Proteolytic regulation of toxin-antitoxin systems by ClpPC in Staphylococcus aureus

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Running Title: ClpPC degrades S. aureus antitoxins
Bacterial toxin-antitoxin (TA) systems typically consist of a small, labile antitoxin that inactivates a specific longer-lived toxin. In *E. coli*, such antitoxins are proteolytically regulated by the ATP-dependent proteases Lon and ClpP. Under normal conditions, antitoxin synthesis is sufficient to replace this loss from proteolysis, and the bacterium remains protected from the toxin. However, if TA production is interrupted, antitoxin levels decrease, and the cognate toxin is free to inhibit the specific cellular component, such as mRNA, DnaB or gyrase. To date, antitoxin degradation has only been studied in *E. coli*, so it remains unclear as to whether similar mechanisms of regulation exist in other organisms. To address this, we followed antitoxin levels over time for the three known TA systems of the major human pathogen *Staphylococcus aureus*, *mazEF*, *axe1/txe1* and *axe2/txe2*. We observed that the antitoxins of these systems, MazE<sub>sa</sub>, Axe1 and Axe2, respectively, were all degraded rapidly (t<sub>1/2</sub> = ~18 min), at rates notably faster the *E. coli* counterpart such as MazE (t<sub>1/2</sub> = ~30-60 min). Furthermore, when *S. aureus* strains deficient for various proteolytic systems were examined for changes in the half-lives of these antitoxins, only strains with *clpC* or *clpP* deletions showed increased stability of these molecules. From these studies, we concluded that ClpPC serves as the functional unit for the degradation of all known antitoxins in *S. aureus*.

Keywords: *Staphylococcus aureus*, toxin-antitoxin, *mazEF*, *clpPC*, *axe/txe*, *relBE*
**INTRODUCTION**

*Staphylococcus aureus* is a versatile human pathogen responsible for an increasing number of hospital- and community-acquired infections (33, 41) ranging from superficial skin lesions to life-threatening sepsis, endocarditis, and toxic shock (29). *S. aureus*’s capacity to cause illness is enhanced by its robust stress response, which allows it to endure adverse conditions such as heat, antibiotics and nutritional deprivation. This is mediated in part by transcriptional regulators like CtsR (11), CodY (31) and the alternative sigma factor $\sigma^B$ (24) that allow the bacteria to rapidly adjust to challenging environments.

The role of chromosomal toxin-antitoxin (TA) modules in environmental and antibiotic stress response has been documented for a variety of organisms, especially *E. coli*, but only recently have they been investigated in *S. aureus* (12, 18, 43). These systems typically consist of a pair of co-transcribed stress-inducible genes (19) that encode a stable toxin and a more labile antitoxin. Depletion of the antitoxin allows activation of its cognate toxin, which is then free to interfere with a specific cellular target, such as mRNA, DNA gyrase or DNA helicase. Depending on the species and the TA system, this activation results in a variety of phenotypes, but those related to growth, stress response, starvation and persistence are often seen (12, 19, 30). For example, *Streptococcus mutans* devoid of its TA systems are more susceptible to changes in nutrient availability and pH changes than their counterpart wild-type strains (26). Furthermore, TA systems are absent in obligate intracellular organisms (37), suggesting that they are not necessary for growth in stable, intracellular environments.
Previous reports have demonstrated that at least three TA systems exist in *S. aureus* (12, 43), annotated as *mazEF* (SAS0167/SA1873), *axe1/txe1* (SA2196-5) and *axe2/txe2* (SA2246-5). Although *mazEF* was named for its similarity to *mazEF* in *E. coli*, its transcriptional regulation (12) and ribonucleic target sequences are considerably different from that of *E. coli* (17). The *axe1/txe1* and *axe2/txe2* TA systems have significant homology to one another (48%/48% amino acid similarity for antitoxins and toxins) as well as to both the *relBE* (37) and *yefM/yoeB* (6) TA systems in *E. coli*. Like the *mazEF* system, both of these systems in *S. aureus* show transcriptional activation in response to select antibiotics (12), and have specific endoribonucleic activities (43).

As a functional family, antitoxins can be either small RNAs (Class I) or proteins (Class II) (20) that specifically bind to a cognate toxin and inhibit its enzymatic activity; in the case of *S. aureus* all three antitoxins appear to belong to the latter group (12, 43). Class II antitoxins are often strongly acidic and are thought to be largely unstructured (28), attributes that facilitate the conformational changes necessary to enable the tight binding needed to repress their positively-charged toxins. The unfolded nature of these antitoxins is also thought to contribute to their degradation (34), as protein unfolding is an important first step in the delivery and processing of the target protein by ATP-dependent proteases.

While TA systems for a variety of bacteria have been shown to respond transcriptionally to environmental and antibiotic stresses (4, 12), little is known about the proteolytic regulation of TA systems in species other than *E. coli* (19). In this organism, two of its four proteolytic systems (Lon, ClpP, FtsH, or HslVU (ClpQY)) are involved in antitoxin degradation: Lon breaks down the antitoxins RelB (9), MazE (10), ParD (39).
and CcdA (42), while the ClpP protease degrades MazE (1), PhD (25) and YefM (6). On its own, ClpP can degrade only small peptides, but would deploy an Hsp100/Clp ATPase chaperone (1, 25) to unfold and translocate proteins with specific amino acid signal sequences (e.g. C-terminal AA and LCN motifs) (7, 36, 38) into its proteolytic core. In E. coli, the ClpA and ClpX chaperones have both been shown to be critical to this ClpP-mediated antitoxin degradation, but their counterparts in S. aureus are less clear. S. aureus has a single homologue to E. coli ClpX, but it also possesses several chaperones that share high similarity to ClpA [ClpB (59%), ClpC (61%) and ClpL (61%)]. Among these, only ClpC and ClpX are thought to contain ClpP recognition sequences (14), suggesting that ClpB and ClpL might not directly bind ClpP (although that may not be a requirement to function as a ClpP chaperone). In terms of function in S. aureus, ClpC appears to mediate resistance to oxidative stress, enhance growth recovery from stationary and post-stationary phases, and contribute to biofilm formation (5). ClpX has a role in osmoprotection, and interestingly, also controls aspects of virulence gene regulation through an unidentified regulator of the agr system (15). Less is known of the two other chaperones ClpB and ClpL, although roles in the thermotolerance have been suggested (14).

While neither of the two other ATP-dependent proteases in E. coli, FtsH and HslVU, have been shown to be involved in antitoxin degradation, S. aureus homologues of these proteases conceivably could compensate for the lack of Lon. Strains lacking FtsH have defects in osmotic and heat shock tolerance, general cell growth and starvation (27), phenotypes associated with TA systems. Little is known of HslVU’s cellular function in S. aureus other than it has a limited role in stress survival (16).
We report here that the antitoxins MazE<sub>sa</sub>, Axe1 and Axe2 from the three known TA systems in <i>S. aureus</i> are each rapidly degraded <i>in vivo</i> with half-lives of approximately 18 minutes, rates that are faster than their <i>E. coli</i> counterparts. Examination of the genetic components involved in <i>S. aureus</i> antitoxin degradation revealed that strains lacking <i>clpP</i> showed greatly decreased degradation rates for all three antitoxins, while rates were unchanged in <i>ftsH</i> and <i>hslV</i>-deficient strains. The chaperone ClpC was also shown to be the only ATPase chaperone necessary for antitoxin breakdown. This indicated that while <i>S. aureus</i> uses ClpP to degrade its antitoxins in a way similar to <i>E. coli</i>, <i>S. aureus</i> TA regulation is distinct from <i>E. coli</i> in that it employs the Gram-positive specific stress-response chaperone ClpC (5, 14) to facilitate antitoxin breakdown.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth media.** Table 1 contains a list of bacterial strains and plasmids used for this study. <i>E. coli</i> was grown in Luria-Bertani (LB) broth or on plates containing LB agar, and <i>S. aureus</i> was grown in Trypticase soy broth (TSB) or agar (TSA). Competent <i>S. aureus</i> were prepared in B2 medium as described (40). Antibiotics were used at the following concentrations: erythromycin (2.5 µg/ml), ampicillin (50 µg/ml), chloramphenicol (10 µg/ml) and rifampicin (200 µg/ml). Xylose induction was performed using 0.1% xylose (v/v).

**DNA manipulations.** <i>E. coli</i> plasmid purification was performed using Qiagen mini-prep kits (Qiagen) according to the manufacturer’s instructions, while plasmid isolation from <i>S. aureus</i> was performed as described (40). Transformations of <i>S. aureus</i>
by plasmids were via electroporation, using the heavily mutagenized RN4220 as an intermediate between \textit{E. coli} and relevant \textit{S. aureus} strains (40). The sequences of primers used (IDT Technologies) are available upon request.

\textbf{Expression vector creation.} To facilitate their detection in cell lysates, the MazEsa, Axe1 and Axe2 antitoxins were epitope tagged with either N- or C-terminal Myc (EQKLISEEDL) or HA (YPYDVPDYA) tags codon-optimized for \textit{S. aureus}. These tag sequences were joined to the antitoxin genes by PCR amplified from the SH1000 chromosome, along with an upstream \textit{EcoRI} site, a \textit{sarA} ribosome-binding site, and a downstream \textit{XbaI} site. Products were digested using \textit{EcoRI} and \textit{XbaI}, ligated into similarly digested pEPSA5 vector, and used to transform competent \textit{E. coli} XL1-Blue. Successful transformants were verified by restriction digest and DNA sequencing.

\textbf{Construction of \textit{clp} knockouts.} The \textit{S. aureus} genome contains genes for five Clp ATPases (\textit{clpB}, \textit{clpC}, \textit{clpL}, \textit{clpX}, and \textit{clpY}) and three ATP-dependent peptidases (\textit{hslV/clpQ}, \textit{clpP} and \textit{ftsH}) (14, 27). Deletion of the first six of these genes was performed in the SH1000 background using the temperature-sensitive allelic replacement plasmid pMAD (3) to create clean, markerless deletions of individual \textit{clp} genes. Verification of these deletions was performed by colony PCR and chromosomal sequencing. For the \textit{clpP} mutant in the SH1000 background, we repaired the 11 bp deletion in \textit{rsbU} in the \textit{ΔclpP} mutant of 8325-4 using a similar pMAD replacement strategy. Since the small size of this replacement made the identification of a successful \textit{rsbU} repair impractical by colony PCR, enhanced pigmentation in putative \textit{rsbU}+ strains was used for our initial selection (24), followed by chromosomal sequencing of pigment-
positive strains for an intact rsbU gene. The SH1000 ftsH::tetM strain was a kind gift from Simon Foster (27).

**Protein lysate preparation.** To prepare protein lysates of SH1000 strains, overnight cultures of *S. aureus* were diluted 1/100 into 100 ml of TSB and grown shaking at 250 rpm at 37°C (flask:media ratio of 2.5:1). For those strains over-expressing antitoxins from the pEPSA5 plasmid, 10 µg/ml chloramphenicol and 0.1% xylose were also added during the initial inoculation. At A_{650} = 1.1, 200 µg/ml of rifampicin was added to inhibit transcription (2), whereupon a 10 ml aliquot was immediately withdrawn. Similar samples were then taken every fifteen minutes thereafter. Cells were pelleted by centrifugation at 1°C, flash-frozen in liquid nitrogen and stored at -80°C. Once all time points were collected, cells were thawed on wet ice and washed twice with ice-cold sample buffer (50 mM Tris-HCl pH 7.6, 50 mM EDTA). Cells were then resuspended in 300 µL of sample buffer, 100 µL of 0.1 mm silica/glass beads added and then mechanically disrupted in a Mini-beadbeater-8 at its maximum setting for two, one-minute pulses punctuated by a one-minute rest on ice. Lysates were centrifuged at 20k rpm for 15 minutes at 1°C, then aprotinin (1 µg/ml), leupeptin (1 µg/ml), and PMSF (300 µM) were added to the clarified supernatants, and their protein concentrations determined by Bradford assay on a FL600 microplate reader (Biotek Instruments).

**Western blot analysis.** 25 µg of lysate from *S. aureus* strains described above were separated by SDS-PAGE, transferred to PVDF membrane and blocked overnight in Tris-buffered saline (150 mM NaCl, 10 mM Tris pH8.0) with 0.1% Tween-20 (v/v) (TBS-T) and 5% dried milk. Membranes were then incubated with either mouse α-HA (Cell Signaling), α- Myc (Cell Signaling), α-SarA (32), or α-σB (8), α-MazFsa (17) or α-MazEsa.
(17) antibodies in TBS-T for two hours, washed three times with TBS-T for five minutes each, treated with horseradish peroxidase-conjugated goat α-mouse secondary antibody (Jackson ImmunoResearch) for thirty minutes, and washed again with TBS-T three times for five minutes each. Membranes were then covered with ECL reagent (GE Healthcare) and exposed to film. Scanned images from non-saturated exposed film were then analyzed densitometrically using ImageJ, and the half-lives calculated using Prism 5 (Graphpad).

RESULTS

Evaluation of protein stability in *S. aureus* following transcriptional inhibition.

MazE degradation in *E. coli* was first shown by comparing time-points of lysates of $^{35}$S labeled cells grown during heat shock at 42°C, a condition that represses MazE production (1). A more specific approach was used in our studies where antitoxin levels were determined by Western blot, a method similar to that used by Christensen *et al.* for RelB (9). To more easily expose the changes in antitoxin levels over time, rifampicin was added to cultures at a level sufficient to stall RNA polymerase (200 µg/ml) and block any further transcription of TA mRNA (2). The use of a transcriptional inhibitor like rifampicin was desirable because antibiotics such as macrolides and tetracyclines block translation and also have the undesirable effect of up-regulating TA transcription in *S. aureus* (12). However, to provide evidence that this treatment did not alter protein levels in *S. aureus* in general, equivalent amounts of lysates from strain SH1000 were separated using SDS-PAGE and either Compassion stained, or Western blotted using α-SarA or α-σB antibodies. As seen in Figure 1A, rifampicin treatment produced no gross changes in
protein over the course of treatment, and both $\sigma^B$ (Fig. 1B) and SarA levels (Fig. 1C) remained steady. This indicated that using rifampicin to transcriptionally stall \textit{S. aureus} does not lead to a significant changes in the general turnover of cellular proteins.

\textbf{MazE}_{sa} is rapidly degraded in \textit{S. aureus} while MazF_{sa} is stable.} As antitoxin degradation is one of the one of the key aspects of \textit{E. coli} TA regulation, we examined whether the \textit{S. aureus} MazE_{sa} antitoxin was regulated in a similar manner. Western blots of lysates made from transcriptionally stalled SH1000 cells over-expressing MazE_{sa} were probed with $\alpha$-MazE_{sa} antibodies and showed that the MazE_{sa} levels rapidly decreased over time after rifampicin treatment to the point where the protein was nearly undetectable after thirty minutes (Fig. 1D). Densitometry of this progression indicated MazE_{sa} has an \textit{in vivo} half-life between eighteen and twenty minutes, a rate faster than that reported for MazE of \textit{E. coli} ($t_{1/2} = 30$ minutes) (1). To determine if the \textit{S. aureus} MazF_{sa} toxin was more stable than its cognate antitoxin, MazF_{sa} levels were tracked by immunoblotting rifampicin-treated SH1000 cell lysates using $\alpha$-MazF_{sa} antibodies. As shown in Figure 1E, in contrast to the antitoxin MazE_{sa}, MazF_{sa} levels remained relatively constant after transcriptional inhibition. This indicated that the \textit{S. aureus} \textit{mazEF} TA system was post-translationally regulated in a manner similar to its homologue in \textit{E. coli}.

\textbf{Axe1 and Axe2 are degraded rapidly \textit{in vivo}.} We next examined whether the two other known antitoxins of \textit{S. aureus} exhibited degradation patterns similar to that of MazE_{sa}. To circumvent the issue that antisera to Axe1 and Axe2 were unavailable, epitome-tagged versions of these antitoxins were created to facilitate their detection. An \textit{in silico} analysis suggested that the addition of HA or Myc tags to either terminus caused
no secondary structure changes, but also did not reveal any known motifs involved in Clp-specific degradation (e.g. LCN, FMLYPK, or AA motifs from \textit{B. subtilis}) \cite{7, 36, 38}. To guard against the possibility such a tag could affect the degradation of these proteins, separate constructs were made that fused either HA or Myc epitope tags to alternatively the N- or C-terminus of each antitoxin. As a related control, we compared the degradation rate of untagged MazEs\textsubscript{sa} to Myc-tagged MazEs\textsubscript{sa}, and determined the half-life to be essentially unchanged (twenty minutes versus eighteen minutes, Fig. 1D and 2). With this information, we proceeded to transcriptionally stall SH1000 wild-type cells containing pEPSA5 plasmids capable of over-expressing either \textit{axe1} or \textit{axe2}, and withdrew samples every fifteen minutes. Western blot analysis of lysates made from these cells revealed that levels of both HA and Myc tagged antitoxins of Axe2 dropped rapidly after rifampicin treatment ($t_{1/2} = 20$ minutes for Myc and HA tagged antitoxins) (Fig. 2 and 6A for Myc-tag and 7A for HA-tag). Similarly, Axe1 tagged with Myc (Fig. 2 and 5A) or HA (data not shown) displayed similar but rapid degradation rates in strain SH1000. From these data, we concluded that the Axe1 and Axe2 antitoxins were rapidly degraded in a fashion similar to MazEs\textsubscript{sa} in \textit{S. aureus}.

\textit{clpP} and \textit{clpC} are required for antitoxin degradation in \textit{S. aureus}. To date, all proteolysis of \textit{E. coli} antitoxins appears to be through Lon or ClpP \cite{19}, with the latter deploying ClpA or ClpX ATPase chaperones for this destruction. However, the mechanism(s) of this degradation in \textit{S. aureus} is less clear, as not only does it lack a Lon homologue, but it also contains several Hsp100/Clp ATPases with similar levels of homology to ClpA [(ClpB (59%), ClpC (61%) and ClpL (61%) similarity]. To clarify the degradative mechanism, SH1000 strains were made lacking either the three known ATP-
dependent proteases: ftsH, clpP, or hslV (clpQ), or their chaperones: clpB, clpC, clpL, clpX, or clpY (hslU). These strains were then transformed with either pALC6188, pALC6486 or pALC6489 for ectopic over-expression of Myc-tagged MazEsa, Axe1, or Axe2, respectively. Lysates were then made from these strains at time-points either immediately following rifampicin treatment or after thirty minutes, and then analyzed by Western blot with α-Myc antibodies. As seen in Fig. 3, only lysates from strains lacking clpC or clpP showed defects in MazEsa, Axe1 and Axe2 degradation after 30 minutes, suggesting that clpP and clpC were involved in breakdown of all three S. aureus antitoxins.

**clpP, but not clpC, is essential for degradation of all S. aureus antitoxins.** We next examined the overall impact of clpC and clpP deletions on antitoxin breakdown. To do so, levels of Myc-tagged MazEsa, Axe1 and Axe2 were followed for almost two hours in transcriptionally stalled wild-type SH1000 or in isogenic strains lacking either clpC, clpP or clpB (as a control). As seen in figures 4E, 5E and 6E, levels of all three antitoxins remained unchanged in a SH1000 ΔclpP strain during these two hours, while the ΔclpB (Figs. 4B, 5B and 6B) and clpP complemented strains (Figs. 4F, 5F and 6F) showed similar degradation rates to those found in wild-type SH1000 (Figs. 4A, 5A and 6A). A slightly different degradation pattern emerged when the SH1000 ΔclpC strain was examined. The MazEsa protein exhibited limited breakdown in the ΔclpC mutant (Fig. 4C) at a rate substantially slower (t_{1/2} = 46 minute) than in the wild type (t_{1/2} = 12 minutes). In contrast, levels of Axe1 and Axe2 remained steady throughout in the ΔclpC mutant (Figs. 5C and 6C). In support of our findings, the half-lives of the respective proteins were restorable to wild-type levels in a clpC complemented strain (Figs. 4D, 5D...
and 6D). To ensure that the Myc tag did not alter the profile of proteolysis of the antitoxin, we substituted the C-terminal Myc tag of Axe2 with an HA tag. A similar pattern of clpC and clpP dependent degradation was seen for these antitoxins with the HA tag when compared to the Myc tag (Fig. 7). Similar data were obtained with Axe1 (data not shown). Collectively, these data indicated that clpP was essential for proteolysis of all three antitoxins in S. aureus, while clpC was involved in degradation of MazE_{sa}, Axe1 and Axe2, but was essential for only the latter two antitoxins.

DISCUSSION

Maintenance of antitoxin protein levels relies on continued synthesis in the host bacterium, whereupon any disruption of the production process causes antitoxin levels to drop, leading to toxin activation. However, as antitoxin proteolysis has only been described for E. coli TA systems, our findings that the S. aureus ATP-dependent protease ClpP and its ATPase chaperone ClpC function to degrade the antitoxins MazE_{sa}, Axe1 and Axe2 provide the first evidence that this mechanism of toxin activation by ClpP is conserved in Gram-positive bacteria as well. Furthermore, the 12 to 20-minute half-lives of the three S. aureus antitoxins were all considerably faster than their closest E. coli homologues [MazE = 30 min (1); YefM = 50-60 min (6)]. These data suggest that the toxins of S. aureus TA systems might become activated much more quickly to stimuli than their E. coli counterparts, attributable to the faster decrease in S. aureus antitoxins levels.

Interestingly, the genetic components necessary for antitoxin breakdown were not absolute. While both ClpP and ClpC were essential for Axe1 and Axe2 degradation, the
MazE<sub>sa</sub> level was found to decrease in the clpC mutant at one-quarter the rate compared to that of the wild-type. This finding suggested several possibilities: 1) a second, unannotated chaperone might function to partially complement ClpC’s breakdown of MazE<sub>sa</sub>; 2) another ATP-dependent protease might also process MazE<sub>sa</sub>; 3) that ClpP may still process MazE<sub>sa</sub> in the absence of ClpC. The third scenario may be the most likely as 1) ClpC is essential for Axe1 and Axe2 degradation, 2) no other clp-related gene deletion in our tested mutant strains resulted in a decreased MazE<sub>sa</sub> breakdown rate, 3) deletions of hslV or ftsH did not alter antitoxin breakdown and 4) ClpP may be capable of slowly degrading proteins independent from an ATPase chaperone (22).

During our study of Axe1 and Axe2, two antitoxins with 48% amino acid similarity, we observed that the addition of either a Myc or HA epitope tag to the N-terminus of Axe2 could block its degradation, while a similar inhibitory pattern in Axe1 could only be obtained when these tags were added to its C-terminus (data not shown). In silico analyses of these fusions suggested that the epitope tags did not alter the antitoxins’ secondary structure, nor did they mask any motif known to be involved in Clp-specific degradation [e.g. LCN, FMLYPK, or AA motifs from B. subtilis (7, 36, 38). We are currently investigating the basis of this proteolytic disparity.

As many TA systems do not have obvious phenotypes, identification of ClpPC as the functional protease in S. aureus antitoxin degradation may increase our understanding of the contributions of TA systems in this and other Gram-positive organisms. Previously, it has been shown that the transcription of clpC and all three of S. aureus’s TA systems was found to be increased upon heat shock (5, 12); the subsequent increase in ClpC levels may enhance toxin activity by virtue of increased capacity for antitoxin degradation.
degradation. Furthermore, Chatterjee et al. (5) showed that a ∆clpC mutant strain survived better over five days of growth compared to wild type, while a sigB mutant grew poorly in comparison. In light of our data, a portion of this increased survival of a ∆clpC strain may be due to the permanent inactivation of toxins that would result from the lack of antitoxin proteolysis. A similar phenotype may also be found in a strain lacking all its TA systems, as they would lack the restraints upon growth that TA systems seem to contribute. The ∆sigB growth phenotype may be in part due to the de-repression of TA transcription that occurs in a ∆sigB strain (12). Such an increase would lead to the accumulation of stable toxins, which would necessitate an ever-increasing level of antitoxins. Such a demand would likely be unsustainable and result in toxin activation and, ultimately, decreased survival and/or slower growth.

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REFERENCES


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<td>pALC6493</td>
<td>pMAD::(clpP) complement This work</td>
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LEGENDS

Fig. 1. Analysis of protein levels of lysates from time-points of *S. aureus* strains grown to *A*₆₅₀ = 1.1 and then treated with rifampicin (200 µg/ml). (A) Coomassie Brilliant Blue stained wild-type SH1000 lysates separated by SDS-PAGE; (B, C) Lysates of wild-type SH1000 lysates separated by SDS-PAGE and immunoblotted using primary antibodies specific to (B) σB and (C) SarA. (D, E) Immunoblots of lysates from SH1000 with pALC4077 grown to *A*₆₅₀ = 1.1 with 0.1% xylose and then treated with rifampicin (200 µg/ml). Immunoblots of these lysates used primary antibodies specific to (D) MazEₕ and (E) MazFₕ. The top of each panel displays the bands from each Western blot, and the corresponding graph represents the calculation of original amount of detected protein by densitometry before addition of rifampicin (t = 0). The experiments were repeated three times and a representative blot is shown.

Fig. 2. Axe1 and Axe2 levels in *S. aureus* SH1000 wild-type cells following transcriptional arrest from rifampicin. Wild-type SH1000 was transformed with pEPSA5::*mazE*ₕ-*c-myc* (pALC6188), pEPSA5::*axe1*ₕ-*c-myc* (pALC6486) or pEPSA5::*axe2*ₕ-*c-myc* (pALC6489), grown to *A*₆₅₀ = 1.1 with 0.1% xylose, and then treated with rifampicin (200 µg/ml). Samples were taken every fifteen minutes, lysed and then separated by SDS-PAGE for immunoblotting with an α-Myc primary antibody. The experiments were repeated three times and a representative blot is displayed.

Fig. 3. Stability of *S. aureus* antitoxins in SH1000 wild type and strains lacking genes in various ATP-dependent proteolytic pathways. Wild-type and mutant strains were
transformed with pEPSA5::mazE<sub>C-myc</sub> (pALC6188), pEPSA5::axe1<sub>N-myc</sub> (pALC6486) or pEPSA5::axe2<sub>C-myc</sub> (pALC6489) grown in inducing conditions (0.1% xylose), treated with 200 µg/ml rifampicin, and harvested either immediately or after thirty minutes. Lysates of the samples were subjected to gel electrophoresis and immunoblotting with an α-Myc primary antibody. The experiments were repeated three times and a representative blot is shown.

Fig. 4. Comparative rates of MazE<sub>sa</sub> degradation in SH1000 wild-type and clp mutant strains. (A) Wild-type, (B) ∆clpB, (C) ∆clpC, (D) clpC complemented, (E) ∆clpP and (F) clpP complemented strains were transformed with pEPSA5::mazE<sub>C-myc</sub> (pALC6188). Cells were induced and transcriptionally stalled similar to those in Fig. 3. The upper panel represent Western blots the respective time-points using a primary α-Myc antibody, while the lower panel depicts the percentage of signal at each time-point compared to t = 0. The experiments were repeated three times and a representative blot is displayed.

Fig. 5. Comparative rates of Axe1 degradation in SH1000 wild-type and clp mutant strains. (A) Wild-type, (B) ∆clpB, (C) ∆clpC, (D) clpC complemented, (E) ∆clpP and (F) clpP complemented strains were transformed with pEPSA5::axe1<sub>N-myc</sub> (pALC6486). Cells were treated and analyzed as those in Fig. 4. The experiments were repeated three times and a representative blot is shown.

Fig. 6. Comparative rates of Axe2 degradation in SH1000 strains wild-type and clp mutant strains. (A) Wild-type, (B) ∆clpB, (C) ∆clpC, (D) clpC complemented, (E) ∆clpP
and (F) clpP complemented strains were transformed with pEPSA5::axe2c-myc (pALC6489). Cells were treated and analyzed as those in Fig. 4. The experiments were repeated three times and a representative blot is displayed.

Fig. 7. Comparative rates of Axe2 degradation in SH1000 strains wild-type and clp mutant strains with an alternate HA epitope tag. (A) Wild-type, (B) ΔclpB, (C) ΔclpC and ΔclpP strains were transformed with pEPSA5::axe2C-HA (pALC6682). The upper panel represent Western blots the respective time-points using a primary α-HA antibody, while the lower panel depicts the percentage of signal at each time-point compared to t = 0. Cells were analyzed as those in Fig. 4. The experiments were repeated twice and a representative blot is displayed.
Fig. 2

<table>
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<td>0, 15', 30', 45', 60', 75', 90', 105'</td>
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</tbody>
</table>
Fig. 4

A. SH1000

B. SH1000 ΔclpB

C. SH1000 ΔclpC

D. SH1000 clpC complement

E. SH1000 ΔclpP

F. SH1000 clpP complement
Fig. 6

A

SH1000

-11 kD

B

SH1000 ΔclpB

-11 kD

C

SH1000 ΔclpC

D

SH1000 clpC complement

E

SH1000 ΔclpP

F

SH1000 clpP complement

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