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DegP Is Involved in Cpx-Mediated Posttranscriptional Regulation of the Type III Secretion Apparatus in Enteropathogenic *Escherichia coli*

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The Cpx envelope stress response facilitates adaptation to envelope stresses that lead to the misfolding of periplasmic proteins. Cpx-mediated adaptation involves elevated expression of periplasmic proteases and chaperones. Previously, we demonstrated that induction of the Cpx envelope stress response in enteropathogenic *Escherichia coli* (EPEC) also results in inhibition of type III secretion (T3S) and that this is correlated with downregulated transcription of the relevant genes. Here, we investigated whether the Cpx stress response might also exert posttranscriptional effects on the T3S apparatus. We show that DsbA is required for T3S, while removal of transcription factor CpxR or the Cpx-regulated folding factor CpxP or PpiA has minimal effects. Conversely, the entire T3S complex is removed from the envelope when the Cpx response is activated. Overexpression of the chaperone/protease DegP mimics the Cpx-dependent inhibition of the T3S complex at a posttranscriptional level, and mutation of *degP* partly abrogates the ability of the Cpx response to inhibit the T3S complex and motility. We present data that suggest that both the protease and chaperone activities of DegP are likely important for the impact on T3S. Altogether, our data indicate that DegP is normally a part of the Cpx-mediated inhibition of virulence determinant expression in EPEC and that additional factors are involved.

The bacterial envelope is a dynamic compartment that houses a multitude of proteins involved in essential cellular processes. Direct contact with the external environment makes its protein content vulnerable to stress-induced misfolding. Signal-specific extracytoplasmic stress response systems have evolved in Gram-negative bacteria to alleviate the potential toxicity associated with the accumulation of misfolded proteins (for recent reviews, see references 39 and 54). One such system is the Cpx three-component signal transduction pathway. It is comprised of the transcription factor CpxR, the inner membrane sensory histidine kinase CpxA, and a small periplasmic inhibitor protein, CpxP (10, 13, 16, 53). CpxA has been shown to respond to a variety of external stressors, believed to generate misfolded periplasmic proteins, through autophosphorylation and subsequent phosphorylation of the response regulator CpxR (10, 11, 26, 33, 45, 52, 53, 58, 62). Phosphorylated CpxR upregulates the expression of protein folding and degrading factors and downregulates expression of certain proteins en route to the periplasm (10, 11, 40, 48, 49, 53, 61).

The Cpx pathway and the genes it regulates are important in pathogenesis (39, 40, 51, 60, 61). The Cpx regulon member DsbA catalyzes disulfide bond formation, a requirement for the proper folding of many virulence factors en route to the outer membrane (19). In *Escherichia coli*, these include structural components of the flagellar apparatus, the type III secretion system (T3SS), and adhesive appendages, as well as several toxins and enzymes secreted to the extracellular matrix (19, 34). Another regulon member, DegP, is a periplasmic protease/chaperone that rids the cell of potentially toxic misfolded proteins. This action is thought to facilitate survival of the bacterial cell within the mammalian host by promoting degradation of proteins that become misfolded as a result of host-induced oxidative damage (25, 37, 51). Conversely, high-level activation of the Cpx pathway results in the repression of select virulence genes, including those encoding the P pilus subunits of uropathogenic *E. coli* (UPEC), structural components and substrates of the T3SS of enteropathogenic *E. coli* (EPEC) (23, 40), and the EPEC type IV bundle-forming pilus (BFP) (61), as well as the master regulator of the motility genes *flhC* (12, 49).

We previously showed that the Cpx pathway inhibits EPEC type III secretion (T3S) by downregulating the expression of key components and substrates at the transcriptional level (40). In the same study, we observed that the decrease in transcription of the locus of enterocyte effacement (LEE) loci encoding these T3S components by the strongest Cpx-activating condition (*cpxA24* allele) was only 3-fold but that the secretion defect was complete. This observation suggests that posttranscriptional mechanisms may be involved in the inhibition of T3S in EPEC. The objective of the present study was to determine whether we could identify Cpx-regulated genes involved in posttranscriptional regulation of the T3S complex.

MATERIALS AND METHODS

Growth conditions. *E. coli* K-12 and EPEC strains were grown overnight with shaking at 37°C in LB broth supplemented with the appropriate antibiotics. Bacterial strains for which secretion assays and/or Western analysis was performed were grown in Dulbecco's modified Eagle's medium (DMEM)–F-12 in 5% CO_2 at 37°C, statically. Antibiotics were used at the following concentrations: kanamycin at 30 μg/ml for *E. coli* K-12 strains and 50 μg/ml for EPEC strains, chloramphenicol at 25 μg/ml, and streptomycin at 50 μg/ml.

Bacterial strains and plasmids. Bacterial strains employed in this study are described in Table 1. Knockout mutants were generated with W3110 by transducing the desired mutant alleles from the Keio collection (2) into wild-type W3110 using standard methods (57). The inducible pCA24N-based plasmids used in this study were obtained from the ASKA collection (30).

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TABLE 1 Strains and plasmids used in this study

Strain/plasmid	Relevant characteristic(s)	Source or reference
E. coli strains		
E2348/69	Prototype O127:H7 EPEC strain	36
W3110	F-1-IN (*rrnD-rrnE*)*1 rph-1*	Lab stock
MC4100	F *araD139* Δ(*argF-lac*)*U169 rpsL150* Str^r *relA1 flbB5301 deoC1 ptsF25 rbsR*	8
ALN88	E2348/69 *cpxR*::*kan*	46
ALN188	E2348/69 *degP*::*kan*	61
ALN190	E2348/69 *ppiA*::*kan*	61
ALN194	E2348/69 *cpxP*::*kan*	61
ALN195	E2348/69 *cpxA24*	40
TR1121	E2348/69 *dsbA*::*kan*	61
Plasmids		
pCA vectors	Gene of interest cloned downstream of the IPTG-inducible promoter on pCA24N	30
pJW17	LEE1-*lux* reporter	40
pJW20	LEE4-*lux* reporter	40
ptir-lux	LEE5-*lux* reporter	40
pJW25	*cpxP-lux* reporter	40
pCS19	Vector control for pCS20 and pCS21	59
pCS20	IPTG-inducible *degP*	59
pCS21	IPTG-inducible *degP*(*S210A*)	59

Secretion assays. Overnight cultures were diluted 1:100 in 2 ml of prewarmed DMEM–F-12 (catalog no. 11330-032; Invitrogen) containing the appropriate antibiotics in a 24-well tissue culture plate. Cultures were incubated statically in 5% CO_2 at 37°C. For strains with plasmids carrying genes controlled by IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoters, after 2 h of growth, IPTG was added to a final concentration of 0.1 mM, and the cultures were incubated for an additional 3 to 5 h to an absorbance measured at 600 nm (A_{600}) of 0.6 to 0.8. One ml of sample was taken from the culture, and cells were pelleted using a tabletop centrifuge. The supernatant was transferred to a fresh tube containing 10% trichloroacetic acid (TCA) and put on ice for at least 1 h. The cell pellet was resuspended in 2× sample buffer (55) and stored at −20°C. The precipitated, secreted proteins were pelleted at 14,000 rpm for 10 min at 4°C. The supernatant was removed, and the protein pellet was washed with acetone at −80°C for at least 2 h. The washed pellet was recentrifuged (10 min, 4°C), and the acetone was removed. The dried pellet was resuspended in 2× sample buffer. Secreted protein was subjected to SDS-PAGE (12% polyacrylamide gel) with Coomassie blue (10% Coomassie blue dye, 10% methanol-H_2O [1:1], 10% acetic acid) staining. Whole-cell lysates were subjected to Western analysis.

Western analysis. Western analysis was performed as outlined by MacRitchie et al. (40). Briefly, bacterial cells were resuspended in 2× sample buffer. The samples were boiled for 5 min, cooled to room temperature, and subjected to 10 to 12% SDS-PAGE. The protein was subsequently transferred to a nitrocellulose membrane and blocked with 5% nonfat milk (Difco). The blots were exposed to anti-Tir (1:400), anti-EscJ (1:300), anti-EscC (1:300), anti-CpxR (1:25,000), or anti-bacterial alkaline phosphatase (BAP) (1:50,000) antiserum. After a wash with 0.05% Tween 20 in 1× Tris solution, the blots were incubated with secondary antibodies conjugated to alkaline phosphatase (Invitrogen) and developed using a chemiluminescence kit (Invitrogen). The secondary antibodies used in our experiments included anti-rabbit (1:25,000) for anti-CpxR and anti-BAP antibodies, anti-mouse (1:12,500) for anti-Tir blots, and anti-rat (1:12,500) for anti-EscJ and anti-EscC Westerns (Invitrogen).

Motility assays. Bacterial strains were grown overnight to saturation. The following day, 2 μl of culture was inoculated into 0.3% agar plates containing 0.1 mM IPTG and 25 μg/ml chloramphenicol. The plates were incubated at 30°C, statically, for 12 to 15 h. The resulting swim diameters were measured and graphed as a percentage of the result for a vector control strain, which was present in the same plate. To determine whether the difference between two values was statistically significant, we performed an independent-samples *t* test on five replicates of each strain. The statistical software used was SSPS version 17 (2008; SSPS, Inc.).

Bioluminescence assays. Overnight cultures of strains harboring *luxCDABE* reporters were subcultured 1:100 into 2 ml of fresh LB broth containing the appropriate antibiotics in triplicate. IPTG (0.1 mM) was added to cultures of strains harboring pCA-based vectors (2) to induce overexpression of relevant proteins. The strains were grown with shaking at 37°C for 2 h. At this point, 200 μl of culture was transferred to a 96-well, white-sided tissue culture plate (Gibco), and the A_{600} and bioluminescence (counts per second [CPS]) were measured using a Wallac 420 multilabel plate reader (Perkin Elmer). The plate was returned to the incubator, and subsequent readings were taken. The final bioluminescence (CPS/A_{600}) values were calculated by dividing the corrected CPS ($CPS_{sample} - CPS_{blank\ control}$) by the corrected A_{600} ($A_{600\ sample} - A_{600\ blank\ control}$). Data presented represent the means and standard deviations from three replicates.

Quantification of bands on SDS-polyacrylamide gels and Western blots. EspB and EscJ bands on Coomassie-stained SDS-polyacrylamide gels and Western blots (respectively) were quantified using the program ImageJ as recommended by the developers (1). Briefly, bands in a lane were converted into a graphical representation, and the area under the peak representing a protein of interest was quantified. Relative amounts of protein were determined by setting the value obtained for the wild-type or vector control strain to 100% and adjusting other values accordingly. In cases where a T3S mutant control lane was present (see Fig. 3), we also subtracted the background value of that lane from that of all other lanes before adjusting the numbers to that of the wild-type or vector control strain.

RESULTS

Cpx regulon members facilitate EPEC virulence structure assembly. We have shown that Cpx-regulated protein folding and degrading factors found in the envelope are required for the efficient assembly of the type IV BFP of EPEC (61). To determine whether the Cpx-regulated genes *dsbA*, *degP*, *ppiA*, and *cpxP*, which encode known envelope protein folding and degradation factors, are important for the elaboration of other virulence determinants in EPEC, we examined T3S and motility in EPEC mutants lacking each of these folding factors. It was observed that mutation of *degP*, *ppiA*, or *cpxP* caused slight but reproducible decreases in T3S and motility (Fig. 1A and B). In addition, a *degP* mutant contained more TCA-precipitated protein in the supernatants than the wild-type strain (Fig. 1A). It has been shown that DsbA facilitates the proper folding of the outer membrane pore-forming proteins of both the T3SS and the flagellar apparatus of *E. coli* (9, 44). Consistent with these findings, we observed dramatically decreased levels of EspB secretion and motility in our *dsbA* mutant (Fig. 1A and B). Despite the fact that deletion of these Cpx-regulated gene products negatively impacted both T3S and motility, mutation of *cpxR* had a modest negative effect on T3S (Fig. 1A). These results suggest that although several Cpx-regulated genes appear necessary for efficient expression of the T3SS and motility, the Cpx response itself is not.

Activation of the Cpx response inhibits elaboration of the T3S apparatus. Previous work demonstrated that Cpx pathway activation dramatically inhibits the process of T3S but only moderately reduces the transcription of T3S effectors and complex components (40) (Fig. 1A). Based on this observation, we wondered whether the T3S defect reflected a loss of T3SS function

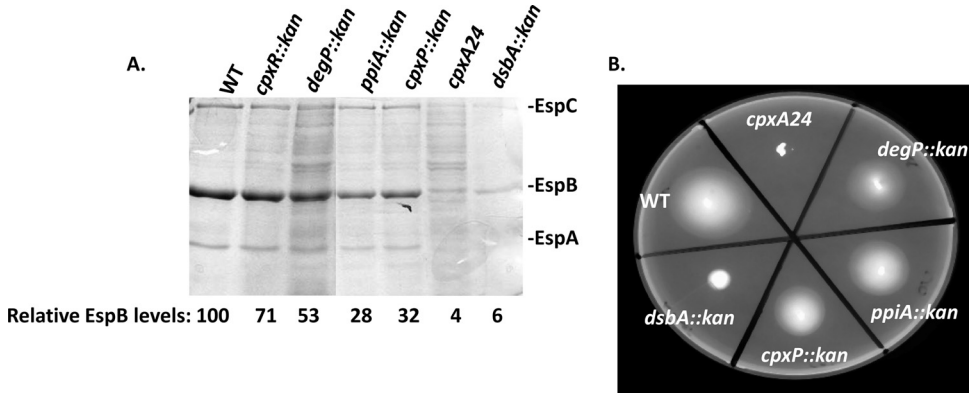


FIG 1 Cpx-regulated genes are required for efficient T3S and motility. (A) Coomassie blue-stained SDS-PAGE gel showing secreted proteins from EPEC and mutant strains with Cpx-regulated genes interrupted by a kanamycin cassette. The *cpxA24* mutant is a gain-of-function mutant. Relative amounts of EspB are indicated under the gel and were determined by using the program ImageJ (1) and setting the wild-type (WT) value to 100. (B) EPEC mutants were stabbed into motility agar and grown at 30°C for 15 h.

and/or a reduction in the number of T3S complexes. To answer this question, we analyzed the levels of the inner and outer membrane T3S components EscJ and EscC by Western blotting after activation of the Cpx response. The Cpx response was induced by mutation or NlpE overexpression, and whole-cell lysates from bacteria grown under conditions that stimulate T3S were analyzed (28). Cpx pathway activity in each strain was confirmed by examining the cellular levels of the response regulator CpxR, which is autoregulated (52) (Fig. 2, lanes 1, 4, and 5). The absence of a functional Cpx pathway in E2348/69 *cpxR*::*kan* had very little effect on EscJ or EscC levels (Fig. 2, lanes 1 and 2), consistent with our finding that mutation of *cpxR* does not alter the secretion of translocator proteins (Fig. 1) (40). In contrast, EPEC cells harboring a constitutively active *cpxA* allele, *cpxA24*, had undetectable levels of EscJ and EscC (Fig. 2, lanes 1 and 5). Similarly, EPEC cells for which the wild-type Cpx pathway was activated by overproduction of the outer membrane lipoprotein NlpE had little to no EscJ or EscC protein compared to the level for the vector control (Fig. 2, lanes 3 and 4). Combined, these studies suggest that, despite the modest effects that induction of the Cpx response has on transcription of the genes encoding the T3S apparatus, the entire EPEC T3S machine is depleted when the Cpx pathway is activated.

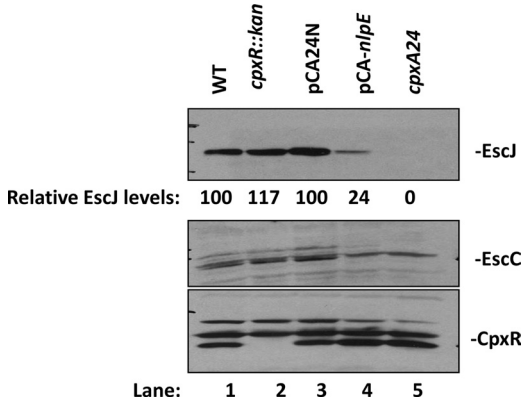


FIG 2 Activation of the Cpx response diminishes the components of the T3S machinery. Whole-cell lysates from E2348/69 (lane 1), E2348/69 *cpxR*::*kan* (lane 2), E2348/69(pCA24N) (lane 3), E2348/69(pCA-*nlpE*) (lane 4), and E2348/69 *cpxA24* (lane 5). EPEC strains were grown in DMEM–F-12 at 37°C in 5% CO_2 for 2 h, induced with 100 μM IPTG, and allowed to grow to an A_{600} of ~0.5 to 0.7. Bacterial cells were collected and subjected to Western analysis probing with anti-EscJ, anti-EscC (more prominent lower band), and anti-CpxR antiserum. Cross-reacting bands present in the anti-CpxR Western served as a loading control. Relative amounts of EscJ are indicated under the top panel and were determined by using the program ImageJ (1) and setting the wild-type and vector control values to 100.

Overexpression of DegP mimics Cpx pathway inhibition of the T3SS. The drastic reduction in T3S under Cpx-activating conditions is correlated with reduced expression of genes carried in the LEE (40). However, the incomplete inhibition of LEE transcription by the Cpx response, together with the inability of overexpression of the positive regulators of LEE transcription, PerABC, to rescue T3S, suggested that the Cpx response might inhibit T3S in EPEC at posttranscriptional levels (40). In order to determine whether any known Cpx-regulated protein folding and degrading factors contributed to this phenotype at the posttranscriptional level, vectors harboring *degP*, *dsbA*, *ppiA*, and *cpxP* under the control of an IPTG-inducible P_{T5}-*lac* promoter were transformed into wild-type EPEC cells, expression of the relevant genes was induced, and the functions of the T3SS and flagella were assessed. In order to confirm that each regulon member was overexpressed, the bacterial cells collected during the secretion assay were subjected to SDS-PAGE and Coomassie blue staining (Fig. 3A). A band of the appropriate size was visible for the strains overexpressing DegP, DsbA, and PpiA (Fig. 3A, compare lanes 4, 5, and 6 to lane 3). CpxP overexpression could not be confirmed by this method. We found that overexpressing the periplasmic protease DegP drastically diminished EspB secretion (Fig. 3A, compare lanes 3 and 4). This is the same effect seen on levels of EscC and EscJ when the Cpx response is activated by mutation or NlpE overexpression (40) (Fig. 2). Overexpression of *dsbA* or *ppiA* had no or very little effect on EspB secretion (Fig. 3A, compare lane 3 to lanes 5 and 6). Curiously, although we could not confirm CpxP overexpression, it appeared to result in a noticeable increase in EspB levels (Fig. 3A, compare lanes 3 and 7). The same observations were made with respect to the impact of overexpressing DegP on motility (Fig. 3B).

To determine whether the effect of DegP overexpression on T3S was posttranscriptional, we assayed the expression of the luminescent reporter genes LEE1-*lux*, LEE4-*lux*, LEE5-*lux*, and

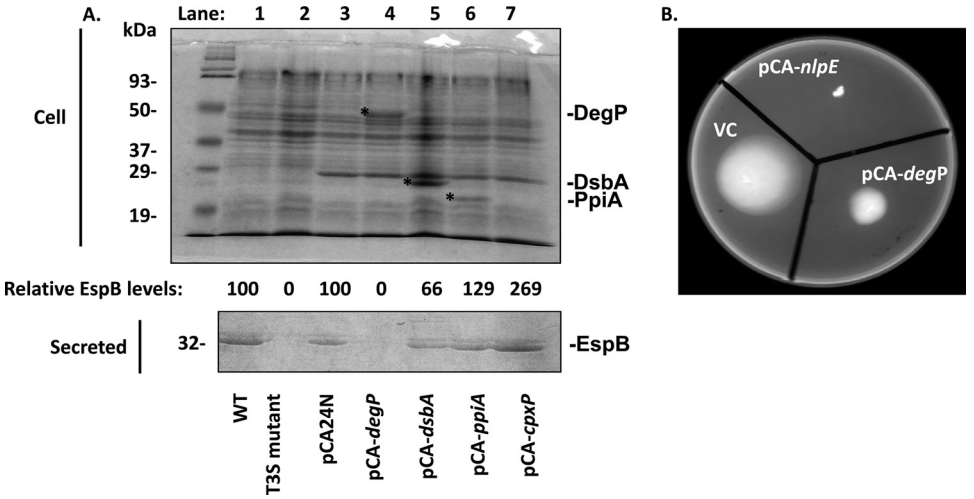


FIG 3 Overexpression of DegP inhibits T3S and motility. Secretion assay of EPEC strains overexpressing individual Cpx regulon members. (A) Cellular protein was subjected to SDS-PAGE with Coomassie blue staining (top). The extracellular level of T3S substrate EspB was examined by SDS-PAGE and Coomassie blue staining of secreted proteins (bottom). Relative amounts of EspB are indicated under the Western blot and were determined by using the program ImageJ (1) and setting the wild-type and vector control values to 100. (B) EPEC strains harboring overexpression vectors were stabbed into motility agar containing the appropriate antibiotics and 100 μM IPTG and grown at 30°C for 15 h. VC, vector control.

cpxP-lux in the presence of pCA24N and pCA-*degP*. DegP overexpression was found to have no discernible effect on any of the promoters tested (Fig. 4). This suggests that the accumulation of the DegP protease affects T3S posttranscriptionally rather than by activating the Cpx pathway and causing a concomitant decrease in the transcription of the T3S genes. To further confirm that DegP overexpression was acting independently of Cpx pathway activation, a secretion assay with a *cpxR* null E2348/69 derivative harboring pCA-*degP* was performed. In the absence of a functional Cpx pathway, DegP overexpression continued to cause a decrease in T3S (data not shown).

To determine whether DegP was responsible for the decrease in T3S observed under Cpx-activating conditions, the T3S profile of an E2348/69 *degP*::*kan* mutant strain harboring the vector control pCA24N or pCA-*nlpE* was examined. In wild-type strains, NlpE overexpression led to a dramatic decrease in the levels of T3 secreted proteins, as previously observed (Fig. 5A, compare lanes 2 and 3). Conversely, in a *degP* mutant, although the amounts of EspB, EspD, and EspA that were secreted were still diminished under Cpx-activating conditions, this decrease was obviously smaller than that seen in the wild-type strain (Fig. 5A, compare lanes 2 and 3 to 4 and 5). Removal of DegP also resulted in a slight restoration of motility under Cpx-activating conditions ($P = 0.000323$) (Fig. 5B). While wild-type EPEC cells overexpressing NlpE had a swim diameter that was 9% of that of the vector control strain, the swim diameter of E2348/69 *degP*::*kan*(pCA-*nlpE*) was 19% of that of its vector control (Fig. 5B). The same experiments were performed for the remaining regulon members. Elimination of *ppiA*, *dsbA*, or *cpxP* failed to alleviate Cpx-mediated inhibition of T3S or motility (data not shown). Based on these findings, we conclude that DegP is a contributing factor in the Cpx-mediated inhibition of EPEC T3S and motility.

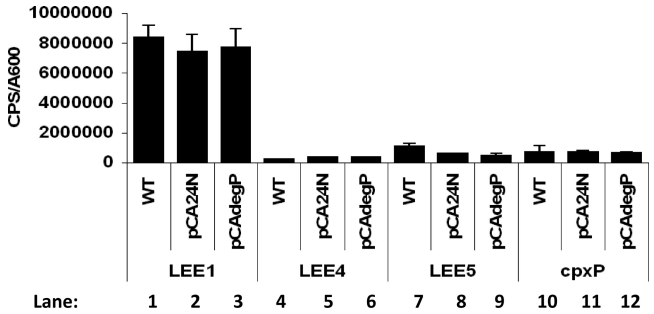


FIG 4 DegP overexpression inhibits T3S at the posttranscriptional level. Expression from the LEE1, LEE4, LEE5, and *cpxP* promoters was measured by monitoring levels of bioluminescence from luminescent reporter genes in wild-type EPEC, E2348/69(pCA24N), and E2348/69(pCA-*degP*). Strains were grown in LB broth at 37°C with shaking, and the A_{600} and bioluminescence (CPS) were measured every 2 h for 10 h. Data represented here are from the 6-h time point (mid-log phase). Bars represent the means from three replicates; error bars represent the standard deviations.

DegP protease and chaperone functions are involved in T3S inhibition. DegP facilitates protein folding homeostasis in the periplasm by means of both protease and chaperone activities (59). We wondered whether the protease activity, the chaperone activity, or both mediated the inhibition of T3S when DegP was overexpressed or the Cpx response activated. To answer this question, we collected secreted proteins from EPEC strains carrying the pCS19 vector control; the pCS20 plasmid, which carries an inducible copy of the wild-type *degP* gene; or the pCS21 plasmid, which carries an inducible copy of the gene encoding a proteolytically inactive DegP, *degP*(*S210A*) (59). Overexpression of a wild-type *degP* gene caused a little over 5-fold reduction in the amount of secreted EspB (Fig. 6, compare lanes 3 and 4). While still mediating a noticeable decrease in the amount of T3S protein, overexpression of the mutant *degP*(*S210A*) allele led to only a 2.4-fold reduction in the amount of secreted EspB (Fig. 6, compare lanes 3 and 4 to lanes 5 and 6). Analysis of Coomassie blue-stained SDS-polyacrylamide gels of whole-cell lysates indicated that the wild-type and DegP(S210A) proteins were expressed at comparable levels (data not shown). Although we cannot rule out that a small difference in protein expression might account for the reduced

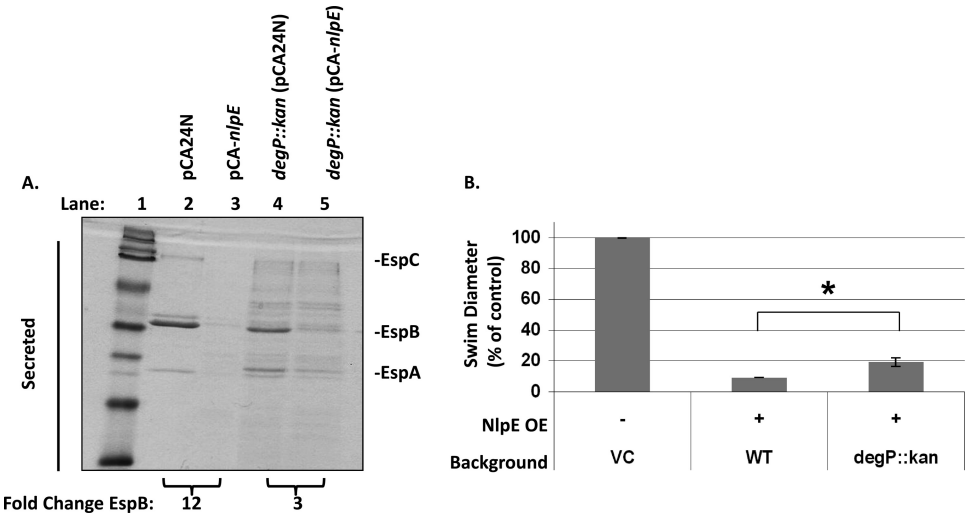


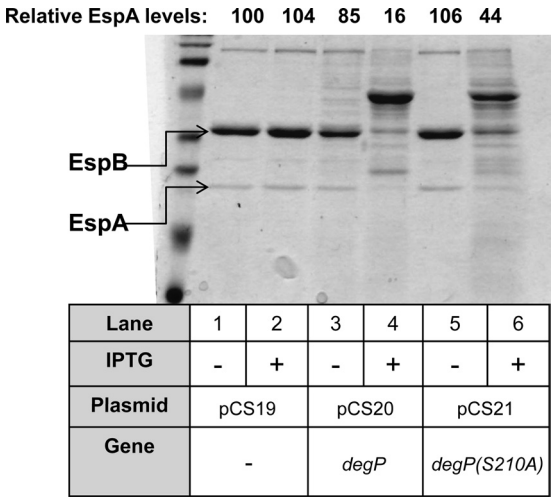
FIG 5 DegP contributes to the Cpx-mediated inhibition of T3S and motility. (A) Secretion profile of EPEC strains. Broad-range molecular weight marker (lane 1), E2348/69(pCA24N) (lane 2), E2348/69(pCA-*nlpE*) (lane 3), E2348/69 *degP*::*kan*(pCA24N) (lane 4), and E2348/69 *degP*::*kan*(pCA-*nlpE*) (lane 5). The amount of EspB in each lane was quantified using the program ImageJ (1), and the values for the vector control strains were set to 100. The fold change was determined by dividing the vector control amount of EspB by the amount present in the pCA-NlpE strain. (B) EPEC strains were stabbed into motility agar, and the swim diameter was calculated as a percentage of the vector control value. Bars represent the means from three replicates; error bars represent the standard deviations. The asterisk indicates a significant difference ($P = 0.000323$). OE, overexpression.

ability of DegP(S210A) to inhibit T3S, our data argue that both the protease and chaperone activities of DegP are likely to be important for the negative impact on T3S.

DISCUSSION

It is well documented that several Cpx-regulated protein folding and degrading factors involved in envelope maintenance also facilitate pathogenesis. Accordingly, we investigated the involvement of key Cpx regulon members (DsbA, PpiA, DegP, and CpxP) in the function of the EPEC T3SS and motility. As previously published, DsbA was essential for both T3S and motility (9, 44) (Fig. 1). *degP*, *ppiA*, and *cpxP* mutants exhibited a slight but reproducible decrease in T3S and motility (Fig. 1). PpiA is a peptidyl-prolyl-isomerase (27) and may facilitate biogenesis of T3S and flagellar components. CpxP, on the other hand, serves as a negative regulator of the CpxA sensor kinase, is an adaptor to target misfolded proteins to DegP for degradation, and has weak chaperone activity (5, 24, 50, 52, 63). Since deletion of *cpxP* leads to higher than normal levels of Cpx pathway activity in the absence of an inducing cue (52), it may be that the E2348/69 *cpxP*::*kan* mutant has an elevated level of Cpx pathway activity which causes the observed decrease in T3S and motility. DegP serves an important cellular function by degrading potentially toxic misfolded envelope proteins in the periplasm and facilitating the folding of certain outer membrane proteins (OMPs) (3, 4, 56). The secretion profile of our E2348/69 *degP*::*kan* mutant differed greatly from that of wild-type EPEC in that it contained a larger variety of protein species although not obviously elevated levels of EspB, EspA, and EspD (Fig. 1 and 5). This observation suggests an envelope defect in the *degP* mutant, as has been observed in other organisms (3), but argues against a major role for DegP in promoting T3S.

Curiously, although these data implicate Cpx-regulated protein folding and degrading factors in the efficient expression of the T3S and motility complexes, deletion of *cpxR* had very little effect on T3S (Fig. 1A), ruling out a major role for the Cpx response in the biogenesis of these structures. These seemingly contradictory data can be explained by the fact that CpxR is not required for basal expression of *dsbA* or *ppiA* (49). Thus, our data argue that while these factors do appear to be involved in T3SS and flagellar biogenesis, their Cpx regulation is not necessary for this function. Following this line of thought, it would appear that the processes



Lane	1	2	3	4	5	6
IPTG	-	+	-	+	-	+
Plasmid	pCS19		pCS20		pCS21	
Gene	-		*degP*		*degP(S210A)*	

FIG 6 Both the proteolytic and chaperone activities of DegP are involved in inhibiting T3S. Secreted proteins were collected from E2348/69 strains carrying the vector control (pCS19), a plasmid that overexpresses wild-type DegP (pCS20), and a plasmid that overexpresses a proteolytically inactive DegP (pCS21), in the absence (−) and presence (+) of the inducer IPTG. TCA-precipitated proteins were electrophoresed on an SDS-polyacrylamide gel and stained with Coomassie blue. Relative amounts of EspA are indicated above the gel and were determined by using the program ImageJ (1) and setting the value for the vector control in the absence of IPTG to 100.

of T3S and motility themselves do not generate an envelope stress that demands the presence of the Cpx response.

We previously showed that Cpx pathway activation downregulates the transcription of genes encoding late-stage structural components and substrates of the T3SS, the *bfp* genes encoding the type IV pilus, and several genes that are part of the flagellar regulatory hierarchy (40, 49, 61). In the present study, we determined that induction of the Cpx response results in almost complete abolition of the major inner and outer membrane T3S complex components EscJ and EscC from the cell (Fig. 2) and causes a dramatic decrease in motility (Fig. 1B and 3B). Induction of the Cpx response has also been shown to negatively affect the expression of the conjugation pilus, the uropathogenic *E. coli* (UPEC) P pilus, and the T3SS and invasin adhesin of *Yersinia pseudotuberculosis* (6, 7, 17, 20). These observations demonstrate that the elimination of protein complexes from the envelope is a conserved role of the Cpx response upon activation and further suggest that minimizing protein traffic in this compartment is a necessary component of adaptation to stresses that cause protein misfolding.

Interestingly, the inhibitory effect of the Cpx response on envelope protein complexes appears to be mediated by multiple mechanisms. In some cases, such as the EPEC type IV BFP and the UPEC P pilus, Cpx-mediated inhibition appears to occur entirely through reduced transcription of the structural and biogenesis genes (20, 22, 61). In other cases, posttranscriptional mechanisms are involved. The TraJ regulator of the conjugation pilus genes is actively degraded by the HslVU chaperone/protease pair upon Cpx response induction (35). Similarly, Carlsson and colleagues showed that Cpx-dependent inhibition of T3S in *Yersinia* spp. involves both transcriptional and posttranscriptional mechanisms (6). Previously, we showed that the T3S genes carried in the LEE as well as genes that are part of the flagellar regulon are inhibited at the transcriptional level upon Cpx response activation (40, 49). Here, we show that both the chaperone and protease activities of DegP are required for full inhibition of T3S and motility (Fig. 3, 4, 5, and 6).

Expression of *degP* is also regulated by the σ^E envelope stress response (14, 38). Interestingly, the σ^E response has been shown to have a positive influence on T3S in other enteric pathogens (6, 47). These findings suggest a stimulatory role for DegP in T3S, as opposed to the inhibitory role that we describe. One explanation for these apparently contradictory findings could be that DegP manifests different roles in T3S that are context dependent. σ^E is expressed intracellularly during *Salmonella enterica* serovar Typhimurium infections (15), while it seems unlikely that the Cpx response would be active at the site of infection, given its demonstrated inhibitory role in virulence (6, 21, 40, 61). Thus, one possibility is that DegP exerts positive effects on T3S at the site of infection, where a different suite of genes are expressed, but negatively impacts T3S at times when the Cpx response is induced. Since DegP has both chaperone and protease activities (59), it is perhaps intuitive that different conditions might alter the relative ratio of these activities to exert alternative outcomes.

DegP acts at a posttranscriptional step (Fig. 4); however, at this point, we cannot say how. DegP must be activated allosterically by misfolded protein substrates in order to form higher-order oligomeric structures which have both protease and chaperone activities (18, 31, 32, 41, 42, 56), so one possibility is that, under conditions when the Cpx response is activated, components of the T3S and/or flagellar apparatuses become misfolded and thus substrates for DegP. If this is the case, misfolding of T3S complex components in the presence of stress is not dependent on the apparatus being functional, since Cpx-mediated inhibition of T3S still occurs in a mutant lacking the EscN ATPase, which is necessary for secretion (data not shown). Another possibility is that proteolytic adapters, such as CpxP (24), are induced upon Cpx response induction and alter the substrate specificity of DegP, ultimately leading to the elimination of protein complexes from the envelope. In this regard, we note that there are reports of DegP targeting individual subunits of higher-order complexes for proteolytic degradation (29). Alternatively, DegP may act on an unidentified component of the envelope, which in turn impacts the stability of the T3S apparatus.

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