Selectivity among Anti- σ Factors by *Mycobacterium tuberculosis* ClpX Influences Intracellular Levels of Extracytoplasmic Function σ Factors

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ABSTRACT Extracytoplasmic function σ factors that are stress inducible are often sequestered in an inactive complex with a membrane-associated anti- σ factor. *Mycobacterium tuberculosis* membrane-associated anti- σ factors have a small, stable RNA gene A (*ssrA*)-like degron for targeted proteolysis. Interaction between the unfoldase, ClpX, and a substrate with an accessible degron initiates energy-dependent proteolysis. Four anti- σ factors with a mutation in the degron provided a set of natural substrates to evaluate the influence of the degron on degradation strength in ClpX-substrate processivity. We note that a point mutation in the degron (X-Ala-Ala) leads to an order-of-magnitude difference in the dwell time of the substrate on ClpX. Differences in ClpX/anti- σ interactions were correlated with changes in unfoldase activities. Green fluorescent protein (GFP) chimeras or polypeptides with a length identical to that of the anti- σ factor degron also demonstrate degron-dependent variation in ClpX activities. We show that degron-dependent ClpX activity leads to differences in anti- σ degradation, thereby regulating the release of free σ from the σ /anti- σ complex. *M. tuberculosis* ClpX activity thus influences changes in gene expression by modulating the cellular abundance of ECF σ factors.

IMPORTANCE The ability of *Mycobacterium tuberculosis* to quickly adapt to changing environmental stimuli occurs by maintaining protein homeostasis. Extracytoplasmic function (ECF) σ factors play a significant role in coordinating the transcription profile to changes in environmental conditions. Release of the σ factor from the anti- σ is governed by the ClpXP2P1 assembly. *M. tuberculosis* ECF anti- σ factors have an *ssrA*-like degron for targeted degradation. A point mutation in the degron leads to differences in ClpX-mediated proteolysis and affects the cellular abundance of ECF σ factors. ClpX activity thus synchronizes changes in gene expression with environmental stimuli affecting *M. tuberculosis* physiology.

KEYWORDS AAA+ ATPase, gene expression

Mycobacterium tuberculosis encounters diverse host microenvironments, including acidification of phagosomes, nitrogen intermediates, reactive oxygen species, nutrient starvation, DNA damage, phosphate deprivation, and hypoxia (1). Extracytoplasmic function (ECF) σ factors are nonessential and stress inducible, and they contribute significantly to bacterial survival, alongside one- and two-component systems (2). *M. tuberculosis* has 10 ECF σ factors, 4 of which are localized in an inactive complex with membrane-associated anti- σ factors (3, 4). The membrane-associated anti- σ factors (RsdA, RsmA, RskA, and RslA in *M. tuberculosis*) share a structural organization comprising an extracytoplasmic domain that is a receptor for environmental stress connected to the cytoplasmic anti- σ domain by a single transmembrane helix (Fig. 1). The stress-induced release of an ECF σ factor from the σ /anti- σ factor complex

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The intracellular release of an ECF σ factor from the inactive membrane-associated

σ /anti- σ complex is governed by a proteolytic cascade referred to as the regulated intramembrane proteolysis (RIP) pathway (6). This cascade is initiated by the action of a so-called site 1 protease that acts on the extracytoplasmic domain of the anti- σ factor (6). This triggers the activity of a transmembrane protease (site 2 protease) that dissociates the σ /anti- σ complex from the membrane. The anti- σ factor is then degraded by energy-dependent proteolytic complexes, releasing the bound ECF σ factor, which can then associate with the RNA polymerase and initiate transcription (Fig. 1A). The intracellular proteolysis of the anti- σ factor RseA is primarily governed by ClpXP in *Escherichia coli*, although other proteolytic assemblies also contribute to this process (7). The specific degradation of *E. coli* RseA from the σ^E /RseA complex is also influenced by an adaptor protein, SspB (8). *E. coli* SspB-mediated interactions are crucial for effective degron recognition: while *E. coli* ClpX interacts with residues 9 to 11 at the C terminus of the small, stable RNA gene A (*ssrA*) degron, SspB interacts with residues 1 to 4 and 7 (9–11). Other *E. coli* ClpX adaptors that have been characterized are RssB and UmuD (12, 13). The presence of different adaptors suggested a mechanism for the specific recruitment of diverse substrates for the ClpX unfoldase to initiate proteolysis with the serine protease ClpP in the ClpXP proteolytic complex (12).

ClpX comprises a small N-terminal domain (NTD) flexibly attached to the unfoldase module, the AAA+ domain (14). The AAA+ domain has multiple conserved sequence features, including Walker A and Walker B motifs for ATP binding, a second region of homology (SRH) segment involved in ATP hydrolysis, and sensor 2 and 3 residues that propagate conformational changes upon ATP hydrolysis to stabilize the ATP binding conformation of the unfoldase (14–18). With the two domains functioning in a concerted manner, ClpX can translocate and unfold a diverse range of substrates (19). Analysis of *E. coli* ClpX substrates suggested five distinct degron motifs (19). Apart from adaptor proteins that enforce specificity, the N-terminal domain of *E. coli* ClpX is also involved in substrate recognition (15). The role of the ClpX N-terminal domain, however, differs across substrates. While the N-terminal domain substantially influences *E. coli* ClpX action on substrates like λ O and MuA, it has much less influence on green fluorescent protein (GFP) substrates with an *ssrA* degron (15).

The *M. tuberculosis* RIP pathway is only partially characterized. The site 1 protease that initiates the proteolytic cascade in the RIP pathway has not been identified in *M. tuberculosis*. One site 2 protease, Rip1 (Rv2869c), acts on all membrane-associated anti- σ factors (RskA, RsmA, RslA, and RsdA) (20, 21). For comparison, in *E. coli*, the first two proteolytic steps are performed by DegS and YaeL (22, 23). However, straightforward extension from the *E. coli* model for the subsequent steps is difficult, as there are four membrane-associated σ /anti- σ complexes in *M. tuberculosis* (σ^D /RsdA, σ^K /RskA, σ^L /RslA, and σ^M /RsmA) as opposed to one (σ^E /RseA) in *E. coli* (Fig. 2A). The cytosolic step of the cascade involving intracellular proteolytic complexes is also substantially different in *M. tuberculosis* than in either *E. coli* or *Bacillus subtilis* (8, 24). For example, *M. tuberculosis* has two ClpP protease components, ClpP1 and ClpP2 (25). Furthermore, targeted protein degradation by the ClpXP complex is also influenced by adaptor proteins in *E. coli*. Unlike in *E. coli*, no SspB homologue or adaptor of *M. tuberculosis* ClpX has been annotated or experimentally identified thus far. Nonetheless, previous studies revealed that the cytoplasmic domain of RsdA was recognized and cleaved by the *M. tuberculosis* ClpXP2P1 complex (26). The degron in *M. tuberculosis* RsdA is VAA, identical to that in *E. coli* RseA. RslA, however, was found to be resistant to ClpXP2P1 degradation, despite having the *ssrA*-like degron (26). Apart from proteolytic degradation, another mechanism(s) modulates the cellular abundance of specific ECF σ factors by altering the rate of release of an ECF σ from an inactive σ /anti- σ complex. For example, *M. tuberculosis* RslA was shown to release σ^L under oxidative stress conditions (27). In this case, the receptor for the redox stimulus was the zinc binding CXXC motif in the anti- σ factor, RslA. The release of zinc under oxidizing conditions was seen to alter the conformation of RslA, thereby releasing σ^L . *M. tuberculosis* RskA also was shown to dissociate from σ^K under reducing conditions, the σ factor σ^K being the redox sensor

FIG 2 SPR sensorgrams of ClpX interactions with the four σ /anti- σ factor complexes. (A) Schematic representation of the *M. tuberculosis* σ factors in complex with the membrane-anchored anti- σ factors. (B) SPR sensorgram of σ^D /RsdA-ClpX interactions. σ^D /RsdA binds most tightly to ClpX, with an affinity of ca. 14.5 nM. R.U., response units. (C) A 10-fold reduction in the binding affinity was seen in the binding of σ^M /RsmA with ClpX (corresponding to a K_D of $1.33 \cdot 10^{-7}$ M). (D) The binding of σ^K /RskA with ClpX is intermediate between those of σ^M /RsmA and σ^L /RslA (ca. 164 nM). (E) Interaction of σ^L /RslA with ClpX reveals substantial reduction in the binding affinity compared to that of σ^D /RsdA. The interaction data are compiled in Table 1. Details of the protein constructs are described in Table SA4 in the supplemental material.

in this case (28). All these anti- σ factors, however, also contain an ssrA-like degron that is exposed upon RIP-1 (site 2 protease) activity.

In the regulated proteolytic cascade, the targeted proteolysis of an anti- σ factor by the ClpXP proteolytic complex is the last step in signal transduction to effect changes in gene expression in response to environmental stress. The cytosolic domains of four *M. tuberculosis* anti- σ factors with the ssrA-like degron provided a set of natural variants to understand the basis for substrate selection in *M. tuberculosis* ClpX. We note that while the N-terminal domain of ClpX is not involved in degron recognition, it influences unfoldase activity. We also describe biochemical experiments which reveal that the degron sequence governs both the substrate binding affinity and the kinetics of unfolding. The variation in the dwell time of the substrate on ClpX was also seen to have a direct bearing on the proteolytic degradation of the anti- σ substrates by the

FIG 3 Sequence and conformational features that influence ClpX substrate recruitment in the absence of adaptor proteins. (A, B) SPR measurements of σ^D /RsdA_{AAA}-ClpX and σ^D /RsdA_{GAA}-ClpX interactions reveal that σ^D /RsdA_{AAA} and σ^D /RsdA_{GAA} bind to ClpX with 10-fold lower affinity than the σ^D /RsdA_{VAA} complex. The binding affinity of the σ^D /RsdA_{AAA} complex is equal to that of the σ^L /RslA complex. (C) Charged residues at the terminal end of the degreon substantially reduce ClpX-substrate interaction. The affinity of the σ^D /RsdA_{VDD} complex is 100-fold lower than that of the σ^D /RsdA_{VAA} complex.

ClpXP2P1 complex to release free ECF σ factors that can initiate transcription. In effect, *M. tuberculosis* ClpX translates variations in the degreon sequence into differential unfoldase activities. These degreon-dependent differences in the last step in the *M. tuberculosis* RIP cascade are thus likely to provide an additional regulatory layer for nuanced changes in the transcriptional profile in response to a stress stimulus.

RESULTS

The *ssrA*-like degreon governs interactions between *M. tuberculosis* ClpX and anti- σ substrates. The binding affinity of *M. tuberculosis* ClpX with the anti- σ substrates containing the C-terminal *ssrA*-like degreon was determined by surface plasmon resonance (SPR) (Fig. 2B to D). As the purified anti- σ factors are prone to aggregation, the purified substrates used in these experiments consisted of the ECF σ factor complexed with an anti- σ factor containing the degreon at the C terminus. Coexpression and copurification of the σ /anti- σ factor complexes significantly improves the yield of homogenous protein samples, as the σ or anti- σ factors in isolation are relatively unstable (29). The segment comprising the last three residues (9–11) of the *ssrA* degreon was shown to interact with *E. coli* ClpX (19). This feature was seen to be retained in the case of *M. tuberculosis* ClpX (26). SPR sensorgrams revealed that deletion of the terminal residues of the degreon abrogated the binding of these substrate proteins to ClpX, a finding similar to the case where the last three residues of the degreon (VAA) were replaced by a negatively charged C terminus (VDD) (Fig. 3C; see Fig. SA1 in the supplemental material). Indeed, the finding that substrates without the degreon do not bind ClpX also suggests that nonspecific binding is unlikely (Fig. SA1). These constructs were employed as control, inactive “degreons” in the subsequent analysis. *M. tuberculosis*

TABLE 1 Kinetic parameters of ClpX interactions with anti- σ factors

σ /anti- σ factor complex	k_a ($M^{-1} s^{-1}$) ^a	k_d (s^{-1}) ^b	K_D (M)	ΔG^c (kJ/mol)
σ^D /RsdA ₍₁₋₉₄₎	$6.4 \cdot 10^4$	$9.2 \cdot 10^{-4}$	$2.8 \cdot 10^{-8}$	-44.66
σ^M /RsmA ₍₁₋₁₁₈₎	$2 \cdot 10^4$	$2.7 \cdot 10^{-3}$	$1.2 \cdot 10^{-7}$	-39.22
σ^K /RskA ₍₁₋₉₈₎	$3.3 \cdot 10^4$	$5.5 \cdot 10^{-3}$	$2.2 \cdot 10^{-7}$	-38.69
σ^L /RslA ₍₁₋₁₂₅₎	$3.0 \cdot 10^4$	$4.9 \cdot 10^{-3}$	$2.3 \cdot 10^{-7}$	-38.02

^a k_a , association rate constant.^b k_d , dissociation rate constant.^cG, Gibbs free energy.

ClpX bound to σ^D /RsdA with the highest affinity (Table 1). We note a 10-fold reduction in ClpX binding to the σ^L /RslA, σ^K /RskA, and σ^M /RsmA complexes compared with its binding to the σ^D /RsdA substrate (Table 1). These SPR measurements were performed using freshly prepared ClpX samples. We note that ATP does not appear to be a prerequisite for substrate binding. This differs from previous reports that suggested nucleotide addition as a trigger for substrate recruitment in this class of unfoldases. A related observation is that while freshly prepared ClpX samples are primarily hexameric, with a small dimeric component, the hexameric species is unstable in the absence of ATP (polydispersity increases after ca. 24 h). SPR sensorgrams reveal that the difference in these ClpX- σ /anti- σ interactions lies in the dissociation rate constant (k_d) (Table 1). A comparison between the standard deviation in these measurements across different substrates is shown in Fig. SA2. The degrons in the four anti- σ factors have different aliphatic residues at the antepenultimate position (Fig. 1C). Mutations in the degon of RsdA where the degon resembled those of RslA (AAA) and RsmA (GAA) shows that the binding affinities of ClpX with the σ^D /RsdA_{AAA} and σ^D /RsdA_{GAA} mutants are lower than that of wild-type σ^D /RsdA, being comparable to ClpX interactions with the σ^L /RslA and the σ^M /RsmA complex, respectively (Fig. 3A and B; Table 2). These observations suggest that although the C-terminal alanines in the degon are involved in ClpX interactions, the antepenultimate residue influences substrate tethering and, consequently, the residence time of the substrate protein on the ClpX hexamer (Table 2).

The degon in substrates determines ClpX ATPase and unfoldase activity.

Processing of protein substrates by ClpX is coupled to the rate of ATP hydrolysis (30). ATPase assays revealed that differences in the ClpX binding affinities of the four anti- σ substrates were correlated with the rates of ATP hydrolysis. That substrate binding directly influences ATPase activity was evident from the observation that the ATPase activity of ClpX was highest in the presence of σ^D /RsdA, almost 5-fold higher than that of ClpX alone. Second, ClpX ATPase activity was not altered in the presence of the substrate with the inactive degon in σ^D /RsdA (VDD). The ATPase activity of ClpX was 3-fold lower in the presence of σ^L /RslA than in the presence of σ^D /RsdA (Fig. 4A; Table 3). Given that the lengths of the anti- σ factors vary from 222 to 375 residues, due to the linker between the transmembrane helix and the cytosolic anti- σ domain (Fig. 1C; Table SA5), the ATPase activity of ClpX was evaluated using one substrate (σ^D /RsdA) with various antepenultimate residues in the degon (Fig. 4A). When the antepenultimate residue was modified to Ala/Gly from Val in σ^D /RsdA, ATPase activity decreased, consistent with the results of SPR experiments that show that ClpX binds this substrate

TABLE 2 Interactions between σ^D /RsdA degon variants and ClpX

σ^D /RsdA degon variant	k_a ($M^{-1} s^{-1}$) ^a	k_d (s^{-1}) ^b	K_D (M)	ΔG (kJ/mol)
VAA	$6.4 \cdot 10^4$	$9.2 \cdot 10^{-4}$	$1.4 \cdot 10^{-8}$	-44.66
GAA	$4.3 \cdot 10^4$	$5.8 \cdot 10^{-3}$	$1.4 \cdot 10^{-7}$	-35.84
AAA	$6.8 \cdot 10^3$	$2.7 \cdot 10^{-3}$	$6.9 \cdot 10^{-7}$	-36.49
VDD	$1.3 \cdot 10^4$	$1.3 \cdot 10^{-2}$	$1.1 \cdot 10^{-6}$	-33.37

^a k_a , association rate constant.^b k_d , dissociation rate constant.

FIG 4 The degren influences the unfoldase activity of *M. tuberculosis* ClpX. (A) The ATPase activity of ClpX is altered by the presence of substrates, with the maximum activity for the $\sigma^D/RsdA_{VAA}$ substrate and the minimum for the $\sigma^D/RsdA_{VDD}$ mutant. (B) The degren attached to polypeptides of equal length also shows degren-dependent gradation in levels of ATPase activity. ATPase activity of ClpX increases only in the presence of the degren peptide ending with VAA. No change in ATPase activity was observed for degrens with either GAA or AAA. (C) GFP chimeras mimic the anti- σ factor substrates in inducing degren-dependent gradation in levels of unfoldase activity. The relative decrease in fluorescence was highest for GFP_{VAA}, followed by GFP_{GAA} and GFP_{AAA} for both full-length ClpX and ΔNCipX. Details of protein constructs are compiled in Table S4 in the supplemental material. (D) Removal of the ClpX N-terminal domain reduces degren-dependent change in ClpX activity. **, $P < 0.005$; ***, $P < 0.0001$.

protein with reduced affinity (Fig. 4A; Table 3). In another experiment to evaluate the effect of variation of substrate size on the binding affinity or ATPase activity, ClpX ATPase activity was also evaluated in the presence of *ssrA* peptide chimeras. As seen by the results in Fig. 4B, the ATPase activity of ClpX was substantially enhanced in *ssrA*_{VAA} (mimicking $\sigma^D/RsdA$) but was less affected by *ssrA*_{AAA} (mimicking $\sigma^I/RslA$) (Fig. 4B; Table 3). Finally, we performed an experiment wherein GFP chimeras with different degrens at the C terminus were subjected to unfolding by ClpX. The degrens in these GFP chimera substrates mimic those in the anti- σ factors. These results show that GFP_{VAA} (mimicking

TABLE 3 ATPase activities of ClpX in the presence of substrates

Substrate	Mean ClpX sp act $\pm$ SD (μ M/min/ μ g)
Anti- σ factors	
None ^a	1.9 $\pm$ 0.1
σ^D /RsdA _{VAA}	9.6 $\pm$ 0.1
σ^M /RsmA _{GAA}	5.5 $\pm$ 0.2
σ^K /RskA _{AAA}	3.7 $\pm$ 0.2
σ^L /RslA _{AAA}	3.4 $\pm$ 0.1
σ^D /RsdA degnon variants	
GAA	5.9 $\pm$ 0.2
AAA	3.5 $\pm$ 0.1
VDD	2.2 $\pm$ 0.1
GFP chimera degnons	
None	1.4 $\pm$ 0.1
VAA	6.4 $\pm$ 0.1
GAA	2.4 $\pm$ 0.1
AAA	2.0 $\pm$ 0.1
ssrA peptides	
None	1.5
VAA	2.7
GAA	1.7
AAA	1.5

^aNone, ClpX alone was used.

σ^D /RsdA) unfolded faster than GFP_{GAA} (mimicking σ^M /RsmA) or GFP_{AAA} (mimicking σ^K /RskA) (Fig. 4C; Table SA1). As in the case of the σ /anti- σ substrates, GFP_{1-855rA} (devoid of the last three residues in the degnon) was not unfolded by ClpX. Taken together, the data suggest that degnon composition, particularly the antepenultimate residue, affects the ATPase and unfoldase activity of *M. tuberculosis* ClpX (Fig. 4A to C).

Role of the NTD of *M. tuberculosis* ClpX in substrate recruitment. Next, we evaluated the role of the N-terminal domain (NTD) in stabilizing ClpX-substrate interactions. ATPase assays were performed with full-length ClpX and the N-terminal deletion construct of ClpX (Δ NClpX). GFP-degnon chimeras were used as substrates in these experiments. The deletion of the N-terminal domain does not substantially affect ATPase activity (which is ~ 1.2 μ M/min/ μ g, lower than with full-length ClpX, ~ 1.4 μ M/min/ μ g). While ClpX activity with the full-length enzyme showed a clear degnon-dependent gradation, highest in the case of GFP_{VAA} (mimicking σ^D /RsdA) and lowest for GFP_{AAA} (mimicking σ^L /RslA), changes in the specific activity of Δ NClpX were less pronounced (Fig. 4D; Tables 3 and 4). The unfoldase activity of Δ NClpX was also monitored for GFP-ssrA chimeras and compared with the activity of full-length ClpX. Consistent with previous observations, the unfoldase activity of Δ NClpX was less than that of ClpX, while the degnon dependence (GFP_{VAA} unfolded faster than GFP_{GAA} or GFP_{AAA}) remained unaltered (Fig. 4C; Table SA1). To determine whether deletion of the N-terminal domain of ClpX affected the binding affinity with the GFP-degnon substrate, we performed SPR experiments with the Δ NClpX construct. There was a 2-fold decrease in the binding affinity of substrates for Δ NClpX compared to their binding to full-length ClpX (Fig. SA4; Table SA2). Despite the lower binding affinity for substrate proteins, the

TABLE 4 ATPase activities of Δ NClpX in the presence of GFP chimeras

GFP chimera degnon	Δ NClpX sp act (μ M/min/ μ g)
None ^a	1.2
VAA	2.3
GAA	1.6
AAA	1.4

^aNone, Δ NClpX alone was used.

Δ NCIpX construct retained the degron-dependent gradation, highest in the presence of GFP_{VAA} and lowest for GFP_{AAA}.

ATP binding was shown to stabilize the ClpX hexamer (10). An interesting observation from these experiments is that ClpX substrate interactions can occur in the absence of nucleotides. Since this observation stands out from the *E. coli* ClpX system and because the possibility that the Δ NCIpX construct is more unstable than the full-length ClpX persists, we experimentally evaluated the binding affinity of GFP_{VAA} for both ClpX and Δ NCIpX in the presence of ATP (Fig. SA5). Although the addition of ATP improved substrate binding for both ClpX and Δ NCIpX constructs, Δ NCIpX bound the substrates with lower affinity than full-length ClpX (Fig. SA5; Table SA3). The N-terminal domain thus influences binding affinity and the residence time of substrate proteins on ClpX.

ClpX links proteostasis with transcription. Under diverse stress conditions, proteolytic degradation of an anti- σ factor releases a free ECF σ factor to bind to the RNA polymerase and initiate transcription. In the case of the *E. coli* anti- σ factor RseA, other cellular proteases apart from ClpX, such as ClpAP, Lon, and FtsH, were shown to mediate proteolytic degradation (7). *M. tuberculosis* has three Clp unfoldases, ClpX, ClpC1, and ClpB. In the case of the cytosolic *M. tuberculosis* σ^E /RseA complex, phosphorylated RseA was shown to be a target for proteolytic processing by the ClpC1P2P1 assembly (31). ClpB was shown to be primarily involved in the prevention of heat-induced aggregation and refolding of denatured proteins (32–34). Homologues of the *E. coli* Lon and HslUV proteases are absent in *M. tuberculosis* (35, 36). In the light of these observations, *M. tuberculosis* ClpC1 appeared to be the other likely unfoldase that could participate in anti- σ factor degradation. Experiments performed with freshly purified *M. tuberculosis* ClpXP2P1 and ClpC1P2P1 complexes reveal that σ^D /RsdA is proteolyzed specifically by the ClpXP2P1 and not by the ClpC1P2P1 complex (Fig. 5A).

To evaluate whether the expression levels of *clpX* and the anti- σ factor genes were correlated, gene expression microarray data sets from different experimental conditions, including hypoxia, stationary phase, oxidative stress, and presence of vitamin C, were examined (compiled in Table 5). While σ^D maintains homeostasis in the late stationary phase of *M. tuberculosis* growth, genes in the σ^M regulon are expressed in the stationary phase (37, 38). The oxidative stress response involves both σ^K and σ^L , as these ECF σ factors together respond to redox stress stimuli (27, 28). The genes encoding each *M. tuberculosis* σ /anti- σ factor pair examined in this study lie in the same operon and are positively regulated by the cognate σ factor. Despite multiple other factors that could influence gene expression, we note a correlation between the expression levels of *clpX* and anti- σ factor genes under specific environmental conditions (Table 5). This finding was further evaluated in an experiment wherein the mRNA levels of the different σ factors upon *clpX* induction were monitored by quantitative real-time PCR (qPCR). The premise for this experiment was that increasing ClpX levels would result in more degradation of the target anti- σ factor, thereby enhancing the cellular abundance of the corresponding free ECF σ factors. An increase in the intracellular levels of ECF σ factors, in turn, would result in upregulation of genes in the corresponding regulon. This hypothesis was examined in *M. tuberculosis* H37Rv under two experimental conditions, at the logarithmic phase and late stationary phase of growth. The stationary phase in particular was evaluated because RsdA (anti- σ^D) shows the highest susceptibility for ClpX-induced proteolysis *in vitro* and σ^D was shown to maintain homeostasis in the late stationary phase of *M. tuberculosis* growth (39). We note that the expression levels of σ^D are most upregulated upon ClpX induction under both experimental conditions (Fig. 5). On the other hand, the mRNA levels of *sigL* were relatively unaffected upon *clpX* induction. Thus, increased levels of ClpX directly influence the intracellular concentrations of free σ factors. We note that in logarithmic growth phase (in which the anti- σ factor is less susceptible to RIP proteolysis), the effect is less pronounced (Fig. SA6 and SA7).

To evaluate downstream effects of changes in σ factor levels, the mRNA levels of representative genes from the regulons of *sigD*, *sigM*, *sigK*, and *sigL* in the late stationary

FIG 5 ClpX governs intracellular levels of membrane-associated ECF σ factors. (A) ClpX and not ClpC1 governs the degradation of an anti- σ factor with an *ssrA*-like degron. For a comparison of the proteolysis of RsdA from the σ^D /RsdA complex by the ClpXP2P1 and ClpC1P2P1 complexes, samples were analyzed for proteolysis at different time points. This comparison suggests that RsdA is selectively proteolyzed by the ClpXP2P1 assembly. Targeted proteolysis was less pronounced with the ClpC1P2P1 assembly. The reaction mixture consisted of 0.3 μM ClpX/ClpC1, 0.8 μM ClpP2P1, 5 mM ATP, 5.0 μM σ^D /RsdA substrate, and an ATP regeneration system (0.32 mg/ml creatine kinase and 16 mM creatine phosphate). Immunoblots were performed using antibodies raised against purified recombinant σ^D and RsdA. The ATPase activities of both unfoldases (ClpX and ClpC1) are similar. (B) qPCR experiments reveal that the mRNA levels of all ECF σ factors increase upon ClpX induction. The differences observed in the mRNA levels of *sigM* and *sigK* upon ClpX induction are broadly consistent with the relative degradation rates of their cognate anti- σ factors. Results shown here depict data from late stationary phase. *, $P < 0.05$; **, $P < 0.005$.

phase were examined by qPCR (40–42). These experiments reveal that (i) the expression of all four ECF σ factors is upregulated upon ClpX induction and (ii) upregulation of the cognate regulon is less clear (Fig. 6A). Some aspects of the nonlinear response upon ClpX induction can be rationalized, however. For example, while RskA and RslA share

TABLE 5 Correlations between the expression of *clpX* and anti- σ factors under different experimental conditions

Gene ^b	<i>r</i> (<i>t</i> test value) under indicated condition ^a				
	Log phase	Stationary phase	Hypoxia	ROS	Vitamin C
<i>rsdA</i>	−0.59 (−1.5)	−0.16 (−0.4)	1 (2.5)	0.96 (2.4)	0.99 (2.5)
<i>rsmA</i>	−0.95 (−2.4)	−0.72 (−1.8)	0.8 (2)	0.32 (0.8)	−0.42 (−1)
<i>rskA</i>	0.99 (2.5)	0.78 (1.9)	0.93 (2.4)	0.38 (0.9)	0 (0.08)
<i>rslA</i>	0.53 (1.3)	0.91 (2.3)	0.54 (1.4)	−0.27 (−0.7)	0.49 (1.3)

^aThese correlations were compiled using microarray data (GEO accession numbers [GSE16146](#), [GSE101048](#), and [GSE8786](#)). Correlation coefficients (*r*) with values of >0.9 are in boldface. ROS, reactive oxygen species.

^b*rsdA*, anti- σ^D ; *rsmA*, anti- σ^M ; *rskA*, anti- σ^K ; *rslA*, anti- σ^L .

FIG 6 Mechanistic model for the gradient mechanism in the RIP pathway. (A) Fold changes in mRNA levels of ECF σ factors and representative genes from their regulons. While the expression of all ECF σ factors was upregulated upon ClpX induction, changes in the expression levels of representative genes (*rpfC*, *esxE*, *mpt70*, and *mpt53*) in the target regulons were less pronounced (34, 44–46). Multiple factors are likely to contribute to this nonlinear response. Activation of σ^K and σ^L is also affected by redox stimuli (25, 26). Other factors governing activation of σ^M remain to be determined. *, $P < 0.05$. (B) A mechanistic model for degron-dependent variation in levels of ClpX activity. The rate of anti- σ factor degradation controls the cellular abundance of free ECF σ factor. Differences in the intracellular levels of free σ factors alter the expression profile.

the same degron sequence (AAA), the expression of *sigK* (and *rskA*) is higher than that of *sigL* (and *rsIA*). Another feature that could contribute to nonlinearity is that the release of σ^K from the σ^K /RskA complex is also governed by redox stimuli, since σ^K is a redox sensor that allows dissociation of RskA under reducing conditions (28). It thus appears likely that differences in the expression of the σ^K regulon are likely to mimic steady-state levels in stationary-phase, low-oxygen *M. tuberculosis* cultures (43). Another parameter that could significantly influence this experiment is that ClpX dependence is preceded by RIP-1 activity on the anti- σ factor in the regulated intramembrane proteolysis (RIP) pathway. RIP-1 activity is also influenced by environmental stimuli (6).

Taken together, these observations suggest that ClpX activity alters intracellular ECF σ factor levels in a degron-dependent manner, thereby influencing the expression profile in *M. tuberculosis* (Fig. 6B).

DISCUSSION

An intriguing feature in ECF σ factors, examined extensively in *E. coli* and *B. subtilis*, is that of overlapping regulons (44–47). This overlap in σ factor function ensures appropriate changes to the transcriptional profile wherein multiple ECF σ factors are activated upon a stress signal (48). Another aspect is the apparent hierarchy among σ factors. Some σ factors (*M. tuberculosis* σ^C or σ^I , for example) are under the control of σ^F , which in turn is regulated by σ^M (49, 50). Both these aspects depend on the cellular concentration of ECF σ factors, which is largely regulated by a posttranslational mechanism involving the release of free σ factor from an inactive σ /anti- σ complex (51). These release mechanisms involve either concerted conformational changes leading to the dissociation of the inactive σ /anti- σ complex or targeted proteolysis of the anti- σ to release the free ECF σ . Targeted proteolysis based on an accessible degron in a substrate effectively controls cellular protein levels. Indeed, this strategy is considered robust enough to be employed for optimizing microbial cell factories for diverse applications (52). It is in this context that the finding that the *M. tuberculosis* ClpX activity is modulated by degron composition becomes relevant.

In the RIP cascade, the signal transduction of environmental stress to the transcription mechanism has multiple temporal checkpoints: (i) the rate at which the extracytoplasmic receptor domain responds to the stress stimulus, (ii) the rate of transmembrane signal transduction involving the site 1 protease(s) and the site 2 protease Rip1 that acts on all membrane-associated anti- σ factors, and (iii) the rate at which the anti- σ domain is selectively degraded by ClpX to release free ECF σ factors to initiate transcription. In the *E. coli* RIP cascade, the proteolytic step initiated by the site 1 protease DegS is the rate-limiting step (half-life [$t_{1/2}$] ≤ 1 min) for RseA degradation, with the other two proteolytic events being at least 3-fold faster (7). The dissociation of RseA from the membrane generates a cytosolic fragment with a degron (sequence ending in VAA) (8). The specific degradation of the cytosolic RseA by ClpXP is rapid ($t_{1/2} \leq 20$ s) and aided by an adaptor, SspB (7, 8). In the event ClpXP is weighed down by competing substrates, other cellular proteases can take over, albeit at lower rates ($t_{1/2} \leq 1.6$ min). Thus, DegS activity on RseA is the rate-limiting step in the *E. coli* RIP pathway governing the cellular levels of σ^E (7). While *E. coli* RseA is potentially a substrate for multiple proteases, like ClpA, HslUV, and Lon, ClpXP was demonstrated to be the major proteolytic complex in this process. It is worth noting in this context that *E. coli* *clpX* and *clpP* are not essential (53). On the other hand, *M. tuberculosis* *clpX*, *clpP1*, and *clpP2* are essential genes (37, 54).

The ability of *M. tuberculosis* ClpX to bind substrates in the absence of ATP suggested that *M. tuberculosis* ClpX alternates between two different states, an observation similar to the case for Hsp60 and Hsp70 chaperones (55). *E. coli* ClpX has also been shown to switch between two conformations, an “open state” with lower binding affinity for substrates in the absence of nucleotides and a “closed state” with higher affinity in response to ATP binding and hydrolysis (56). The finding that variations in substrate interaction kinetics elicited correlated changes in unfoldase activity suggested that concerted conformational changes could be a prominent feature of adaptor-independent ClpX activity. Indeed, in *E. coli*, conformational changes in AAA+ proteases were shown to provide a mechanism to correlate ATP hydrolysis with denaturation of target protein substrates (10). ATPase assays and SPR interaction studies performed with both wild-type ClpX and Δ NClpX suggest that the N-terminal domain plays a role in substrate recruitment by affecting both ATPase activity and substrate binding in ClpX. The correlation between interaction kinetics (monitored by surface plasmon resonance) and levels of ATPase activity was significantly dampened in Δ NClpX compared to their correlation in full-length ClpX. This observation that the interaction kinetics obtained from SPR experiments are consistent with the results of

both ATPase activity and unfoldase assays (significant differences only in the presence of cognate substrates) suggests that nonspecific interactions are unlikely. On the other hand, the observation that the dissociation trajectories of substrates do not return to the baseline indicates a slow dissociation process, an aspect that has been reported previously in the case of *E. coli* ClpX. A plausible rationale for this comes from the low-frequency normal modes in the *M. tuberculosis* ClpX model that suggest flexible tethering of the N-terminal domain (NTD) in ClpX could enable conformational changes for stronger substrate interactions (see Fig. SA3 in the supplemental material). This finding is similar to the case for *E. coli* ClpX, wherein nucleotide-dependent movements of the NTD facilitate the entry of substrates inside the ClpX ring (57). Put together, these data are consistent with an induced fit model for ClpX that can be described as follows:

where k_1 is the rate at which the substrate (S) binds, k_2 and k_{-2} are the relative rates of conformational changes induced by substrate binding in the forward and backward directions, k_3 is the rate of chemical reaction, and k_4 is the rate of product (P) release. O and C stand for the open (before conformational change) and closed (after conformational change) states of the enzyme. The increase in ATPase activity upon *E. coli* ClpX interaction with a substrate was shown to precede a series of conformational changes in this enzyme (58, 59). These transitions were seen to initiate conformational changes in the pore 1 (GYVG) and pore 2 (RKSENPSITRD) loops to accelerate ATP hydrolysis (59, 60). With a favorable amino acid at the antepenultimate position, conformational changes engage the substrate and the enzyme switches from an open to a closed state, leading to increased ATPase activity and the consequent unfolding of the substrate. When k_{-2} becomes $\ll k_3$, the substrate is committed for the reaction after the conformational change. The observation that the ATPase activity of ΔNClpX is less than that of full-length ClpX agrees well with the above-described model (Fig. 4D; Tables 3 and 4).

The finding that the last step in the RIP proteolytic cascade involving *M. tuberculosis* ClpX varies across substrates—fastest for RsdA and slowest for RslA—suggests significant differences from the *E. coli* model. In *E. coli*, the expression of the σ^E regulon in *clpX* and *sspB* null mutants is reduced, suggesting a correlation between proteolysis and transcription (8). The presence of multiple anti- σ factors in *M. tuberculosis* and degron-dependent variations in ClpX unfolding suggests a broader application of this link between targeted protein degradation and the expression profile. Previous reports on *sigA* transcript levels in *M. tuberculosis* H37Rv suggest that *sigA* expression is not altered at different phases of growth and exposure to stresses *in vitro* (45, 60, 61). However, upon ClpX overexpression, we observe differences in the levels of *sigA*; the levels are lower in the stationary phase than in the logarithmic phase (Fig. SA8). σ^A modulates the expression of essential genes and virulence in *M. tuberculosis* (61). ClpX overexpression is thus likely to affect the ability of *M. tuberculosis* H37Rv to respond to stress. The degron-dependent differences in ClpX–anti- σ factor interactions and subsequent release of free ECF σ factors from the inactive complex are thus expected to lead to a measured change in transcription in response to a stress signal in *M. tuberculosis* (Fig. 6). An analysis using annotated σ /anti- σ pairs suggests that this feature is likely to be applicable in other bacteria (62). Indeed, a large proportion of ECF anti- σ factor sequences having one transmembrane helix (similar to the *M. tuberculosis* membrane-associated anti- σ factors) have an *ssrA*-like degron (567 of 722 anti- σ factors) (Fig. 1B). Put together, these data suggest that the *M. tuberculosis* ClpX function is more nuanced than a simple on/off switch in releasing ECF σ factors from an inactive σ /anti- σ complex. It appears likely that this variation in the unfoldase activity of ClpX is, in effect, a regulatory layer coordinating environmental stimuli to elicit calibrated changes in gene expression.

MATERIALS AND METHODS

Cloning, expression, and purification of recombinant proteins. *M. tuberculosis* *clpX*, *clpC1*, and *clpP2* genes were cloned in the *E. coli* expression vector pET28a, while *clpP1* was cloned in multiple cloning site I (MCS-I) of the pETDuet-1 vector (Novagen, Inc.). In the case of the σ /anti- σ factor complex substrates, the full-length σ factors (σ^D , σ^K , σ^L , and σ^M) were cloned in MCS-I, whereas the anti- σ factor constructs (ending at the *ssrA*-like motif at the C-terminal end), were cloned in MCS-II of the pETDuet-1 expression vector. GFP-*ssrA* from *E. coli* was obtained as a gift from Tania Baker's laboratory. Mutants of the σ /anti- σ substrates and the GFP-*ssrA* mutants were prepared following a standard site-directed mutagenesis (SDM) protocol (Table SA4 in the supplemental material lists the details of the constructs used in this study). The plasmids were transformed into a ClpP knockout strain of *E. coli* (obtained from Tania Baker's laboratory). *E. coli* cultures were grown in Luria broth with appropriate antibiotic markers to an optical density at 600 nm (OD_{600}) of 0.4 to 0.6 at 37°C, whereupon they were induced with 0.8 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG). Postinduction, the cells were grown at a temperature of 18°C for 12 to 14 h and harvested at 600 nm by centrifugation at 4,500 rpm. Cell pellets containing Clp protein were resuspended and sonicated in lysis buffer (buffer L) containing 50 mM Tris-HCl, pH 7.6, 300 mM NaCl, 100 mM KCl, 1 mM dithiothreitol (DTT), 10 mM imidazole, and 10% (vol/vol) glycerol, while cell pellets containing σ /anti- σ factor complexes were resuspended in buffer L devoid of DTT (except for the σ^L /RslA complex). After sonication, the cell debris was separated from the crude cell lysate by centrifugation for 30 min at 15,000 rpm. The cell-free lysate was then incubated with Ni^{2+} -nitrilotriacetic acid (NTA) affinity beads (Sigma-Aldrich, Inc.) for 1 h at 4°C. The bound proteins were eluted by imidazole prepared at a gradient of concentrations (50 mM to 250 mM) in buffer L. The pure fractions were pooled, concentrated, and loaded on to a PD-10 desalting column (GE Healthcare) and were desalted in buffer D (50 mM HEPES-KOH, pH 7.5, 25 mM $MgCl_2$, 100 mM KCl, 0.1 mM EDTA, and 10% [vol/vol] glycerol) for Clp proteins or buffer S (50 mM HEPES-KOH, pH 7.5, 100 mM KCl, and 10% [vol/vol] glycerol) for the substrate proteins.

Surface plasmon resonance. Interaction studies were performed on a Biacore 2000 instrument (Biacore, Uppsala, Sweden). ClpX was covalently immobilized on a CM5 sensor chip (Biacore) using the standardized protocol in replicates. The SPR buffer (50 mM HEPES, 200 mM KCl with 10% glycerol at pH 7.5), filtered through 0.45- μ m membrane filters (Millipore) and degassed, was used in these experiments. Experiments were carried out at 25°C. Carboxymethyl groups on the chip were activated by injecting a freshly prepared 1:1 ethyl-3-(3-dimethylaminopropyl)-carbodiimide-*N*-hydroxysuccinimide (EDC-NHS) mixture (1 M each). ClpX diluted in 10 mM sodium acetate (pH 4.0) was then passed over the active surface until the required immobilization was achieved. The unreacted activated sites were blocked with 1 M ethanolamine. Amounts of 50 μ l of each substrate at various concentrations were passed over the flow cells (flow rate, 30 μ l/min) and allowed to dissociate for 200 s. The sensor surface was regenerated using multiple injections of 4 M $MgCl_2$ and/or 0.05% to 0.1% SDS whenever required. The response curves, with reference values subtracted, obtained for substrate binding to ClpX were evaluated using BIA evaluation software. The data obtained were fit to the Langmuir 1:1 interaction model to obtain rates of association (k_a) and dissociation (k_d). The standard deviation across replicates was used to calculate the fitting error (Table SA5; Fig. SA2) (63, 64). The equilibrium dissociation constant (K_D) is defined as the ratio of the dissociation rate constant (k_d) and the association rate constant (k_a).

ATPase assay. ATPase assays were performed using malachite green to calculate the specific activity of ClpX. The assays were performed with 100 nM ClpX and 5 μ M substrate protein or 20 μ M *ssrA* peptides in buffer containing 25 mM HEPES (pH 7.6), 200 mM KCl, 20 mM $MgCl_2$, 10% glycerol. The reaction was initiated by the addition of 1 mM ATP and was carried out at 30°C for 25 min. Malachite green dye buffer containing 0.045% malachite green, 4.2% ammonium molybdate, and 1% Triton X-100 was added to the reaction mixtures at suitable time points. After 1 min, 34% citric acid was added and the reaction mixture mixed well and further incubated for 40 min for color development. Absorbance was measured at 660 nm. The amount of inorganic phosphate released was calculated based on the absorbance standard curve established by H_3PO_4 standards.

Unfoldase assay. The catalytic unfolding of GFP-*ssrA* substrate by ClpX was monitored using an experimental protocol identical to that used in the case of *E. coli* ClpX (57). Unfolding of GFP-*ssrA* was monitored using a Varioskan plate reader with an excitation wavelength of 488 nm and emission wavelength of 520 nm. The reaction was monitored over a period of 30 min (Fig. SA9). The reaction mixture was comprised of 1 $\times$ unfoldase buffer (25 mM HEPES, pH 7.5, 20 mM $MgCl_2$, 10% glycerol), 1 $\times$ ATP regeneration system (creatine phosphate [16 mM] and creatine kinase [0.32 mg/ml]), 200 mM KCl, 100 nM ClpX, and 5 μ M of GFP-*ssrA* substrate.

Molecular modeling and normal modes analysis. The molecular model for *M. tuberculosis* ClpX was constructed using Modeller (65). The crystal structure of *E. coli* ClpX (3HWS) was used as the template for comparative modeling. In this procedure, care was taken to ensure that the asymmetry observed in the ClpX hexamer was retained in the model in which energy was minimized. Both the C and F chains of the model obtained had the closed conformation as observed in the *E. coli* ClpX crystal structure. Molecular graphics, energy minimization of the model, and analyses were performed with the UCSF Chimera package (66). Normal modes analysis was performed using Anisotropic Network Model Web Server 2.1 (67).

RNA isolation and qPCR analysis. *M. tuberculosis* H37Rv cells transformed with pNit-3F vector and pNit-3F-ClpX were grown in the presence of 5 μ M isovaleronitrile (IVN) as the inducer. Amounts of 10 ml of bacterial cells at an OD_{600} of $\sim$ 1.0 were processed to extract RNA using the TRIzol method.

Briefly, cells were lysed in 1 ml TRIzol using three 30-s cycles of bead beating with intermittent ice treatment for 2 min. The cell debris was removed from the lysate by centrifugation at 13,000 rpm for 10 min. The lysate was treated with 400 μ l chloroform and centrifuged to separate the three phases. The top layer, containing RNA, was carefully extracted and the RNA was precipitated by the addition of 1 ml isopropanol. The RNA pellet thus obtained was washed with 70% ethanol to remove excess salts. The dried RNA pellet was dissolved in 30 μ l RNase-free water and kept overnight at 4°C to ensure complete dissolution. The RNA was quantified using a NanoDrop instrument (NanoDrop 2000c; Thermo Scientific), and the sample was run on a 1% formaldehyde-agarose gel to estimate integrity. The RNA sample was further processed by being passed through an RNeasy minicolumn (Qiagen). An amount of 1 μ g of purified RNA of each test and control sample was then treated with DNase I (Thermo Scientific, Inc.) to remove contaminating DNA and was used for cDNA synthesis, employing a one-step cDNA synthesis kit from Bio-Rad. The cDNA synthesis was performed in 20- μ l reaction mixture volumes according to the manufacturer's protocol using 500 ng of pure DNase I-treated RNA as the template. A reaction mixture without reverse transcriptase was processed simultaneously as a control. For the final qPCRs, the 20 μ l of cDNA reaction mixture was diluted to 100 μ l, and 2 μ l per reaction mixture was utilized as the template. Oligonucleotide sequences for qPCR were designed using Primer3 software (Table SA6 lists primers, melting temperatures [T_m], and the GC content of primers used). The 16S rRNA amplicon was used as the reference gene. Two-step SYBR green PCRs were performed in a MasterCycler RealPlex4 machine (Eppendorf, Germany), using 10- μ l reaction mixture volumes in triplicates. The following conditions were used for amplification with SYBR green: 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Melt curve analysis was done for each primer pair. Reverse transcriptase minus and plus cDNA samples were subjected to qPCR with 16S rRNA primers for analysis of gDNA contamination. For each individual gene analyzed using qPCR, the quantification cycle (C_q) values were normalized with respect to the C_q values of the 16S rRNA amplicon. After normalization of C_q values, the fold changes in the expression of various genes upon ClpX overexpression were calculated by the cycle threshold ($2^{-\Delta\Delta C_T}$) method as described previously. Relative quantification allowed us to relate the PCR signal of our target transcripts in the ClpX overexpression group to that of the vector control group. The $2^{-\Delta\Delta C_T}$ method was used to analyze the relative changes in gene expression (68).

Protease assays. Protease assays were performed in buffer containing 50 mM HEPES-KOH (pH 7.5), 150 mM KCl, 20 mM MgCl₂, and 10% glycerol at 37°C. Clp proteases ClpP2 and ClpP1 (1 μ M each) were preincubated with 0.1 mM Z-LL dipeptide for 30 min at 37°C. This step was followed by the preincubation of ClpC1 or ClpX (0.5 μ M), ClpP2P1 (1 μ M), σ^D /RsdA_{VAA} substrate (5.0 μ M)-PyrB (1 μ M) substrate, and an ATP regeneration system (0.32 mg/ml creatine kinase and 16 mM creatine phosphate) for 10 min to allow the formation of stable ClpXP2P1 or ClpC1P2P1 complexes. The reaction was initiated by the addition of 5 mM ATP. Samples were removed after specific time intervals, and a Western blot analysis was performed using anti-RsdA and anti- σ^D antibodies at 1:7,000 dilution while ClpX, the Clp proteases (ClpP2 and ClpP1), and PyrB were probed with antihistidine monoclonal antibodies (GE Healthcare) at 1:10,000 dilution. Immunoblots were developed with Luminata Forte Western horseradish peroxidase (HRP) substrate for peroxidase-attached secondary antibodies.

Expression levels and correlation analysis. The expression levels of *clpX* and the four antisigma genes were obtained from previously published microarray data sets (GEO accession numbers GSE16146, GSE101048, and GSE8786). Each data set corresponds to a different experimental condition, including logarithmic phase ($n = 3$), stationary phase ($n = 3$), hypoxia ($n = 3$), oxidative stress (reactive oxygen species [ROS]; $n = 3$), and vitamin C ($n = 3$). Pearson's correlation coefficients (r) and t values (Student's t test) between a given anti- σ factor and *clpX* were calculated for all experimental conditions using the following formulas: $r = [\Sigma(x - x_i)(y - y_i)] / \sqrt{\Sigma(x - x_i)^2 \Sigma(y - y_i)^2}$ and $t = r/[1 - (0.92^2)]^{0.5}$.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00748-18>.

SUPPLEMENTAL FILE 1, PDF file, 1.7 MB.

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We declare no competing interests.

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