflhBΔ975;flhT· flhL5100::MudJ clpX79 (ΔZBD (AA1–60)) | | |
| E. coli | | |
| DH5αZ1 | DH5α lacP | Laboratory collection |
| CS5121 | DH5αZi carrying pTYK610 | |
| CS6757 | MC1400 ΔclpPX-Cm ΔflhDC·Km | Tomoyasu et al. (14) |
| CS6764 | MC1400 ΔclpPX·λom·Cm ΔhilVII·Tc carrying pHCX1 | |
| CS6828 | DH5α carrying pGex6p1-FliT | |
| CS6840 | SG1146 ΔclpC·Cm ΔflhDC·Km carrying pHCl1 | |
| CS6453 | MC1400 Δλom·ΔclpPX·proEX htb carrying gfp·ara/A | |
| CS6530 | DH5α carrying pTYK630 | |

**TABLE 2**

Plasmids used in this study

| Plasmid | Relevant characteristics | Reference or source |
|---------|--------------------------|---------------------|
| pTKY610 | pHE21–2 Δflh12 carrying clpP gene, Ap | Takaya et al. (12) |
| pHCX1 | pTrcHisA carrying clpP gene | This study |
| pTKY630 | pHE21Δclc1 carrying ΔZBD (1–60)·clpX gene | This study |
| pDHCl | pGex6p1 carrying flhD·Hns·flhC gene | This study |
| pGex6p1·FlT | pGex6p1·carrying FlT gene | Imada et al. (17) |
| pUHE1–Δflh12 | pUHE1·Δflh12·system vector, Ap | Garner et al. (19) |
| pProx htb·GFP·sorA | pProx htb carrying gfp·sorA | Woytira et al. (20) |
| pZA4lacP | lacP·Sp | Lab collection |

onstrate that FlhD4C2 bound to the promoter DNA for class II genes in the flagellar regulon is resistant to ClpXP-proteolysis.

A *Salmonella* protein FiT is a multifunctional protein that regulates flagellar biogenesis. FiT functions as an anti-FlhD4C2 factor in transcriptional control (i.e. it inhibits the binding of FlhD4C2 complex to the promoter DNAs of class II genes in flagellar regulon, leading to the negative regulation of flagellar gene expression) (15). FiT is also an export chaperone specific for the filament-capping protein FliD; it binds to FliD to prevent its premature aggregation in the cytoplasm and control its export (16, 17). FiT also associates with ATPases of flagellar apparatus, FliI and FliJ (18).

In the present study, we demonstrate that FiT selectively enhances the proteolysis of FlhC subunit in FlhD4C2 complex by ClpXP. Our analyses show that FiT in *vitro* forms a ternary complex of ClpXP–FiT–FlhD4C2 through interactions with ClpX and FlhD4C2 and between FiT and FlhC. We also show that the DNA-bound form of FlhD4C2 is resistant to ClpXP-dependent proteolysis. These findings indicate that the role of FiT as an anti-FlhD4C2 factor for flagellar expression includes (i) inhibition of FlhD4C2 binding to the promoter DNA, resulting in an increase of the form of FlhD4C2 sensitive to ClpXP-proteolysis, and (ii) enhancement of selective proteolysis of the FlhC subunit in the complex with ClpXP, decreasing the level of functional FlhD4C2.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Media**—Bacterial strains and plasmids are listed in Tables 1 and 2 (12, 17, 19, 20). Bacteria were grown in L broth (1% Bacto tryptone, 0.5% Bacto yeast extract, 0.5% sodium chloride, pH 7.4) and L agar. When necessary, 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).

**In Vivo Transcription of flh. Promoter—**In vivo transcription of the FlhD4C2-dependent *flil* promoter was monitored using a mini-Mu lac operon reporter MudJ (21). Transposition of MudJ into the chromosome of *Salmonella* Typhimurium strain LT2 was performed as described, selecting for MudJ-encoded kanamycin resistance (22). Selection plates also contained bacteriophage χ (Chi), which kills flagellated cells (23) and provided a positive selection for MudJ insertions that had disrupted flagellar genes. MudJ 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 *flhS1000::MudJ* allele used here determined that the MudJ 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,3 the *clpX* gene was amplified from the chromosome of strain χ306, using the primers clpX-NheTEV-f (GGTATGGCTAGCCAAAACCTGTACTTCCAAATGCACTAAACGCAAAG) and clpX-Xho-r (GCAAGAGTGAAAGTTGGGCGATATGCTATCCGCCCCAGGAGGAG). The fragment generated was cleaved with Ndel at the 5' end and XhoI at the 3' end, and cloned into pTrcHisA. To construct plasmid pHC1 encoding FlhD-His₆-TEV site-FlhC, the *flhD* gene was amplified from the chromosome with *flhD*-Neo-f (CTTCCTCCATGGAACACTGATCATACA) and *flhD*-Xho-r (GCTAGCTCGATTGATCCCTTTTCTTACG). The fragment generated was cleaved with Ncol at the 5' end and XhoI at the 3' end and cloned into pTrcHisA. To construct plasmid pHC1 encoding FlhD-His₆-TEV site-FlhC, the *flhD* gene was amplified from the chromosome with *flhD*-Neo-f (CTTCCTCCATGGAACACTGATCATACA) and *flhD*-Xho-r (GCTAGCTCGATTGATCCCTTTTCTTACG).

To purify ClpP, 1 liter of cultured DH5αZi derivative carrying pTYK610 (CS5121) was incubated at 37 °C until the cells reached an A₆₀₀ of 0.4. Isopropyl 1-thio-beta-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

3 The abbreviations used are: TEV, tobacco etch virus; Ni-NTA, nickel-nitrilotriacetic acid; ATP-Y, adenosine 5'-O-(thiotriphosphate); BLI, biolayer interferometry; ZBD, zinc-binding domain; AMP-PNP, 5'-adenylyl-beta,gamma-imidodiphosphate.
lysis by sonication, the supernatant after centrifugation was loaded onto a HiTrap Q HP (1 ml; GE Healthcare) and washed with buffer A. 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) with buffer C (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 20 mM imidazole, 0.02% Triton X-100, 20% glycerol). 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 7.6, 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). For concentration, the fraction containing FlhD4-(His6FlhC)2 was dialyzed against buffer G (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM DTT, 10% glycerol). For concentration, the fraction containing FlhD4-(His6FlhC)2 was 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, ΔclpXP-lon::Cm, ΔhisA∆IvhV-Δc::Cm 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 3 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 G (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). For concentration, the fraction containing FlhD4-(His6FlhC)2 was dialyzed against buffer F (50 mM NaHPO4, pH 8.0, 300 mM NaCl, 20 mM imidazole, 1 mM DTT) 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 slurry of Ni-NTA-agarose (Qiagen) and washed with buffer I. Bound proteins were eluted with buffer I containing 250 mM of imidazole. The fractions containing FlhD4-(His6FlhC)2 were dialyzed against buffer J (50 mM HEPES-NaOH, pH 7.5, 50 mM NaCl, 1 mM DTT) and loaded onto HiTrap heparin column (1 ml; GE Healthcare). The proteins were eluted with 20 ml of a 0–1000 mM NaCl linear gradient. To purify intact FlhD4C2, the His-TEV site tag was removed by incubating with His-TEV protease during dialysis in buffer J for 4 h and loaded onto a heparin column as described above. His-GFP-SsrA was purified as described previously (20).

To purify FlIT, 1-liter cultures of E. coli BL21, carrying the plasmid pGex6p-1-GST-FlIT, were grown to an A600 of 0.4, induced by adding 1.0 mM isopropyl 1-thio-β-D-galactopyranoside, incubated for 3 h, collected, and frozen. Thawed cells were suspended to 40 ml of lysis buffer (50 mM Tris-HCl, pH 7.5 150 mM NaCl, 10% glycerol, 1 mM DTT) containing 1 tablet of protease inhibitor (Complete EDTA-free, Roche Applied Science). The cells were lysed by sonication, and the lysate was centrifuged at 10,000 × g for 20 min at 4 °C. The supernatant was batch-bound to 1 ml of pre-equilibrated GST-Sepharose 4FF as slurry, and the sample was rotated for 20 min at 4 °C. After centrifugation, the beads were washed twice with 5 ml of lysis buffer. Bound GST-FlIT was eluted in 1 ml of 20 mM reduced glutathione-containing lysis buffer. To purify GST-FlIT, the eluted fraction was passed through a Superdex 200 10/300G column equilibrated with lysis buffer. For the purification of the intact version of FlIT, the eluted fraction was dialyzed with lysis buffer containing 5 units of Precision protease overnight at 4 °C. The dialyzed sample was bound 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).

**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) using 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 FlhD4C2 complex was preincubated with FlIT for 3 min at 37 °C and then proteolyzed 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 ClpX, 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 coeluted 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 ClpX, 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 mM 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-(His6FlhC)2 and DNA, a biolayer interferometry assay was done in an Octet RED96 instrument. Purified FlhD4-(His6FlhC)2 was captured on an Ni-NTA sensor (ForteBio). Purified flhB promoter DNA was serially diluted in Clp assay buffer and bound to captured FlhD4-(His6FlhC)2. One-shot kinetics analysis was done with parallel sensors, and a local Rmax model was fitted to estimate Kd and koff/kon. The first 10 s of dissociation time is omitted from the curve fitting analysis to minimize the buffer effect. To prepare DNA fragments of FlhD4C2 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 FlhD4C2 as a negative control, a DNA mixture (3:1:1 volumes of water, 2 mM malachite green, 50 mM ammonium molybdate, and 2.5% polyvinyl alcohol), and 25 μM 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

FlhD4C2 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 FlhD4C2 complex through the direct interaction with the FlhC subunit (17, 26). We recently established that overexpression of YdiV promotes degradation of FlhD4C2 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 (PtetA) inhibits transcription of FlhD4C2-dependent gene expression in a ClpXP-dependent manner, yet the expression of YdiV from a stronger arabinose promoter (ParaBAD) inhibits FlhD4C2-dependent transcription in a ClpXP-independent pathway (12). The ClpXP-independent role of YdiV in suppressing gene expression was due to its interaction with FlhD4C2 bound to the class II promoter DNA to release FlhD4C2 from the DNA-protein complex (12). We thus investigated whether the effect of FlIT on FlhD4C2-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 ParaBAD in strain TH18233 (ParaBAD:flIT::flIL::MudJ) 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 FlhD4C2 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 FlhD4C2 by ClpXP. Thus, the effects of FlIT on the degradation of FlhD4C2 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 FlhD4C2 (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 FlhD4C2 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 FlhD4C2. Purification of individual FlhC has so far not been successful (27, 28).

In vitro degradation assays, using purified FlhD4C2 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 this His-tagged FlhD4C2 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-(His6FlhC2) 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

TABLE 3

| Strain background | Miller units (mean ± S.D.) | Relative activity (with arabinose/without arabinose) |
|-------------------|----------------------------|-----------------------------------------------|
| clpX+            | 2495.0 ± 107.4             | 31.1 ± 6.3                                    |
| ΔclpX            | 3022.5 ± 268.4             | 357.0 ± 16.1                                  |
| clpXΔa,b         | 3164.6 ± 163.0             | 359.2 ± 12.6                                  |

**END**
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 araBAD. Depletion of the ZBD domain of ClpX partially suppressed the inhibitory effect of the overproduction of FlhD relative to FlhC by FliT (Fig. 2, c and d). These results suggest that the N-terminal ZBD of ClpX is essential for the enhancement of ClpXP proteolysis of FlhC by FliT.

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Figure 1. Degradation of FlhC in FlhD4C2 by ClpXP is accelerated by FliT in vitro. Effect of FliT on the ClpXP-dependent degradation of the FlhD4C2 (a) and FlhD4-(His6FlhC)2 (b) was examined. Each sample of 0.75 μM FlhD4C2 was preincubated for 3 min in the presence or absence of 1.0 μM FliT2 and mixed with 0.2 μM ClpX and ATP to start the proteolysis. Samples were taken at the indicated times. Each protein was separated in a 16% SDS-polyacrylamide gel and was detected by Coomassie Brilliant Blue staining. c, quantification of the FlhD and FlhC relative to the value at time 0 in the degradation assay using FlhD4-(His6FlhC)2. Mean and S.D. values (error bars) of the results of triplicate independent experiments are given. Triangles and squares show the band intensities of FlhD and FlhC, respectively. The solid line and the dashed line show the data of FliT(–) and FliT(+) respectively. The band intensity of ClpX in each lane was used for the internal control.
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, His₆GFP-SsrA and LambdaO were degraded at similar rates (Fig. 3, a and b). As shown in the degradation of the FlhD₄C₂ 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, FlhD₄C₂, and ClpX Form Ternary Complex in Vitro—To understand the mechanism of action of FliT on FlhD₄C₂ 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 FlhD₄C₂ 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 FlhD₄-(His₆FlhC)₂ generated a ternary complex of the three com-
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nenents (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 complex 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 the 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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FIGURE 7. DNA-bound form of FlhD4C2 is resistant to proteolysis. a, sensorgrams in the BLI interaction assay between FlhD4C2-(His6FlhC)2 captured on Ni-NTA sensor chip and fliB promoter DNA. DNA concentrations used in the assay are shown. b, sensorgrams in the BLI interaction assay between FlhD4C2-(His6FlhC)2 captured on Ni-NTA sensor chip and control unrelated DNA fragment. c, degradation assay of FlhD4C2-(His6FlhC)2 by ClpXP in the presence or absence of Flit and/or DNA. 1 μM Flit, 0.75 μM fliB promoter DNA, or 0.75 μM control DNA was mixed with 0.75 μM FlhD4C2-(His6FlhC)2 as indicated and incubated for 3 min at 37°C. 1 μM Flit, or 0.75 μM fliB promoter DNA and 0.2 μM ClpX P14 were added as indicated and incubated for 5 min. FIT → DNA and DNA → FIT; the addition of Flit before DNA incubation and DNA before Flit incubation, respectively. ATP (3 mM) was added to start proteolysis. Each protein was separated in a 16% SDS-polyacrylamide gel and was detected by Coomassie Brilliant Blue staining. d, mean and S.D. values (error bars) of the FlhC band intensity estimated from the results of triplicate independent experiments are given. Solid lines with circle, square, and triangle symbols indicate the Flit(−) assays of no DNA, fliB promoter DNA, and control DNA, respectively. Dashed lines with asterisk and diamond symbols indicate the Flit(+) assays of DNA → FIT and FIT → DNA, respectively.

between FlhC and ClpX (data not shown), confirming that there was no effect of His tagging at the N terminus of FlhC on FlhC-ClpX interaction.

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-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 almost no interaction with FlhD4C2-(His6FlhC)2 (Fig. 7b). Kd of 2.1 ± 1.0 nM and t1/2 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 fliB 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 FlhC and FlhD, 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 Flit 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 is 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(−)/fliB 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 + 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 ClpX 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 FlhD4C2 Complex Is Selectively Enhanced by FliT—The FliT effect of enhancing the degradation by ClpXP was found only for the FlhC subunit in the FlhD4C2 complex (Fig. 1), despite FliT promoting the binding of ClpX to FlhD4C2 (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 FlhD4C2 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 FlhD4C2 complex (Fig. 1), it seems that both subunits of the FlhD4C2 complex have their own individual degrons. Hence, it is of interest that ClpX-FlhD4C2 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 FlhD4C2 complex does not proceed simultaneously. Therefore, the individual subunits in FlhD4C2 are likely to be independently recognized and processed by ClpXP. Dissociation of the FlhC 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 FlhD4C2 (42), but it enhanced ClpXP-dependent proteolysis of both subunits, FlhC and FlhD, of FlhD4C2 (12). Rapid degradation of the FlhD subunit in the FlhD4C2 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, FlhD2 and FlhD4C2 (43). For efficient binding of FlhD4C2 to class II promoter DNA, both subunits of FlhD4C2 are required (44), suggesting that enhanced degradation of only the FlhC subunit is sufficient to control FlhD4C2 activity. The fact that FlhD2 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 FlhD4C2 activity. FlhD2 also has roles in stabilizing FlhC and promoting the DNA binding ability of FlhC by forming stable hetero-oligomer FlhD4C2. Thus, the cellular pool of individual FlhD2 might affect FlhD4C2 activity and support rapid construction of active FlhD4C2 when cells require flagella. Enhancement of FlhC degradation by FliT-ClpXP may be involved in adjusting the cellular pool of FlhD2.

Multiple Degradation Enhancement Factors for FlhD4C2—We found a second factor that enhances FlhD4C2 degradation in addition to YdiV. Intriguingly, the ClpX-dependent effect of FliT in vivo was stronger than that of YdiV (data not shown), suggesting that both work on the ClpX-dependent pathway but in dissimilar ways. The two factors interact with different partners; YdiV interacts with FlhD, whereas FliT interacts with

affinity by adaptor molecules (6, 7), and enhancement of peptidase activity of ClpP by ADEP (34). We have shown that FliT selectively enhances FlhC degradation by adaptor-like mechanisms, through ternary complex formation of FliT-ClpX-FlhD4C2, and promotion of ClpX-FlhD4C2 interaction (Figs. 4, 6, and 8). However, the action of FliT was different from those of representative ClpX adaptors, such as SspB and UmuD; these adaptors enhance substrate degradation by a so-called tethering mechanism. The adaptor itself in the mechanism directly interacts with N-domain of ClpX and promotes ClpX-substrate interaction. FliT did not directly interact with ClpX, suggesting different mechanisms. Several adaptor-like factors are known to enhance substrate degradation without directly interacting with ClpX (37, 38). For example, degradation of the Mu Rep protein is enhanced by Vir, which does not interact directly with ClpX. Binding of Vir to Rep substrate leads to a conformational change of Rep degron, resulting in enhanced degradation of Rep (38). FliT also increased the interaction between ClpX and FlhD4C2, making affinity promotion by local conformational change possible for the FliT-ClpXP system. Conformational change or oligomeric state affects the susceptibility to AAA⁺ proteases; for example, polymerized FtsZ interacts with
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the FlhC subunit in FlhD₄C₂. Another notable difference relates to the DNA binding state of FlhD₄C₂. We previously showed that YdiV stripped off FlhD₄C₂ that was prebound to DNA (12). On the other hand, FliT could not interact with FlhD₄C₂ bound to DNA (36). ClpXP moderately degrades both subunits of FlhD₄C₂ even without FliT; however, the DNA-bound form of FlhD₄C₂ becomes insensitive to proteolysis by ClpXP (Fig. 7). Furthermore, the FlhD₄C₂-DNA complex is very stable, as shown from the slow dissociation of FlhD₄C₂ from FlhD₄C₂-DNA complex (t₁/₂ = 12.8 min from our BLI assay and >40 min as reported by Clare and Hughes (43)). These results suggest that control of FlhD₄C₂ activity by FliT or ClpXP is mostly restricted by the state of the DNA-unbound free form of FlhD₄C₂. The FliT effect, shifting the equilibrium from the DNA-bound to the DNA-unbound form by inhibiting DNA binding of FlhD₄C₂, may have an additional role in the ClpXP-dependent pathway (i.e. increasing the amount of recognizable FlhD and FlhC in FlhD₄C₂, 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 FlhD₄C₂, 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 FlhD₄C₂ activity; to accomplish a complete shut-off, YdiV acts even on the DNA-bound form of FlhD₄C₂ (i.e. on all states of FlhD₄C₂). 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 fliT 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 FlhD₄C₂ (Fig. 7) (i.e. the DNA-bound state of FlhD₄C₂ becomes resistant to FliT and to ClpXP). This moderate negative regulation dependent on the DNA binding state of FlhD₄C₂ 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 FlhD₄C₂ to FliT inhibition and the long half-life of DNA-bound FlhD₄C₂ indicate the importance of the stringent control of the cellular level of the free form of FlhD₄C₂ in the regulation of flagellar biogenesis. The fact that only a very small amount of FliT sufficiently 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 FlhD₄C₂. From these results, the ClpXP pathway of the FliT-dependent anti-FlhD₄C₂ effect can be considered as having a substantial role in the negative feedback loop that controls flagellar biogenesis.

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