Dual regulatory pathways of flagellar gene expression by ClpXP protease in enterohaemorrhagic *Escherichia coli*

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In enterobacteria such as *Escherichia coli* and *Salmonella* species, flagellar biogenesis is strictly dependent upon the master regulator *flhDC*. Here, we demonstrate that in enterohaemorrhagic *E. coli* (EHEC), the flagellar regulon is controlled by ClpXP, a member of the ATP-dependent protease family, through two pathways: (i) post-translational control of the FlhD/FlhC master regulator and (ii) transcriptional control of the *flhDC* operon. Both FlhD and FlhC proteins accumulated markedly following ClpXP depletion, and their half-lives were significantly longer in the mutant cells, suggesting that ClpXP is responsible for degrading FlhD and FlhC proteins, leading to downregulation of flagellar expression. ClpXP was involved in regulating the transcription of the *flhD* promoter only when the cells had entered stationary phase in a culture medium that markedly induced expression of the locus of enterocyte effacement (LEE). Comparative analyses of transcription from the *flhD* promoter in EHEC cells with different genetic backgrounds suggested that the downregulation of *flhDC* expression by ClpXP is dependent on the LEE-encoded GrlR–GrlA system. We have also shown that the degradation of FlhD and FlhC by ClpXP is responsible for downregulating flagellar expression even when LEE expression is induced.

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INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7 is a causative agent of bloody diarrhoea, non-bloody diarrhoea and haemolytic uraemic syndrome in humans (Akashi *et al.*, 1994). EHEC colonizes the intestine and causes a destructive lesion of the intestinal enterocyte, referred to as the attaching and effacing (A/E) lesion. The genes responsible for the formation of A/E lesions are located on a pathogenicity island called the locus of enterocyte effacement (LEE). The LEE genes are organized into five operons which are under strict control (Friedberg *et al.*, 1999). Three regulators, Ler, GrlR and GrlA, encoded in the LEE have been well characterized: Ler is a central activator of the transcription of most LEE genes, and GrlA and GrlR are positive and negative regulators, respectively, for *ler* transcription (Iyoda *et al.*, 2006). In addition to the control by those regulatory elements encoded in the LEE, various regulatory elements encoded outside the locus, such as BipA (Grant *et al.*, 2003), IHF (Friedberg *et al.*, 1999), H-NS (Bustamante *et al.*, 2001; Umanski *et al.*, 2002) and QseA (Sperandio *et al.*, 2002), are

involved in the control of LEE expression. We have previously reported that the ATP-dependent protease, ClpXP, is a positive regulator of LEE expression (Tomoyasu *et al.*, 2005). ClpXP protease is composed of a ClpP proteolytic component and a ClpX component that binds substrate proteins, denatures them and translocates the unfolded polypeptides into the ClpP degradation chamber (Ortega *et al.*, 2000). ClpXP is a bipartite protease responsible for degrading certain key regulatory proteins such as RpoS, which is a stationary-phase-specific sigma factor for RNA polymerase, and aberrant translation products bearing the SsrA degradation tag, added co-translationally to nascent polypeptides when ribosomes stall (Gottesman *et al.*, 1998; Zhou *et al.*, 2001). Our previous studies demonstrated that positive regulation of LEE expression by ClpXP is via two pathways, namely the RpoS-dependent and RpoS-independent pathways (Tomoyasu *et al.*, 2005). ClpXP is involved in the expression of LEE at the control of the levels of the negative regulator GrlR in EHEC which could be the RpoS-independent pathway (Iyoda & Watanabe, 2005).

In the course of phenotypic characterization of a ClpXP-depleted mutant derived from EHEC O157:H7 Sakai, we have found that depletion of ClpXP results in overproduction of FlhC, a major component of the flagellar filament, whereas there is a marked decrease in LEE-related proteins. The flagellum is an organelle consisting of three

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Abbreviations: EHEC, enterohaemorrhagic *Escherichia coli*; LEE, locus of enterocyte effacement.

A supplementary table of primer sequences is available with the online version of this paper.

distinctive structural parts, the basal body consisting of a central rod and several rings, the short curved structure called the hook, and the long helical filament which consists of an assembly of ~20 000 subunits of a single protein, FlhC (Macnab, 2003). Over 40 genes are specifically required for the flagellar formation and function in *E. coli*, and these are organized into a transcriptional hierarchy that underlies temporal and spatial control of the biogenesis program (Macnab, 1996). At the apex of the hierarchy is the class 1 *flhDC* operon. The two operon products, FlhD and FlhC, form a hetero-oligomer to activate the σ^{70} promoter of class 2 genes that encode hook–basal body proteins, as well as the alternate sigma factor, FlhA. FlhA transcribes class 3 genes involved in motor, chemotaxis and filament structures. Since the FlhD/FlhC is essential for transcription of all the genes in the flagellar cascade, it is also called a master regulator. Transcription of the *flhDC* operon is known to be regulated by a number of global regulatory signals in *E. coli* and related bacteria (Iyoda *et al.*, 2006; Shi *et al.*, 1993; Soutourina *et al.*, 2002). In addition, the FlhD/FlhC is modulated at the post-transcriptional and post-translational levels (Tomoyasu *et al.*, 2003; Wozniak *et al.*, 2009).

Here we provide evidence showing that ClpXP protease tightly regulates the flagellar expression by degrading the FlhD/FlhC master regulator in EHEC. We propose that the flagellar regulon in EHEC is controlled by ClpXP protease through two pathways, namely post-translational control of the FlhD/FlhC master regulator by degrading them and transcriptional control of the *flhDC* operon through the LEE-encoded GrlR–GrlA regulatory system.

METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are shown in Table 1. Unless otherwise specified, bacteria were grown in L-broth (1 % Bacto tryptone, 0.5 % Bacto yeast extract, 0.5 % sodium chloride, pH 7.4) or L-agar.

Construction of plasmids pTKY802 and pTKY753. Plasmid pTKY802, in which the expression of *flhDC* operon is controlled by the $P_{A1lacO-1}$ promoter, was constructed by amplifying a *Bam*HI–*Hind*III fragment carrying the *flhDC* operon by PCR with *flhDC*–*Bam*HI-F and *flhDC*–*Hind*III-R primers (Supplementary Table S1, available with the online version of this paper) and subsequent cloning of the fragment into pUHE21-2Δfd12 cleaved with *Bam*HI and *Hind*III. In plasmid pTKY753 containing the *flhA* promoter, the locus was amplified by PCR with the primers *flhA*pro–*Hind*III-F and *flhA*pro–*Bam*HI-R (Supplementary Table S1). The 320 bp fragment generated was cleaved with *Bam*HI at the 5' end and *Hind*III at the 3' end before being cloned into the vector pCB192 cleaved with *Bam*HI and *Hind*III.

Construction of Δ*flhDC*, Δ*grlR* and Δ*grlA* mutants, and *flhD*–*lacZ* fusion strain. To construct the Δ*flhDC* mutants from O157:H7 Sakai strain, the DNA fragment in the *flhDC* region was initially amplified by PCR from the chromosome of strain CS5295, using the primers *flhDC*–*Bam*HI-F and *flhDC*–*Hind*III-R (Supplementary Table S1). The 1067 bp fragment was cleaved with *Bam*HI and *Hind*III, and cloned into the vector pHSG399. The resulting plasmid was digested with *Hinc*II to eliminate the 600 bp central fragment of *flhDC* and was

self-ligated. The resultant plasmid was cleaved at the *Hind*III site and the overhanging end was filled in with T4 DNA polymerase, and then cleaved at the *Sac*I site. The generated fragment was ligated to the suicide vector pCVD442 cleaved with *Sma*I and *Sac*I. A suicide feature of pCVD442 is based on one of the replication origins of R6K that becomes functional only when the π protein is encoded by the *pir* gene. The resultant mutator plasmid, pTKY883, was introduced into strain SM10Δ*pir*, which can mobilize the plasmid by the conjugative function provided *in trans*. Conjugative crosses with strain CS5295 (*clpPX*⁺) or CS5297 (Δ*clpPX*) were carried out. The chromosomal *flhDC* operon was replaced by the disrupted *flhDC* operon on the suicide vector by double recombination. The destruction of the *flhDC* operon in the resulting strains, CS5835 or CS5836, was verified by amplification of the chromosomal DNA by PCR and Southern blot analysis. To measure the levels of transcription from *flhD* promoter, strains carrying a transcriptional *flhD*–*lacZ* fusion at the *lac* locus on the chromosome were constructed. A fragment carrying *flhD* promoter was amplified by PCR with the primers *flhD*pro–*Hind*III-F and *flhD*pro–*Bam*HI-R (Supplementary Table S1) from CS5295 chromosomal DNA, and the PCR product was ligated upstream of the *lacZ* gene in plasmid pZA3*lacZ*. The resultant plasmid, pTKY884 was cleaved with *Sac*I and *Sac*II, and the overhanging end was blunted with T4 DNA polymerase. The generated fragment was ligated to the suicide vector pCVD442 cleaved with *Sma*I. The resulting plasmid, pTKY885 was introduced into SM10Δ*pir*. Conjugative crosses with CS5295 (*clpPX*⁺) or CS5297 (Δ*clpPX*) were then carried out. The resultant strains, CS6181 and CS6183, carry the *flhD* promoter–*lacZ* fusion on the chromosome with the disrupted *lacI* gene. Allelic exchange was checked by Southern blot analysis and PCR amplification of the chromosomal DNA with *lacI*-F and *lacZ*-R (Supplementary Table S1). To construct a series of O157:H7 Sakai strains with *grlR* and *grlA* mutations, the one-step method of chromosomal gene inactivation with λ Red recombinase was used. PCR products containing kanamycin-resistance cassettes flanked by 45 or 50 bp of homology to the 5' and 3' termini of *grlR* or *grlA* genes were electroporated into competent cells of parental strains carrying plasmid pKD46 encoding the λ Red gene. To construct strain CS6270 and CS6271, PCR fragments were amplified from the pKD4 encoding kanamycin-resistance cassette, using primers *grlR*– λ red-F and *grlR*– λ red-R (Supplementary Table S1). To construct strain CS6272 and CS6273, primers *grlA*– λ red-F and *grlA*– λ red-R (Supplementary Table S1) were used to amplify the fragment from pKD4. Kanamycin-resistant colonies were selected and the resultant clones were streaked at 37 °C to isolate those that had lost pKD46. PCR and Southern blot analysis confirmed mutation of the *grlR* or *grlA* gene. The FRT-flanked kanamycin cassette was removed after transformation with pCP20, as previously described (Datsenko & Wanner, 2000).

SDS-PAGE and immunoblotting analysis. Gel electrophoresis and immunoblotting analysis were carried out according to previously described methods (Takaya *et al.*, 2006).

RESULTS

ClpXP protease is responsible for the turnover of the FlhD/FlhC master regulator in EHEC

In a previous study, we constructed a Δ*clpPX* mutant from O157:H7 Sakai by removing the central region of the *clpPX* operon, followed by insertion of a chloramphenicol resistance cassette (Tomoyasu *et al.*, 2005). The insertion did not block the expression of the downstream gene, *lon*, by its own promoter. Fig. 1(a) shows that FlhD and FlhC proteins accumulate in EHEC cells when the *clpPX* operon is disrupted, indicating that ClpXP protease negatively

Table 1. Bacterial strains and plasmids used in this study

NA, nalidixic acid resistance; Km, kanamycin resistance; Cm, chloramphenicol resistance.

Strain or plasmid	Relevant characteristics	Reference or source
<i>E. coli</i>		
SM10 λ pir	<i>thi thr leu tonA lacY supE recA</i> ::RP4-2, Tc::Mu, Km, λ pir	Laboratory collection
O157:H7 Sakai	<i>E. coli</i> O157:H7 derived from Sakai outbreak (RIMD 0509952)	Akashi <i>et al.</i> (1994)
CS5295	O157:H7 Sakai NA	Tomoyasu <i>et al.</i> (2005)
CS5297	CS5295 <i>clpPX</i> ::Cm	Tomoyasu <i>et al.</i> (2005)
CS5693	CS5295/pTKY753	This study
CS5698	CS5297/pTKY753	This study
CS5829	CS5295/pZA4 <i>lacI</i> ^q and pTKY802	This study
CS5830	CS5297/pZA4 <i>lacI</i> ^q and pTKY802	This study
CS5835	CS5295 Δ <i>flhDC</i>	This study
CS5836	CS5297 Δ <i>flhDC</i>	This study
CS6181	CS5295 Δ <i>lacI</i> 820 bp fragment containing <i>flhD</i> promoter– <i>lacZ</i> transcriptional fusion on chromosome	This study
CS6183	CS5297 Δ <i>lacI</i> 820 bp fragment containing <i>flhD</i> promoter– <i>lacZ</i> transcriptional fusion on chromosome	This study
CS6229	CS5835/pTKY753	This study
CS6230	CS5836/pTKY753	This study
CS6292	CS5835/pZA4 <i>lacI</i> ^q and pTKY802	This study
CS6293	CS5836/pZA4 <i>lacI</i> ^q and pTKY802	This study
CS6270	CS5295 <i>grrL</i> ::FRT	This study
CS6271	CS5297 <i>grrL</i> ::FRT	This study
CS6272	CS5295 <i>grrA</i> ::FRT	This study
CS6273	CS5297 <i>grrA</i> ::FRT	This study
CS6281	CS6270/pTKY753	This study
CS6282	CS6271/pTKY753	This study
CS6283	CS6272/pTKY753	This study
CS6284	CS6273/pTKY753	This study
CS6320	CS6181 <i>grrL</i> ::FRT	This study
CS6321	CS6183 <i>grrL</i> ::FRT	This study
CS6322	CS6181 <i>grrA</i> ::FRT	This study
CS6323	CS6183 <i>grrA</i> ::FRT	This study
Plasmids		
pUHE21-2 Δ fd12	P _{A1lacO-1} system vector	Gamer <i>et al.</i> (1992)
pZA4 <i>lacI</i> ^q	<i>lacI</i> ^q	Laboratory collection
pZA3 <i>lacIZ</i>	Carrying fragments of <i>lacI</i> and <i>lacZ</i>	Laboratory collection
pHSG399	Cloning vector	Laboratory collection
pCB192	Promoter cloning vector	Schneider & Beck (1986)
pKD4	Carrying kanamycin-resistance gene	Datsenko & Wanner (2000)
pKD46	λ Red (γ , β , exo)	Datsenko & Wanner (2000)
pCP20	Carrying the yeast Flp recombinase gene, <i>flp</i>	Cherepanov & Wackernagel (1995)
pCVD442	Carrying <i>oriγ</i> of R6K, <i>mobRP4</i> , <i>bla</i> , <i>sacB</i>	Donnenberg & Kaper (1991)
pTKY753	pCB192 with 320 bp fragment containing <i>fliA</i> promoter	This study
pTKY802	pUHE21-2 Δ fd12 carrying <i>flhDC</i> operon	This study
pTKY883	pCVD442 with <i>flhDC</i> operon disrupted by <i>HincII</i> digestion	This study
pTKY884	Carrying fragments of <i>lacI</i> , <i>lacZ</i> and 820 bp fragment containing <i>flhD</i> promoter	This study
pTKY885	pCVD442 with <i>SacI</i> – <i>SacI</i> fragment containing <i>lacI</i> , <i>lacZ</i> and <i>flhD</i> promoter	This study

controls the cellular levels of the FlhD/FlhC master regulator for flagellar gene expression. To examine whether ClpXP controls the turnover of FlhD and FlhC proteins, leading to the negative regulation of flagellar gene expression in EHEC, we determined the *in vivo* half-lives of these two proteins. Expression of the *flhDC* operon was

initiated from the P_{A1lacO-1} promoter on plasmid pTKY802 in a Δ *flhDC* background on the chromosome by adding IPTG. Tetracycline was added to prevent *de novo* synthesis of proteins and the cellular levels of FlhD and FlhC at the indicated times after tetracycline addition are shown in Fig. 1(b). Whereas FlhD and FlhC decayed with half-lives of

Fig. 1. Cellular contents (a) and *in vivo* stabilities (b) of FlhC and FlhD proteins in EHEC cells with *clpPX*⁺ or Δ *clpPX* background. (a) Whole-cell extracts were prepared from strains CS5295 (*clpPX*⁺) and CS5297 (Δ *clpPX*), and then separated by using SDS-PAGE. The separated proteins were immunostained with anti-FlhC or anti-FlhD antibodies. (b) Strain CS6292 (*clpPX*⁺) and CS6293 (Δ *clpPX*) were grown to OD₆₀₀ 0.5 at 37 °C in L-broth containing 20 μ M IPTG to induce *flhDC* expression for 1 h. Cultures were washed once with fresh medium, followed by medium containing tetracycline (100 μ g ml⁻¹) and glucose (2%) to block translation and transcription of *flhDC*. Aliquots of cells were taken at the indicated times and mixed with pre-chilled TCA (final concentration, 10%). (i) The proteins were separated by using 15% (for FlhC) or 17% (for FlhD) SDS-PAGE, before being immunostained with anti-FlhC antibody or anti-FlhD antibody. (ii) Coomassie blue-stained gel patterns of the same samples used for immunoblotting. (iii) Quantification of the precipitated proteins in *clpPX*⁺ (○) and Δ *clpPX* (●) cells using Quantity One (BioRad) relative to the values at 5 min. Mean $\pm$ SD values of at least three independent experiments are shown.

27.7 min and 13.8 min, respectively, in *clpPX*⁺ cells after the arrest of *de novo* synthesis, they had not decayed in the Δ *clpPX* cells after a 60 min chase, indicating that they were stabilized after depletion of ClpXP. These results suggest that ClpXP could be involved in the turnover of FlhD and FlhC.

To determine whether ClpXP regulates the expression of the flagellar regulon only by the FlhD/FlhC-dependent pathway, the effect of *clpPX* disruption on the expression of

fliA, which belongs to the class 2 genes, was examined using *lacZ* fusion strains with a *flhDC*⁺ or Δ *flhDC* background on the chromosome. Whereas disruption of the *clpPX* operon increased *fliA* transcription $\sim$ 2.5-fold in *flhDC*⁺ cells, the stimulation of *fliA* transcription in Δ *clpPX* cells was completely suppressed by the Δ *flhDC* mutation (Fig. 2). Since the *fliA* product, σ^{28} , is responsible for transcribing all class 3 genes, ClpXP could control the expression of the flagellar regulon through this single pathway.

ClpXP protease controls the transcription of the *flhDC* operon in EHEC under conditions in which LEE expression is induced

To determine whether ClpXP is involved in the transcriptional control of *flhDC*, we examined the levels of transcription of the *flhDC* operon in EHEC strains with a *clpPX*⁺ or Δ *clpPX* background. EHEC cultures were also grown in L-broth and a tissue culture medium such as Dulbecco's modified Eagle's medium (DMEM), in which LEE genes are known to be strongly expressed (Abe *et al.*, 2002), and the efficiencies of transcription of the *flhDC* operon were compared.

The overnight EHEC cultures grown in L-broth were diluted 1:100 with fresh L-broth or DMEM and incubated at 37 °C. The cells were grown to exponential phase (OD₆₀₀ 0.5) or stationary phase (~16 h after initiation of growth). The levels of transcription of the *flhDC* operon in cells with the Δ *clpPX* background were compared with *clpPX*⁺ cells by measuring the activities of the *flhD-lacZ* transcriptional fusion. In *clpPX*⁺ cells grown to exponential phase, *flhD-lacZ* fusion activity was lower in DMEM than in L-broth (Fig. 3a). When the cultures had entered stationary phase, the *flhD-lacZ* fusion activity in cells grown in DMEM was also lower than in cells grown in L-broth, but disruption of the *clpPX* operon increased the *flhD-lacZ* activity to the same levels seen in L-broth (Fig. 3b). In contrast, the increase following disruption of *clpPX* did not occur in exponential phase cultures grown in DMEM (Fig. 3a). The results also showed that when cells were grown in L-broth,

Fig. 2. Effect of depletion of ClpXP protease on the expression of the transcriptional *flhA-lacZ* fusion in the absence of *flhDC* operon in EHEC cells. The levels of β -galactosidase activity of bacterial cells of strains CS5693 (*clpPX*⁺ *flhDC*⁺), CS5698 (Δ *clpPX* *flhDC*⁺), CS6229 (*clpPX*⁺ Δ *flhDC*) and CS6230 (Δ *clpPX* Δ *flhDC*) were assayed according to the previously described method (Takaya *et al.*, 2006). The enzyme units are mean $\pm$ SD of at least three independent assays. ***P* < 0.001.

Fig. 3. Effect of depletion of ClpXP protease on the expression of transcriptional *flhD-lacZ* fusion in the EHEC cells grown under various growth conditions. The activities of *flhD* promoter-*lacZ* fusion in EHEC cells of strains CS6181 (*clpPX*⁺; white bars) and CS6183 (Δ *clpPX*; grey bars) were assayed. Bacterial cells were grown in L-broth or DMEM to OD₆₀₀ 0.5 (a; exponential phase) or for 16 h (b; stationary phase) at 37 °C. The values represent the mean $\pm$ SD of samples tested in triplicate. ***P* < 0.001.

clpPX disruption did not affect the transcription of the *flhDC* operon in either exponential or stationary phase. These results suggest that ClpXP is responsible for controlling *flhDC* expression at the transcriptional level in bacterial cells specifically grown in DMEM and when they enter the stationary phase, i.e. conditions under which LEE expression is induced.

Regulation of flagellar gene expression by ClpXP protease via dual pathways

Expression of the flagellar regulon in *E. coli* has been shown to be under the control of various regulatory elements, including CRP and H-NS (Soutourina *et al.*, 1999), OmpR

(Shin & Park, 1995), CsrA (Wei *et al.*, 2001), IHF (Yona-Nadler *et al.*, 2003), RcsB (Francez-Charlot *et al.*, 2003), LrhA (Lehnen *et al.*, 2002), QseD (Habdas *et al.*, 2010), CadA (Vazquez-Juarez *et al.*, 2008) and GrlA (Iyoda *et al.*, 2006). Among these, GrlA acts as a negative regulator for transcription of the *flhDC* operon, but the mechanism remains unknown (Iyoda *et al.*, 2006). GrlA, which was originally identified as an activator of LEE operon expression, is antagonized by GrlR. The levels of GrlR are regulated in a growth-phase-dependent manner; GrlR levels are minimal when EHEC cells enter stationary phase, thereby facilitating expression of the LEE operon (Iyoda & Watanabe, 2005). To define the role of the GrlR–GrlA system in the control of flagellar expression by ClpXP, we measured the *flhD*–*lacZ* fusion activities in EHEC cells with $\Delta clpPX$, $\Delta grlR$ and/or $\Delta grlA$ mutations as genetic backgrounds when they had entered stationary phase in DMEM. *clpPX* disruption increased the *flhD*–*lacZ* activity by 1.8-fold (Fig. 4a; compare *clpPX*⁺ and $\Delta clpPX$ in a *grlR*⁺ *grlA*⁺ background). In contrast, disruption of *grlR* diminished the effect that *clpPX* disruption had on increasing the *flhD*–*lacZ* activity; the *clpPX* disruption increased activity by 1.2-fold in a $\Delta grlR$ background (Fig. 4a; compare *clpPX*⁺ and $\Delta clpPX$ in a $\Delta grlR$ background). Furthermore, disruption of *grlA* abolished the increase due to *clpPX* disruption (Fig. 4a; compare *clpPX*⁺ and $\Delta clpPX$ in a $\Delta grlA$ background). Therefore, the regulation of transcription of the *flhDC* operon by ClpXP could be through the GrlR–GrlA pathway. When the *flhD*–*lacZ* activity was compared in the *clpPX*⁺ background, disruption of *grlR* decreased *flhD* transcription moderately (Fig. 4a; see *grlR*⁺ and $\Delta grlR$ in a *clpPX*⁺ background). On the other hand, disruption of *grlA* had no effect on the efficiency of transcription from the *flhDC* operon promoter in the *clpPX*⁺ background (Fig. 4a; see *grlA*⁺ and $\Delta grlA$ in a *clpPX*⁺ background). This is similar to the results described in the previous report, in which the negative effect of *grlA* on *flhD* transcription could be shown only in cells overexpressing plasmid *grlA* (Iyoda *et al.*, 2006). In contrast, when GrlA, reputedly a negative regulator for *flhD* transcription was depleted in the $\Delta clpPX$ background, the *flhD*–*lacZ* activity significantly decreased (Fig. 4a; see *grlA*⁺ and $\Delta grlA$ in a $\Delta clpPX$ background). This suggests that an additional factor(s) for regulating the transcription of the *flhDC* operon would be included in the GrlR–GrlA pathway.

We also monitored the transcriptional regulating activity of the FlhD/FlhC complex in EHEC cells grown under different conditions by measuring the efficiencies of the *fliA* promoter in cells with different genetic backgrounds. Fig. 4(b) shows the activities of the *fliA*–*lacZ* transcriptional fusion in EHEC cultures that had entered stationary phase in DMEM. Disruption of *clpPX* strongly increased transcription by the *fliA* promoter; the activity of the *fliA*–*lacZ* transcriptional fusion was increased fivefold (Fig. 4b; see *clpPX*⁺ and $\Delta clpPX$ in a *grlR*⁺ *grlA*⁺ background). Although *grlR* or *grlA* disruption mostly abolished the increase of *flhDC* transcription by *clpPX* disruption (Fig.

Fig. 4. Effect of depletion of ClpXP protease on the expression of transcriptional *flhD*–*lacZ* (a) and *fliA*–*lacZ* (b) fusions in the absence of *grlR* or *grlA* in EHEC cells. (a) The expression levels of a chromosomal *flhD*–*lacZ* fusion were assayed in CS6181 (*clpPX*⁺ *grlR*⁺ *grlA*⁺), CS6183 ($\Delta clpPX$ *grlR*⁺ *grlA*⁺), CS6320 (*clpPX*⁺ $\Delta grlR$ *grlA*⁺), CS6321 ($\Delta clpPX$ $\Delta grlR$ *grlA*⁺), CS6322 (*clpPX*⁺ *grlR*⁺ $\Delta grlA$) and CS6323 ($\Delta clpPX$ *grlR*⁺ $\Delta grlA$). (b) The *fliA* promoter activities were assayed using pTKY753 carrying a transcriptional *fliA*–*lacZ* fusion in CS5693 (*clpPX*⁺ *grlR*⁺ *grlA*⁺), CS5698 ($\Delta clpPX$ *grlR*⁺ *grlA*⁺), CS6281 (*clpPX*⁺ $\Delta grlR$ *grlA*⁺), CS6282 ($\Delta clpPX$ $\Delta grlR$ *grlA*⁺), CS6283 (*clpPX*⁺ *grlR*⁺ $\Delta grlA$) and CS6284 ($\Delta clpPX$ *grlR*⁺ $\Delta grlA$). Bacterial cells were grown in DMEM for 16 h at 37 °C. The data are the mean $\pm$ SD for each strain tested in triplicate. **P* < 0.05; ***P* < 0.01.

4a), neither disruption completely abolished the stimulating effect of *clpPX* disruption on *fliA* transcription; the *fliA*–*lacZ* transcriptional fusion in *grlR* and *grlA* disruption backgrounds was stimulated fourfold (Fig. 4b; compare *clpPX*⁺ and $\Delta clpPX$ in a $\Delta grlR$ background) and 3.6-fold (compare *clpPX*⁺ and $\Delta clpPX$ in a $\Delta grlA$ background), respectively. Immunoblotting demonstrated that the cellular levels of FlhC actually increased after ClpXP depletion in EHEC cells with different *grlA* and *grlR* backgrounds that were grown in DMEM and entered stationary phase (Fig. 5). These results indicate that the regulatory effect of ClpXP on *fliA* transcription under the LEE-expressing

condition could depend on not only transcriptional control of the *flhDC* operon via the GrlR–GrlA-dependent pathway but also the post-translational control of the FlhD and FlhC proteins.

To determine this possibility, we compared the levels of FlhD and FlhC proteins in *clpPX*⁺ and Δ *clpPX* cells where *flhDC* was expressed from the P_{A1lacO-1} promoter, to exclude the effect of transcriptional control of the *flhDC* operon. For this purpose, strains with *clpPX*⁺ and Δ *clpPX* were transformed with the plasmid pTKY802, in which the *flhDC* promoter had been replaced by the P_{A1lacO-1} promoter. EHEC cells were harvested when cultures entered stationary phase in DMEM and FlhD and FlhC proteins were detected by immunoblotting. Fig. 6 shows that FlhD and FlhC were detectable in the absence of IPTG, a condition in which *flhD* and *flhC* were expressed by read-through from the P_{A1lacO-1} promoter. The results show that disruption of *clpPX* significantly increased the levels of FlhD and FlhC even when the corresponding gene was expressed by an external promoter system, suggesting that FlhD and FlhC are controlled post-translationally by ClpXP in EHEC cells under conditions where LEE-encoded GrlA and GrlR are induced.

Fig. 5. Cellular levels of FlhC protein in EHEC cells with different genetic backgrounds grown in DMEM. The bacterial strains used were CS5295 (*clpPX*⁺ *grlR*⁺ *grlA*⁺), CS5297 (Δ *clpPX* *grlR*⁺ *grlA*⁺), CS6270 (*clpPX*⁺ Δ *grlR* *grlA*⁺), CS6271 (Δ *clpPX* Δ *grlR* *grlA*⁺), CS6272 (*clpPX*⁺ *grlR*⁺ Δ *grlA*) and CS6273 (Δ *clpPX* *grlR*⁺ Δ *grlA*). Cells were grown for 16 h at 37 °C, collected, lysed and separated by using 12.5% SDS-PAGE gels. The separated proteins were transferred to a membrane and immunostained with anti-FlhC antisera (a). Coomassie brilliant blue-stained gels of the samples used for immunostaining are shown in (b).

Fig. 6. Cellular levels of FlhD and FlhC proteins by a P_{A1-lacO-1} promoter–*flhDC* fusion in EHEC cells with *clpPX*⁺ or Δ *clpPX* background that had entered stationary phase in DMEM. Bacterial cells of strain CS5829 (*clpPX*⁺) and CS5830 (Δ *clpPX*) were grown for 16 h at 37 °C, collected, lysed and separated by using 15% SDS-PAGE gels. The separated proteins were transferred to a membrane and immunostained with anti-FlhC and anti-FlhD antisera (a). Coomassie brilliant blue-stained gels of the samples used for immunostaining are shown in (b).

These findings suggest that the heightened *flhA* transcription following ClpXP depletion can be attributed to the additive effects of decreased FlhDC proteolysis and increased *flhDC* transcription via the GrlR–GrlA-dependent pathway.

DISCUSSION

We have demonstrated that flagellar gene expression in EHEC is strictly regulated by ClpXP protease through dual pathways (summarized in Fig. 7), i.e. (i) post-translational control of the FlhD/FlhC master regulator by degrading them and (ii) transcriptional control of the *flhDC* operon through the GrlR–GrlA system under conditions in which LEE expression is induced. Concerning the relative importance of ClpXP regulation, it is indicated that post-translational regulation is a greater contributor to the control of flagellar biogenesis than are transcriptional controls. For example, in the analysis of the *in vivo* stabilities of FlhD and FlhC proteins (Fig. 1b) it would appear that after only 60 min, 80% of FlhD and 90% of FlhC have been degraded within the cell, while measuring the activities of the *flhD*–*lacZ* transcrip-

Fig. 7. Diagram of the dual regulatory pathways of flagellar gene expression by ClpXP protease in EHEC. The cellular concentration of FlhD/FlhC master regulator, which is essential for transcription of all the genes in the flagellar cascade is tightly controlled at the post-translational level by ClpXP protease. ClpXP also exerts transcriptional control of the *flhDC* operon through the GrlR–GrIA system. The role of GrIA as a negative regulator for *flhDC* transcription and GrlR as an antagonist of GrIA has been demonstrated by Iyoda *et al.* (2006). The present results support ClpXP as the major cause of FlhD/FlhC downregulation via post-translational degradation, but the contribution to transcription is very modest.

tional fusion shows that *flhD* transcriptional activity increases from ~60 to 85 Miller units, a modest 1.4-fold increase in the absence of ClpXP (Fig. 4a). We originally demonstrated the tight regulation of flagellar biogenesis due to the control of turnover of FlhD/FlhC proteins by ClpXP in *Salmonella* (Tomoyasu *et al.*, 2002). Furthermore, we have demonstrated that ClpXP degrades both proteins in the FlhD/FlhC complex but does not seem to recognize the individual subunits (Tomoyasu *et al.*, 2003). Both FlhD and FlhC proteins share high amino acid sequence homology between EHEC (FlhD, NP_310629; FlhC, NP_310628) and *Salmonella* (FlhD, NP_460882; FlhC, NP_460881). Moreover, ClpX, which is known to be involved in the recognition of protein substrates of the ClpXP protease, is highly conserved in EHEC (NP_308519) and *Salmonella* (NP_459445). We therefore speculated that both FlhD and FlhC are specifically recognized and degraded by ClpXP in EHEC in manner similar to that in *Salmonella*.

ClpXP downregulated the transcription of *flhDC* in EHEC only when the cells entered stationary phase in DMEM, in which LEE-regulated genes including *grlR* and *grlA* are known to be strongly expressed (Fig. 3). The analysis of transcription efficiency from the *flhD* promoter in the *ΔgrlR*

or *ΔgrlA* background (Fig. 4) was consistent with regulation through the GrlA–GrlR system, as previously reported, in which ClpXP represses GrlR to antagonize GrlA, which may be repressing the expression of the *flhDC* operon (Iyoda *et al.*, 2006). Although GrlA has a putative helix–turn–helix DNA-binding motif (Deng *et al.*, 2004), attempts to prove the DNA binding activity of GrlA have been unsuccessful. Therefore, the molecular mechanism by which GrlA represses *flhDC* transcription remains unknown. How does ClpXP alter the transcription of the *flhDC* operon? It is unlikely that ClpXP controls the transcriptional activity in a direct manner because of the proteins that form a protease. It is more likely that the effect is indirect, e.g. ClpXP controls the stability of a regulatory protein for the transcription of the *flhDC* operon. Iyoda & Watanabe (2005) studied the possibility that ClpXP may be directly involved in controlling the stability of the GrlR protein by comparing the levels of FLAG-tagged GrlR at different times after arresting protein synthesis in *clpXP*⁺ and *ΔclpXP* cells. The GrlR–FLAG level in the *clpXP*⁺ strain was unstable in stationary phase, suggesting that ClpXP is involved in controlling the stability of GrlR.

A very recent report has demonstrated that flagellar expression is much lower when *Salmonella* is grown in nutrient-poor medium (MGC) than in rich medium (LB) (Wada *et al.*, 2011). This nutritional control was shown to be executed at a step after *flhDC* transcription by YdiV, which is enhanced in *Salmonella* grown in poor medium. The flagellar cascade is known to be very similar in *E. coli* and *Salmonella* (Macnab, 1996). However, flagellar expression seems to respond quite differently to nutritional conditions in the two species, though *E. coli* also possesses *ydiV* (Zhou *et al.*, 2008). It was anticipated that this difference reflects a difference in *ydiV* expression (Wada *et al.*, 2011). Although no relationship between increased expression of *ydiV* and reduced expression of the flagellar genes has yet been established in *E. coli*, it was assumed that YdiV, if expressed in large enough amounts, could repress flagellar expression.

A study of the function of flagella in the interaction of EHEC O157:H7 with bovine intestinal epithelium has demonstrated that the H7 flagellum acts as an epithelial adhesion device (Mahajan *et al.*, 2009). Furthermore, its involvement in this crucial step in colonization indicated that H7 flagella could be an important target for intervention strategies. However, in EHEC and enteropathogenic *E. coli*, most of the bacterial cells attached to the actin pedestals formed on the target cell surfaces were flagellated at an early stage of infection of HeLa cells, while many cells associated with actin pedestals at later stages of infection did not form flagella (Yona-Nadler *et al.*, 2003). Therefore, it is speculated that the GrlR–GrlA system constructed under conditions in which the LEE genes for internalization strategies are expressed could be involved in the downregulation of flagellar formation. Since H7 flagellins from EHEC O157:H7 are the main triggers inducing expression of the neutrophil chemoattractant interleukin-8 in human intestinal epithelial cells (Berin *et al.*, 2002; Zhou *et al.*, 2003),

EHEC may need to downregulate flagellar synthesis to evade the host immune responses at a specific stage of infection. In support of this hypothesis, expression of *fliC* and *fliA* is repressed during intracellular growth of *Salmonella*, which depends on functioning of ClpXP (Cummings *et al.*, 2006).

Flagellar synthesis is an energy-consuming process, requiring 2 % of the cell's biosynthetic resources and 0.1 % of the cell's energy is consumed in their rotation (Fontaine *et al.*, 2008). Therefore, flagella are likely to be expressed only when they are needed and their biogenesis should be strictly regulated. The control of turnover of the FlhD/FlhC master regulator by ClpXP could be central to ensuring a limited number of flagella are formed to cope with survival of EHEC, *Salmonella* and related bacteria in their environments.

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