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FliT Selectively Enhances Proteolysis of FlhC Subunit in $FlhD_4C_2$ 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, $FlhD_4C_2$.
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 activator complex, $FlhD_4C_2$, to negatively control flagellar biogenesis. The flagellum-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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TABLE 1

Bacterial strains used in this study

Unless designated otherwise, these strains were constructed during the course of this study.

Strain	Relevant properties	Reference or source
***S. enterica* serovar Typhimurium**		
TH18233	Δ*araBAD975*::*fliT*$^+$ (P_{araBAD}-*fliT*$^+$) *fliL5100*::MudJ	
TH18236	Δ*araBAD975*::*fliT*$^+$ *fliL5100*::MudJ Δ*clpX*	
TH18237	Δ*araBAD975*::*fliT*$^+$ *fliL5100*::MudJ *clpX79* (ΔZBD (AA1–60))	
E. coli		
DH5αZ1	DH5α *lacI*^{q}	Laboratory collection
CS5123	DH5αZI carrying pTKY610	Tomoyasu *et al.* (14)
CS6757	MC4100 Δ*clpPX*::Cm Δ*flhDC*::K_m	
CS6764	MC4100 Δ*clpPX-lon*::Cm Δ*hslVU*::Tc carrying pHCX1	
CS6828	DH5α carrying pGex6p1-FliT	
CS6840	SG1146 Δ*clpP*::Cm Δ*flhDC*::K_m carrying pDHC1	
CS6453	MC4100 Δ*lon* Δ*clpPX*/pProEX htb GFP-*ssrA*	
CS6530	DH5α carrying pTKY630	

TABLE 2

Plasmids used in this study

Plasmid	Relevant characteristics	Reference or source
pTKY610	pUHE21–2Δfd12 carrying *clpP* gene, Ap	Takaya *et al.* (12)
pHCX1	pTrcHisA carrying *clpX* gene	This study
pTKY630	pZA4*lacI*^{q} carrying ΔZBD (1–60)-*clpX* gene	This study
pDHC1	pTrcHisA carrying *flhD*-His_6-*flhC* gene	This study
pGex6p1-*fliT*	pGex6p-1 carrying *fliT* gene	Imada *et al.* (17)
pUHE21–2Δfd12	$P_{A1/lacO1}$ system vector, Ap	Gamer *et al.* (19)
pProEx htb GFP-SsrA	pProEx htb carrying *gfp-ssrA*	Wojtyra *et al.* (20)
pZA4*lacI*^{q}	*lacI*^{q} Sp	Lab collection

onstrate that $FlhD_4C_2$ bound to the promoter DNA for class II genes in the flagellar regulon is resistant to ClpXP-proteolysis.

A *Salmonella* protein FliT is a multifunctional protein that regulates flagellar biogenesis. FliT functions as an anti-$FlhD_4C_2$ factor in transcriptional control (*i.e.* it inhibits the binding of $FlhD_4C_2$ complex to the promoter DNAs of class II genes in flagellar regulon, leading to the negative regulation of flagellar gene expression) (15). FliT 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). FliT also associates with ATPases of flagellar apparatus, FliI and FliJ (18).

In the present study, we demonstrate that FliT selectively enhances the proteolysis of FlhC subunit in $FlhD_4C_2$ complex by ClpXP. Our analyses show that FliT *in vitro* forms a ternary complex of ClpX-FliT-$FlhD_4C_2$ through interactions with ClpX and $FlhD_4C_2$ and between FliT and FlhC. We also show that the DNA-bound form of $FlhD_4C_2$ is resistant to ClpXP-dependent proteolysis. These findings indicate that the role of FliT as an anti-$FlhD_4C_2$ factor for flagellar expression includes (i) inhibition of $FlhD_4C_2$ binding to the promoter DNA, resulting in an increase of the form of $FlhD_4C_2$ sensitive to ClpXP-proteolysis, and (ii) enhancement of selective proteolysis of the FlhC subunit in the complex with ClpXP, decreasing the level of functional $FlhD_4C_2$.

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 fliL Promoter—In vivo transcription of the $FlhD_4C_2$-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 *fliL5100*::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 His_6-TEV-ClpX,[3] the *clpX* gene was amplified from the chromosome of strain χ3306, using the primers clpX-NheTEV-f (GGTATGGCTAGCGAAAACCTGTACTTC-CAAATGACAGATAAACGCAAAG) and clpX-Xho-r (GCA-GATCTCGAGTTATTCGCCAGAAGCCT). The fragment generated was cleaved with NheI at the 5′ end and XhoI at the 3′ end, and cloned into pTrcHisA. To construct plasmid pDHC1 encoding FlhD-His_6-TEV site-FlhC, the *flhD* gene was amplified from the chromosome with flhD-Nco-f (CTTCCCCATG-GGAACAATGCATACA) and flhD-Xho-r (CTAGACTCGA-GTTATGCCCTTTTCTTACG). The fragment generated was cleaved with NcoI at the 5′ end and XhoI at the 3′ end and cloned into pTrcHisA. To generate the *flhC*-containing plasmid, the gene was amplified by flhC-NdeI-TEV-f (CACGTG-CATATGCACCATCACCACCATCATGAAAACCTGTAC-TTCCAAATGAGTGAAAAAAGCATTG) and flhC-XhoI-r (ACACCGCTCGAGTTAAACAGCCTGTTCGAT) primers from the chromosome of the strain χ3306. The fragment generated was cleaved with NdeI and XhoI and initially cloned into pCold. The region of His_6-TEV site-flhC on pCold was amplified by using the primers flhC-Xho-f (GCCATCTCGAGGAA-AGGCGCACTTAATTAT and flhC-Pst-r (GATCCCTGCAG-TTAAACAGCCTGTTCGATC). The fragment generated was cleaved with XhoI at the 5′ end and PstI at the 3′ end and cloned into pTrcHisA-FlhD constructed as described above.

Purification of ClpP, ClpX, FliT, and $FlhD_4$-$(His_6FlhC)_2$ Complex—To purify ClpP, 1 liter of cultured *E. coli* DH5aZ1 derivative carrying pTKY610 (CS5123) was incubated at 37 °C until the cells reached an A_{600} 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

[3] The abbreviations used are: TEV, tobacco etch virus; Ni-NTA, nickel-nitrilotriacetic acid; ATPγS, adenosine 5′-*O*-(thiotriphosphate); BLI, biolayer interferometry; ZBD, zinc-binding domain; AMP-PNP, 5′-adenylyl-β,γ-imidodiphosphate.

lysis by sonication, the supernatant after centrifugation was loaded onto a HiTrap Q HP column (1 ml; GE Healthcare) and washed with buffer A. The proteins were eluted with 20 ml of a 150–500 mM KCl linear gradient. The fractions containing ClpP were dialyzed into buffer B (50 mM sodium acetate, pH 5.3, 1 mM DTT, 10% glycerol). The sample was loaded onto a Resource S column (1 ml; GE Healthcare) and washed with buffer B. The proteins were eluted with 20 ml of a 0–1000 mM KCl 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, 1 mM $MgSO_4$, 1 mM DTT, 40 mM NaCl, 10% glycerol).

To purify ClpX, 1 liter of a culture of *E. coli* MC4100, Δ*clpXP-lon*::Cm, Δ*hslUV*::Tc carrying pHCX1 (CS6764) was incubated at 37 °C until the cells reached an A_{600} 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 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). 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 the $FlhD_4$-$(His_6FlhC)_2$, 1 liter of a culture of *E. coli* SG1146 Δ*clpP*::Cm, Δ*flhDC*::Km carrying pDHC1 (CS6840) was incubated at 37 °C until the cells reached an A_{600} of 0.6. Isopropyl 1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM for 3 h, and the cells were collected by centrifugation. The pelleted cells were resuspended in 80 ml of buffer I (50 mM $NaHPO_4$, 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 by centrifugation was batch-bound to a 1-ml 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 $FlhD_4$-$(His_6FlhC)_2$ 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 $FlhD_4C_2$, 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 A_{600} 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 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).

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 $MgCl_2$, and 1 mM DTT) using purified proteins. $FlhD_4$-$(His_6FlhC)_2$ 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 $FlhD_4$-$(His_6FlhC)_2$ complex or intact $FlhD_4C_2$ 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 $FlhD_4$-$(His_6FlhC)_2$ (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 $MgCl_2$, 5% glycerol, 1 mM DTT, 1 mM ATP, or AMP-PNP, 10 mM imidazole). 0.1 μM $ClpX_6$ and 1 μM $FliT_2$ were preincubated with $FlhD_4$-$(His_6FlhC)_2$ for 5 min. Bound beads were washed twice with 500 μl of Clp binding buffer. ClpX and FliT bound to $FlhD_4$-$(His_6FlhC)_2$ 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-$FlhD_4C_2$-ClpX. 1 μM purified $(GST\text{-}FliT)_2$ was batch-bound to GST resin in 100 μl of Clp binding buffer with 1 mM ATPγS. 0.1 μM $ClpX_6$ and 1 μM $FlhD_4$-$(His_6FlhC)_2$ were preincubated with GST-FliT for 5 min. Bound beads were washed twice with 500 μl of Clp assay buffer. ClpX and $FlhD_4$-$(His_6FlhC)_2$ 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 $FlhD_4C_2$, 0.5 μg of ClpP, and 0.5 μg of FliT, respectively, were added to the samples when required. The reactions

were stopped by the addition of 5 μl of 500 mM EDTA. The reaction mixture was mixed with 200 μl of malachite green mixture (3:1:1:1 volumes of water, 2 mM malachite green, 50 mM ammonium molybdate, and 2.5% polyvinyl alcohol), and 25 μl of 1 M 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 $FlhD_4$-$(His_6FlhC)_2$ and DNA, a biolayer interferometry assay was done in an Octet RED96 instrument. Purified $FlhD_4$-$(His_6FlhC)_2$ was captured on an Ni-NTA sensor (ForteBio). Purified *flhB* promoter DNA was serially diluted in Clp assay buffer and bound to captured $FlhD_4$-$(His_6FlhC)_2$. 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 $FlhD_4C_2$ 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 $FlhD_4C_2$ as a negative control, a DNA fragment with similar sized 278-bp region is amplified from the *Salmonella* genome using the following primers, neg-dna-f (AAGGATCCGAAAACCTGTATTTTCAGATGAAACCTCT-TCGCCAGCAAA) and neg-dna-r (TTTCTGCAGTTAG-TCAGCGATTTCGTTTTCCTG).

RESULTS

$FlhD_4C_2$ 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 $FlhD_4C_2$ complex through the direct interaction with the FlhC subunit (17, 26). We recently established that overexpression of YdiV promotes degradation of $FlhD_4C_2$ 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_{tetA}) inhibits transcription of $FlhD_4C_2$-dependent gene expression in a ClpXP-dependent manner, yet the expression of YdiV from a stronger arabinose promoter (P_{araBAD}) inhibits $FlhD_4C_2$-dependent transcription in a ClpXP-independent pathway (12). The ClpXP-independent role of YdiV in suppressing gene expression was due to its interaction with $FlhD_4C_2$ bound to the class II promoter DNA to release $FlhD_4C_2$ from the DNA-protein complex (12). We thus investigated whether the effect of FliT on $FlhD_4C_2$-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_{araBAD} in strain TH18233 (P_{araBAD}::*fliT*$^+$ *fliL*::Mu*dJ*) 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.

TABLE 3
Effect of FliT overexpression on transcription of class 2 *fliL* gene in the *clpX*$^+$ and Δ*clpX* backgrounds

Strain background	Miller units (mean ± S.D.)		Relative activity (with arabinose/ without arabinose)
	Without arabinose	With arabinose	
			-fold
clpX$^+$	2495.0 ± 107.4	31.1 ± 6.3	0.012
Δ*clpX*	3022.5 ± 268.4	357.0 ± 16.1	0.118
$clpX_{\Delta 1-60}$	3146.4 ± 163.0	350.2 ± 12.6	0.111

FliT Directly Accelerates the Degradation of FlhC Subunit in $FlhD_4C_2$ 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 $FlhD_4C_2$ by ClpXP. Thus, the effects of FliT on the degradation of $FlhD_4C_2$ by ClpXP were tested *in vitro*. Purified ClpXP slowly degraded FlhC and FlhD subunits (Fig. 1*a*). *In vitro* degradation with purified FliT showed that it enhanced the ClpXP-dependent degradation of FlhC subunit but not the FlhD subunit in $FlhD_4C_2$ (Fig. 1*a*). 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 $FlhD_4C_2$ 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 $FlhD_4C_2$. Purification of individual FlhC has so far not been successful (27, 28).

In vitro degradation assays, using purified $FlhD_4C_2$ complex whose FlhC subunit was His_6-tagged at the N terminus, were also done to test the effect of N-terminal tagging of FlhC (Fig. 1*b*). ClpXP-dependent degradation of the FlhC subunit of this His-tagged $FlhD_4C_2$ 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 $FlhD_4$-$(His_6FlhC)_2$ 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*

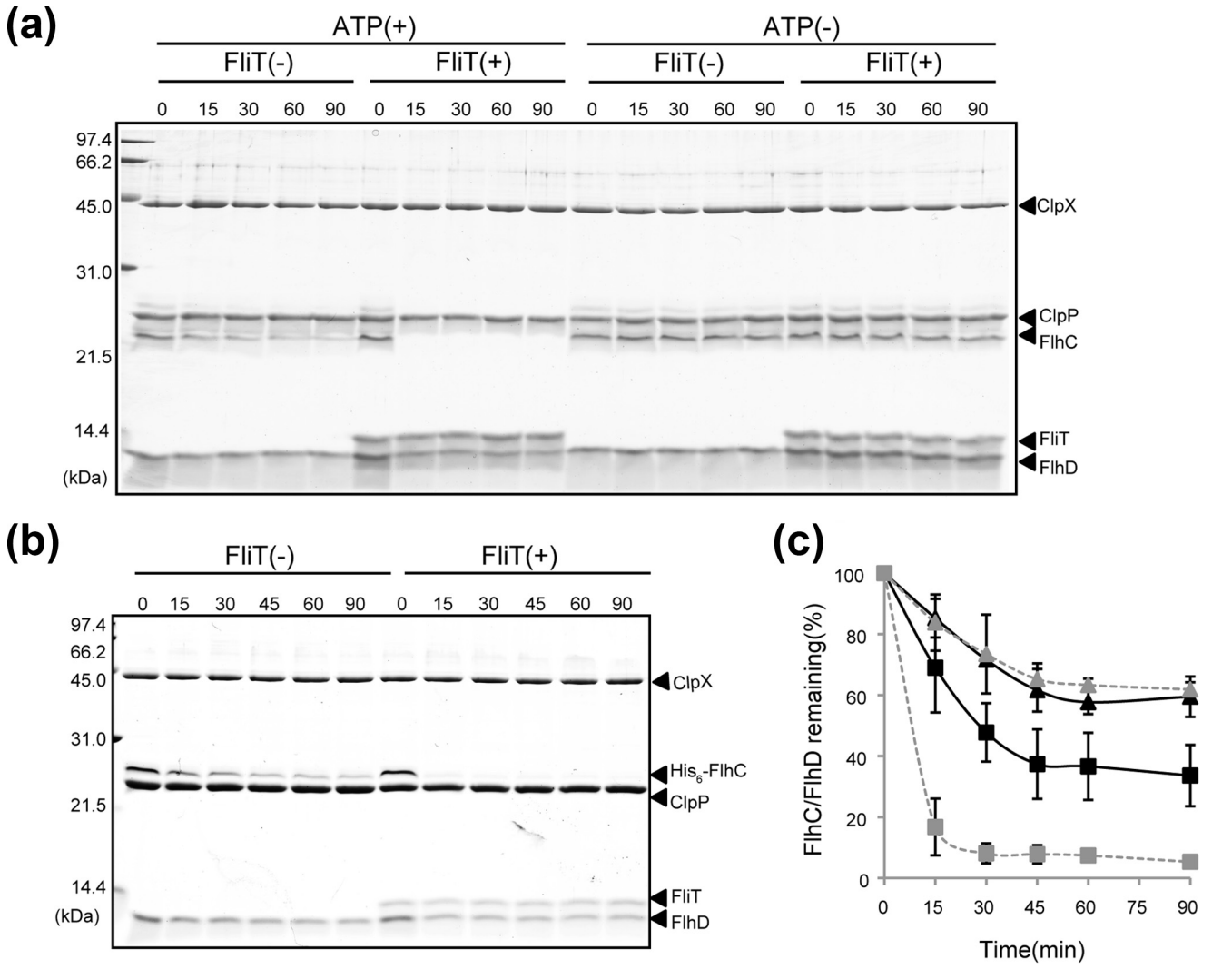

FIGURE 1. **Degradation of FlhC in $FlhD_4C_2$ by ClpXP is accelerated by FliT *in vitro*.** Effect of FliT on the ClpXP-dependent degradation of the $FlhD_4C_2$ (*a*) and $FlhD_4$-$(His_6FlhC)_2$ (*b*) was examined. Each sample of 0.75 μM $FlhD_4C_2$ was preincubated for 3 min in the presence or absence of 1.0 μM $FliT_2$ and mixed with 0.2 μM $ClpX_6ClpP_{14}$ and 0 or 3 mM 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 $FlhD_4$-$(His_6FlhC)_2$. 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.

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 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^+ 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 $FliT_2$ 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 $FliT_2$ (30-fold molar excess). Moreover, the effect of $FliT_2$ 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 $FlhD_4C_2$ 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_6GFP-SsrA and LambdaO were degraded at similar rates (Fig. 3, *a* and *b*). As shown in the degradation of the $FlhD_4C_2$ 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, $FlhD_4C_2$, and ClpX Form Ternary Complex in Vitro—To understand the mechanism of action of FliT on $FlhD_4C_2$ 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. 2*a*), 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 $FlhD_4C_2$ was detected by the pull-down assay (Fig. 4*a*, *lanes 1–3*). No direct interaction between FliT and ClpX could be detected (Fig. 4*a*, *lanes 4–6*). On the other hand, preincubation of ClpX, GST-FliT, and $FlhD_4$-$(His_6FlhC)_2$ generated a ternary complex of the three compo-

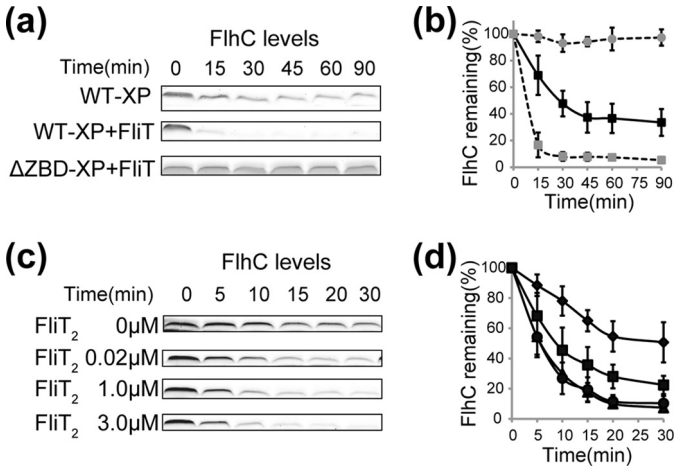

FIGURE 2. **Effect of FliT to enhance *in vitro* degradation of FlhC requires N-terminal zinc binding domain of ClpX, and a very small amount of FliT efficiently enhances the degradation of FlhC.** The degradation assay using N-terminal zinc-binding domain-depleted mutant ClpX was performed as described 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 $FlhD_4C_2$ 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 $ClpX_6ClpP_{14}$.

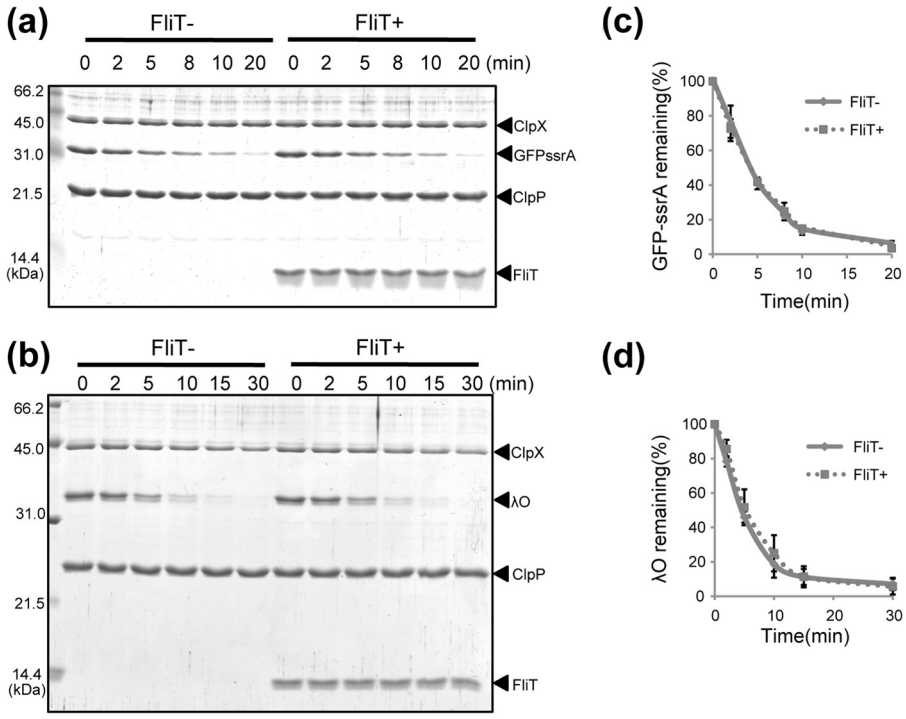

FIGURE 3. **Effect of FliT on ClpXP-catalyzed proteolysis of GFP-SsrA and of LambdaO *in vitro*.** Degradation of 1.5 μM GFP-SsrA (*a*) and LambdaO (*b*) in the presence or absence of 1 μM $FliT_2$ was assayed. The same reaction buffer with the $FlhD_4C_2$ degradation assay was used. Mean and S.D. values (*error bars*) of the results of triplicate independent experiments for GFP-SsrA and LambdaO are given in *c* and *d*, respectively.

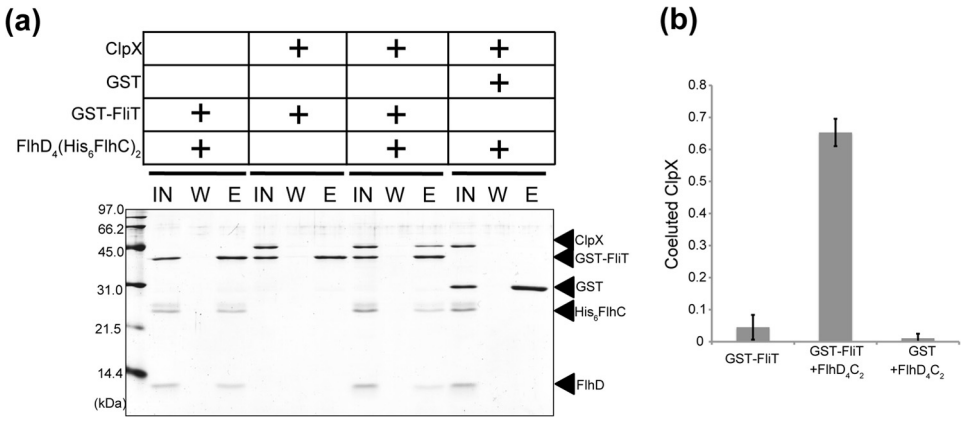

FIGURE 4. **Detection of ternary complex of GST-FliT-ClpX-FlhD$_4$C$_2$ by *in vitro* pull-down assay.** 1 μM purified (GST-FliT)$_2$ was batch-bound to GST resin in 100 μl of Clp assay buffer containing 1 mM ATPγS. The (GST-FliT)$_2$ was incubated with 0.1 μM ClpX$_6$ and 1 μM FlhD$_4$-(His$_6$FlhC)$_2$ for 5 min. GST resin was washed twice by 500 μl of Clp assay buffer. ClpX and FlhD$_4$-(His$_6$FlhC)$_2$ bound to GST-FliT were coeluted with 100 mM GSH-containing Clp assay buffer. Input (*IN*), wash (*W*), and elution (*E*) fractions were loaded onto the SDS-PAGE gel as shown in *a*. Mean and S.D. values (*error bars*) of the band intensities of ClpX estimated from triplicate independent experiments are given in *b*.

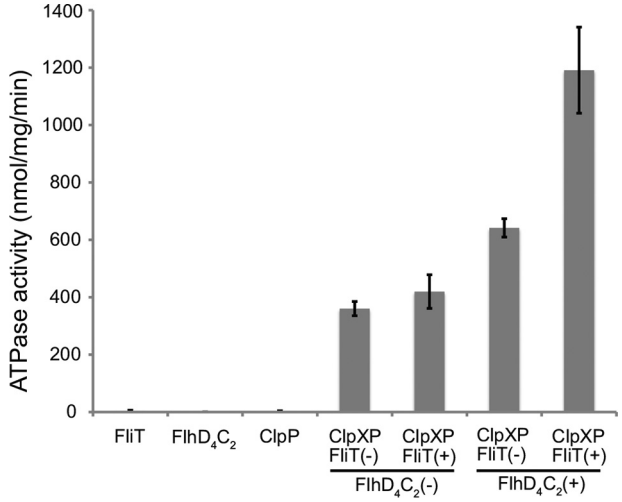

FIGURE 5. **FliT promotes the ATPase activity of ClpX in the presence of FlhD$_4$C$_2$.** ATPase activity of ClpX (0.5 μg) was measured in the absence or presence of FliT (0.5 μg), ClpP (0.5 μg), and/or FlhD$_4$-(His$_6$FlhC)$_2$ (2.0 μg). Mean and S.D. values (*error bars*) were estimated from at least three independent experiments.

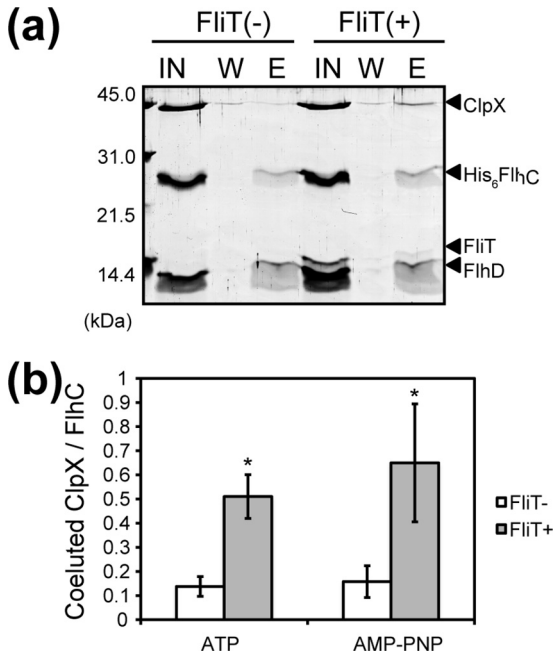

FIGURE 6. **FliT promotes the binding between ClpX and FlhD$_4$C$_2$.** 1.0 μM purified N terminally His-tagged FlhD$_4$C$_2$ was batch-bound to Ni-NTA resin in 100 μl of Clp assay buffer with 10 mM imidazole. 1 μM FliT$_2$ was preincubated with FlhD$_4$-(His$_6$FlhC)$_2$ for 5 min. Bound beads were washed twice in 500 μl of Clp assay buffer. ClpX and FliT bound to FlhD$_4$-(His$_6$FlhC)$_2$ were coeluted with 500 mM imidazole-containing Clp assay buffer. Input (*IN*), wash (*W*), and elution (*E*) fractions were loaded on the SDS-PAGE gel as shown in *a*. Quantification of the ClpX coeluted with FlhD$_4$C$_2$ is shown in *b*. The result of quantification for the pull-down assay using the non-hydrolyzable ATP analog AMP-PNP is also shown in *b*. ClpX band intensity was normalized by the intensity of eluted FlhC in the same *lane*. *, statistical significance of the difference between FliT$^-$ and FliT$^+$ ($p < 0.01$, Student's *t* test). The mean and S.D. values (*error bars*) were estimated from triplicate independent experiments.

nents (Fig. 4, *a* (*lanes 7–9*) and *b*). When purified GST instead of GST-FliT was incubated with ClpX and FlhD$_4$C$_2$, 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 FlhD$_4$C$_2$. Whereas FliT stimulated ATPase activity of ClpX in the presence of FlhD$_4$-(His$_6$FlhC)$_2$ and ClpP (Fig. 5, $p < 0.01$, Student's *t* test), there was no stimulation in the absence of FlhD$_4$-(His$_6$FlhC)$_2$. The data also suggest that FliT does not directly interact with ClpX but does so with FlhC, forming a FliT-FlhD$_4$C$_2$-ClpX ternary complex.

Binding of FlhD$_4$C$_2$ to ClpX Is Promoted by FliT—The formation of a ternary complex of FliT-FlhD$_4$C$_2$-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 FlhD$_4$C$_2$ complex, (ii) alteration of the accessibility of the recognition region on FlhC, and thereby (iii) increase of the affinity between ClpX and FlhD$_4$C$_2$. To test this hypothesis, the binding between ClpX and FlhD$_4$-(His$_6$FlhC)$_2$ complex was assessed by an *in vitro* pull-down assay using Ni-NTA-agarose beads. Coeluted ClpX bound to His-tagged FlhD$_4$C$_2$ complex in the presence or the absence of FliT was examined. In the presence of FliT, binding of ClpX to FlhD$_4$C$_2$ complex increased (Fig. 6, *a* and *b*, $p < 0.01$, Student's *t* test), suggesting that the affinity between ClpX and FlhD$_4$C$_2$ complex is promoted by FliT. A pull-down assay using a different FlhD$_4$C$_2$ protein with a His tag at the N terminus of FlhD also promoted interaction

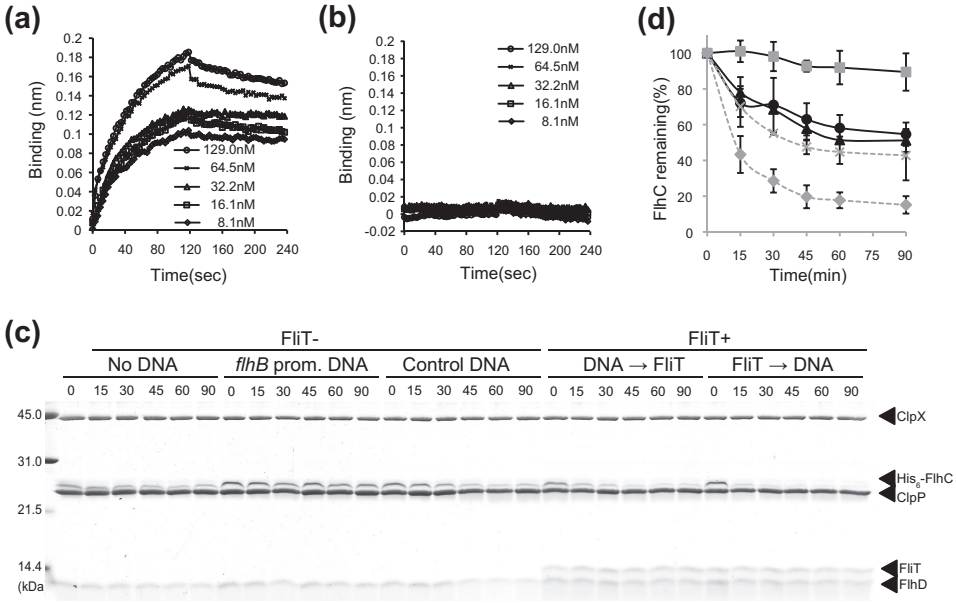

FIGURE 7. **DNA-bound form of $FlhD_4C_2$ is resistant to proteolysis.** *a*, sensorgrams in the BLI interaction assay between $FlhD_4$-$(His_6FlhC)_2$ captured on Ni-NTA sensor chip and *flhB* promoter DNA. DNA concentrations used in the assay are shown. *b*, sensorgrams in the BLI interaction assay between $FlhD_4$-$(His_6FlhC)_2$ captured on Ni-NTA sensor chip and control unrelated DNA fragment. *c*, degradation assay of $FlhD_4$-$(His_6FlhC)_2$ by ClpXP in the presence or absence of FliT and/or DNA. 1 μM $FliT_2$, 0.75 μM *flhB* promoter DNA, or 0.75 μM control DNA was mixed with 0.75 μM $FlhD_4$-$(His_6FlhC)_2$ as indicated and incubated for 3 min at 37 °C. 1 μM $FliT_2$ or 0.75 μM *flhB* promoter DNA and 0.2 μM $ClpX_6P_{14}$ were added as indicated and incubated for 5 min. *FliT → DNA* and *DNA → FliT*, 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, *flhB* promoter DNA, and control DNA, respectively. *Dashed lines* with *asterisk* and *diamond symbols* indicate the FliT(+) assays of DNA → FliT and FliT → 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 $FlhD_4C_2$ complex by ClpX and/or promotes the unfolding of $FlhD_4C_2$ 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 $FlhD_4C_2$ complex by FliT was also obtained with AMP-PNP (Fig. 6*b*). Thus, the FliT effect works at a very early stage of substrate processing by ClpX that does not require ATPase activity.

$FlhD_4C_2$ Is Protected from Degradation by ClpXP When Bound to Class II Promoter DNA—$FlhD_4C_2$ is a DNA-binding protein that strongly binds to the class II promoter region. To determine whether the FliT-ClpXP system degrades $FlhD_4C_2$ bound to the promoter DNA, interaction between *flhB* promoter DNA and His-tagged $FlhD_4$-$(His_6FlhC)_2$ was initially assessed by the BLI method. Sensor responses for $FlhD_4C_2$-DNA interaction showed concentration dependence, with responses reaching a plateau around maximum concentration (Fig. 7*a*), indicating a specific interaction between $FlhD_4$-$(His_6FlhC)_2$ and DNA (Fig. 7*a*). Control unrelated DNA fragments showed only faint or almost no interaction with $FlhD_4$-$(His_6FlhC)_2$ (Fig. 7*b*). K_d of 2.1 ± 1.0 nM and $t_{1/2}$ of 12.8 min ± 4.8 (decay of $FlhD_4C_2$-DNA complex) were estimated from the sensorgrams of triplicate independent BLI assays. Hence, purified $FlhD_4C_2$ 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 $FlhD_4C_2$ 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 $FlhD_4C_2$ is resistant to proteolysis. Control DNA fragment unbound to $FlhD_4C_2$ did not affect the degradation of $FlhD_4C_2$, suggesting that this inhibition is due to specific interaction between $FlhD_4C_2$ and its binding site on class II promoter DNA. On the other hand, when 1 μM $FliT_2$ was given before the addition of DNA, FlhC was rapidly degraded by ClpXP, from which we can infer that inhibition of the $FlhD_4C_2$-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 $FlhD_4C_2$, 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 $FlhD_4C_2$ binds to DNA, interaction between FliT and $FlhD_4C_2$ is decreased due to the hindered access to the interaction site of FliT on $FlhD_4C_2$, 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 $FlhD_4C_2$ 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

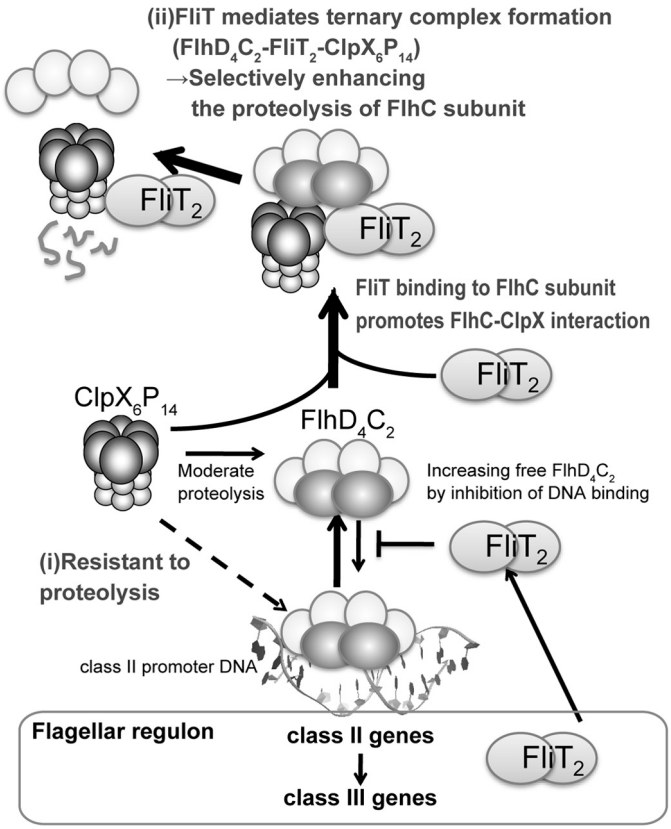

FIGURE 8. **Model of ClpXP-dependent FliT inhibition of $FlhD_4C_2$ activity.** In this study, we revealed two novel ClpXP-dependent routes of FliT effect in inhibiting $FlhD_4C_2$ activity. *i*, the inhibition of DNA binding of $FlhD_4C_2$ by FliT increases the free form of DNA-unbound $FlhD_4C_2$ sensitive to ClpXP-dependent proteolysis, thus leading to enhanced degradation of $FlhD_4C_2$. *ii*, FliT binding to FlhC subunit of $FlhD_4C_2$ promotes FliT-$FlhD_4C_2$-ClpXP ternary complex formation and enhances selective degradation of FlhC subunit of $FlhD_4C_2$. FliT is a flagellum-related gene whose transcription is regulated by both class II and class III promoters. Therefore, concerted action of FliT and ClpXP is considered to be important for the stringent control of $FlhD_4C_2$ activity in a manner of negative feedback control.

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-$FlhD_4C_2$ and promotion of ClpX-$FlhD_4C_2$ 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 $FlhD_4C_2$, 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 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 $FlhD_4C_2$ Complex Is Selectively Enhanced by FliT—The FliT effect of enhancing the degradation by ClpXP was found only for the FlhC subunit in the $FlhD_4C_2$ complex (Fig. 1), despite FliT promoting the binding of ClpX to $FlhD_4C_2$ (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 $FlhD_4C_2$ 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 $FlhD_4C_2$ complex (Fig. 1), it seems that both subunits of the $FlhD_4C_2$ complex have their own individual degrons. Hence, it is of interest that ClpX-$FlhD_4C_2$ 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 $FlhD_4C_2$ complex does not proceed simultaneously. Therefore, the individual subunits in $FlhD_4C_2$ 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 $FlhD_4C_2$ (42), but it enhanced ClpXP-dependent proteolysis of both subunits, FlhC and FlhD, of $FlhD_4C_2$ (12). Rapid degradation of the FlhD subunit in the $FlhD_4C_2$ 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 $FlhD_4C_2$ (43). For efficient binding of $FlhD_4C_2$ to class II promoter DNA, both subunits of $FlhD_4C_2$ are required (44), suggesting that enhanced degradation of only the FlhC subunit is sufficient to control $FlhD_4C_2$ 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 $FlhD_4C_2$ activity. $FlhD_2$ also has roles in stabilizing FlhC and promoting the DNA binding ability of FlhC by forming stable hetero-oligomer $FlhD_4C_2$. Thus, the cellular pool of individual $FlhD_2$ might affect $FlhD_4C_2$ activity and support rapid construction of active $FlhD_4C_2$ 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 $FlhD_4C_2$—We found a second factor that enhances $FlhD_4C_2$ 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 $FlhD_4C_2$. Another notable difference relates to the DNA binding state of $FlhD_4C_2$. We previously showed that YdiV stripped off $FlhD_4C_2$ that was prebound to DNA (12). On the other hand, FliT could not interact with $FlhD_4C_2$ prebound to DNA (36). ClpXP moderately degrades both subunits of $FlhD_4C_2$ even without FliT; however, the DNA-bound form of $FlhD_4C_2$ becomes insensitive to proteolysis by ClpXP (Fig. 7). Furthermore, the $FlhD_4C_2$-DNA complex is very stable, as shown from the slow dissociation of $FlhD_4C_2$ from $FlhD_4C_2$-DNA complex ($t_{½}$ = 12.8 min from our BLI assay and >40 min as reported by Claret and Hughes (43)). These results suggest that control of $FlhD_4C_2$ activity by FliT or ClpXP is mostly restricted by the state of the DNA-unbound free form of $FlhD_4C_2$. The FliT effect, shifting the equilibrium from the DNA-bound to the DNA-unbound form by inhibiting DNA binding of $FlhD_4C_2$, may have an additional role in the ClpXP-dependent pathway (*i.e.* increasing the amount of recognizable FlhD and FlhC in $FlhD_4C_2$, 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 $FlhD_4C_2$, 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 $FlhD_4C_2$ activity; to accomplish a complete shut-off, YdiV acts even on the DNA-bound form of $FlhD_4C_2$ (*i.e.* on all states of $FlhD_4C_2$). 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 *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 $FlhD_4C_2$ (Fig. 7) (*i.e.* the DNA-bound state of $FlhD_4C_2$ becomes resistant to FliT and to ClpXP). This moderate negative regulation dependent on the DNA binding state of $FlhD_4C_2$ 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 $FlhD_4C_2$ to FliT inhibition and the long half-life of DNA-bound $FlhD_4C_2$ indicate the importance of the stringent control of the cellular level of the free form of $FlhD_4C_2$ 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 $FlhD_4C_2$. From these results, the ClpXP pathway of the FliT-dependent anti-$FlhD_4C_2$ effect can be considered as having a substantial role in the negative feedback loop that controls flagellar biogenesis.

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