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Supplementary Information for

Staphylococcal ClpXP protease targets the cellular antioxidant system to eliminate fitness-compromised cells in stationary phase

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Datasets S1 to S7

Supplementary Information Text

Supplemental Experimental Procedures.

Bacterial strains, plasmids, and growth conditions

The *S. aureus* strain JE2 and its isogenic mutants used in this study were obtained from the Nebraska Transposon Mutant Library (1), except for *clpX* and *clpX*$_{I265E}$ (2). Strains and plasmids used in this study are listed in (*SI Appendix*, Table S3). Transposon mutants were re-transduced into JE2 using ϕ11, and tryptic soy agar (TSA) plates containing 5 µg/ml erythromycin were used for selection (1). After re-transduction, all transposon mutants were confirmed by PCR using gene-specific primers listed in (*SI Appendix*, Table S4). We complemented *clpX*$_{I265E}$ chromosomally with the full-length *clpX* under the control of its native promoter by transducing the SaPI integrating plasmid pAQ21 as described previously (2).

To assess stationary phase viability, bacteria were grown aerobically from an initial OD_{600} of 0.06, with agitation at 245 rpm at 37 °C in Tryptic Soy Broth (TSB; Difco Laboratories) using a 10:1 (flask: volume) ratio. TSB was supplemented with 45 mM glucose, either unbuffered or buffered to pH 7.3 with 100 mM 3-(N-morpholino) propanesulfonic acid, 4-morpholinepropanesulfonic acid (MOPS). Cell viability was monitored by subjecting bacterial cells to serial dilution with 0.9% saline, followed by plating on TSA plates. Plates were then incubated aerobically at 37 °C for at least 24 h before counting bacterial colonies; the limit of detection was 10 cfu/ml. To perform experiments involving cross-substitution of cell-free media, 24 h-old cultures from wild type and *clpP* grown as described above were centrifuged at 4100 x g for 10 minutes in 50 ml conical tubes to pellet bacterial cells. Culture supernatants were filtered using 0.2 µm filters (Sartorius) and cross substituted into bacterial cell pellets that were washed with 0.9% saline. The bacterial cell pellets were resuspended in the corresponding supernatant and stationary phase viability was assessed by counting colony-forming units (cfu/ ml) from serially diluted cultures.

To assess survival following antibiotic challenge, overnight bacterial cultures were inoculated in 25 ml of TSB supplemented with 14 mM glucose in 250 ml flask to a final OD_{600} of 0.06, and cultures were grown at 37 °C shaking at 245 rpm. At 5.5 h of growth, cfu's were enumerated as described above (Time 0) and cultures were challenged with chloramphenicol, tetracycline, trimethoprim, kanamycin, erythromycin, vancomycin, oxacillin, or gentamycin (400, 25, 12.5, 87.5, 200, 50, 25, and 25 µg/mL, respectively). Aliquots containing 200 µl of antibiotic-challenged cultures were collected and centrifuged at 16,000 x g, and the pellet was washed by resuspending it in 1 ml 0.9% saline. After two rounds of thorough washing, the pellets were resuspended in 200 µl saline, and then serially diluted and plated on TSA as described above to enumerate survivors (3).

Protein aggregation

Bacterial cells were grown aerobically in 120 ml TSB-45 mM glucose with and without MOPS, as previously described for survival assay. At the specified time points, 5 ml to 10 ml of bacterial culture were collected for the isolation of protein aggregates. The aggregates were isolated from bacterial cells as described by Engman et al. with minor modifications (4). Bacterial cultures were centrifuged at 4000 x g for 10 min, and the cell pellets were washed twice with 1X phosphate-buffered saline (PBS) before storage at – 80 °C. On the day of the experiment, cells were resuspended in 1 ml of cold 50 mM potassium phosphate buffer at pH 7. Cells were disrupted by shaking with glass beads for 4 cycles at 4 °C with 5 s intervals. Each cycle was programmed for 23 sec at 6100 rpm in a Precellys Evolution homogenizer (Berting Instruments). Glass beads were pelleted by centrifugation at 200 x g for 10 minutes. The protein concentration of the crude extract was determined using Bradford protein assay according to the manufacturer's instructions (Biorad). The crude extract containing 250 µg of total protein was transferred into a 1.7-ml Eppendorf tube and centrifuged at 21,000 x g for 40 min at 4 °C. The pellet was resuspended in buffer A (50 mM Tris, 150 mM NaCl, pH = 8) with 1% Triton X-100 and incubated

at 4 °C on a rotating platform for 2 h followed by centrifugation at 16,000 x g for 40 min at 4 °C. After centrifugation, the pellet was resuspended in buffer A and centrifuged at 16,000 x g for 40 min at 4 °C. The resulting pellet was resolubilized in 70 µl of rehydration buffer consisting of 7 M urea, 2 M thiourea, 4% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1- propanesulfonate (CHAPS), and 100 mM dithiothreitol (DDT). 30 µl of the insoluble fraction was loaded on 10% SDS polyacrylamide gels, and the resolved protein aggregates were stained with Coomassie blue G-250. The stained gel was imaged and then densitometrically quantified using ImageJ software (NIH, Bethesda, MD).

Protein carbonylation

Bacterial cells were grown in 115 ml of TSB in one-liter flasks, and 2 ml of culture was collected at the specified time points. Cells were centrifuged at 15,000 x g for 5 min and the pellet was washed twice with 1 ml PBS. Washed cells were resuspended in 1.5 ml of PBS with 50 mM dithiothreitol (DTT) and disrupted by glass beads as described above. After centrifugation at 15,000 x g for 10 min, protein concentration was determined by a Nanodrop 1000 and 1 mg of protein was used to determine carbonyl content as follows. DNA was precipitated using 1% streptomycin sulfate and isolated by centrifugation at 11,000 x g for 10 min. Proteins from the supernatant were precipitated by adding 10% trichloroacetic acid (TCA), vortexed, and incubated for 30 mins on ice. Precipitated proteins were centrifuged for 30 min at 12,000 x g at 4 °C. Carbonyl-containing proteins were derivatized by incubating with 500 µl of 10 mM 2,4-dinitrophenylhydrazine (DNPH) dissolved in 2 M HCl for 1 h with vortexing every 10-15 min. After derivatization, samples were mixed with 500 µl of 20% TCA and centrifuged at 11,000 x g for 3 min. Pellets were washed three times by adding 1 ml of 1:1 ethanol-ethyl acetate, incubating at room temperature for 10 min, and then centrifuged at 11,000 x g for 3 mins. Finally, pellets were dissolved in 6 M guanidine in a 20 mM potassium phosphate buffer adjusted to pH 2.3 with trifluoracetic acid; absorbance was measured at 370 nm and carbonyl content was determined using an extinction coefficient of 22,000 M-1cm-1 (5).

Quantitative real-time PCR

RNA was extracted using RNeasy Bacteria kit according to the manufacturer's instructions (Qiagen) from 1 ml of culture for all experiments that required a quantification of gene expression. To lyse the cells, we used the Precellys Evolution homogenizer as described above but set to 2 cycles of cell disruption. Following RNA extraction, cDNA was synthesized using QuantiTect reverse transcription kit (Qiagen). Quantitative PCR was assessed using LightCycler 480 SYBR green master mix (Roche) according to the manufacturer's instruction on 1:20 diluted cDNA. We used the comparative threshold cycle (C_T) method to calculate relative transcript levels which were normalized to the amount of the *sigA* transcripts present in RNA samples (6).

Western blot analysis

Western blot analysis was carried out using proteins from cell lysates of appropriate mutants. Briefly, 10 µg protein was resolved on a 15% SDS-PAGE gel and transferred using a semi-dry method by Pierce Power Blot Cassette system (Thermo-Fisher) onto a nitrocellulose membrane (7). To detect Spx, cross-reactive antibodies raised against *B. subtilis* Spx (8) were diluted to 1:1,500 in PBS containing 0.01% Tween-20 and 5% skim milk (8). Goat anti-rabbit secondary antibodies (Invitrogen) were diluted 1:40,000. Immunodetection was performed using the Super Signal West Femto chemiluminescence kit (Thermo Scientific). Immunoblot images were acquired using an iBright CL1000 (Invitrogen). To detect SodA in cell lysates, 10 µg of cytoplasmic protein was resolved on a Tris-Glycine 4-20% Mini-PROTEAN gel (Biorad) under reducing conditions and processed as described above. The SodA antibody was obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID (Bethesda, MD, USA): Polyclonal anti–*Bacillus anthracis* Superoxide Dismutase SODA-1 (Locus_Tag:BA_4499), Immunoglobulin G, Rabbit), NR-10506. The anti-SodA antibody was used in a dilution of 1:10,000 in PBS containing 0.01% Tween-20 and 5% skim milk. The dilution of secondary antibodies and the development of the blot were performed as described above for the detection of Spx. Enolase was detected as a loading control. Anti-enolase polyclonal antibodies

were purchased from a commercial vendor (Thermo Scientific) and used at a dilution of 1:2,500 in PBS containing 0.01% Tween-20 and 5% skim milk.

LC-MS/MS analysis

For proteomics, 100 µg of protein per sample from three biological replicates per group was resuspended in 100 mM ammonium bicarbonate. The samples were digested with MS-grade trypsin (Pierce) overnight at 37°C following reduction with 10 mM DTT at 56 ° C for 30 mins and alkylation with 50 mM iodoacetamide was carried out for 20 mins in the dark. The resulting peptides were cleaned using PepClean C18 spin columns (Thermo) and resuspended in a solvent containing 2% acetonitrile (ACN) and 0.1% formic acid (FA). 500 ng of each sample was loaded onto trap column Acclaim PepMap 100 75µm x 2 cm C18 LC Columns (Thermo Scientific™) at a flow rate of 4 µl/min then separated with a Thermo RSLC Ultimate 3000 (Thermo Scientific™) on a Thermo Easy-Spray PepMap RSLC C18 75 µm x 50 cm C-18 2 µm column (Thermo Scientific™) with a step gradient of 4–25% solvent B (0.1% FA in 80 % ACN) from 10-100 min and 25–45% solvent B for 100–130 min at 300 nL/min and 50°C with a 155 min total run time. The eluted peptides were analyzed by a Thermo Orbitrap Fusion Lumos Tribrid (Thermo Scientific™) mass spectrometer in a data-dependent acquisition mode. A full scan MS survey (from m/z 350–1800) was acquired in the Orbitrap with a resolution of 120,000. The AGC target for MS1 was set as 4×10^5 and ion filling time was set as 100 ms. The most intense ions with charge state 2-6 were isolated in a 3 s cycle and fragmented using HCD fragmentation with 35% normalized collision energy and detected at a mass resolution of 30,000 at 200 m/z. The AGC target for MS/MS was set as 5×10^4 and ion filling time set 60 ms dynamic exclusion was set for 30 s with a 10 ppm mass window. Protein identification was performed by searching MS/MS data using PEAKS Studio X+ software against an in-house database created from the predicted proteins of *S. aureus* USA300 FPR3757. The search was set up for full tryptic peptides with a maximum of two missed cleavage sites. Oxidized methionine was included as variable modifications and carbamidomethylation of cysteine was set as fixed modification. The precursor mass tolerance threshold was set 10 ppm for and maximum fragment mass error was 0.02 Da. The significance threshold of the ion score was calculated based on a false discovery rate of ≤ 1%. Qualitative analysis was performed using Progenesis QI proteomics 4.2 (Nonlinear Dynamics). Statistical analysis was performed using ANOVA and the Benjamini-Hochberg (BH) method was used to adjust *P* values for multiple-testing using the false discovery rate. The adjusted $p \leq 0.05$ was considered significant.

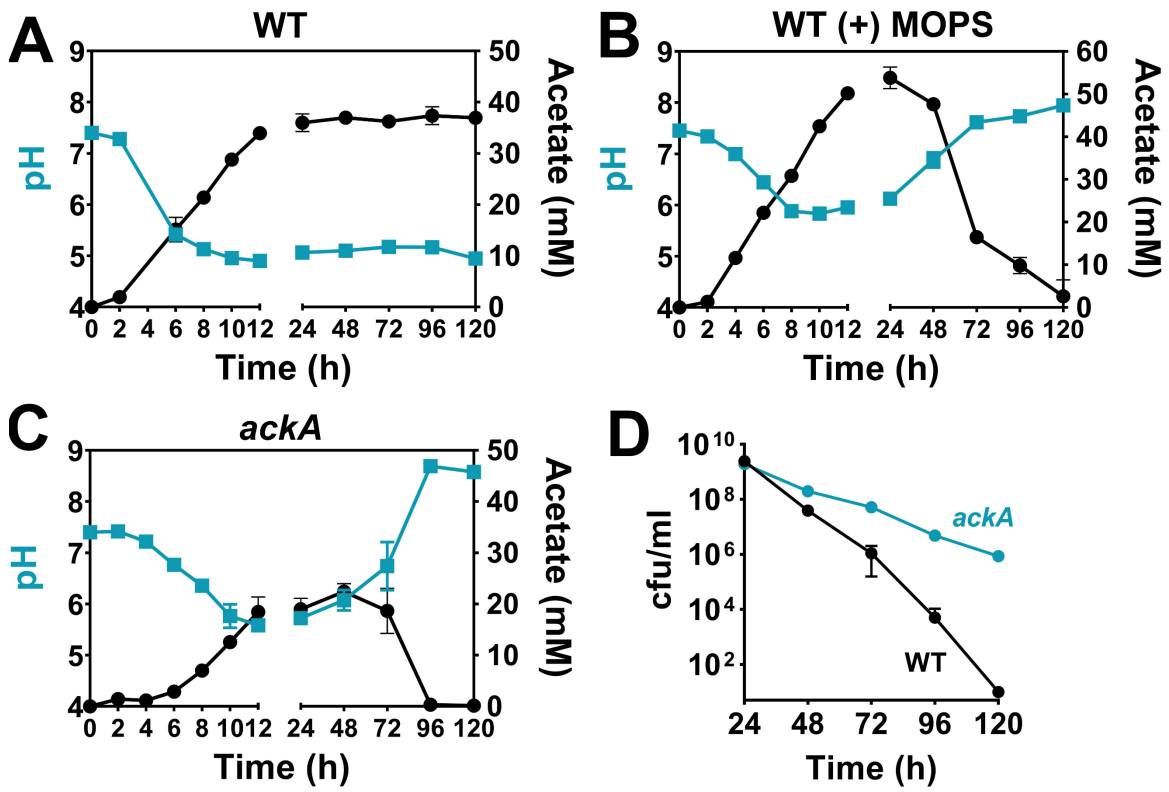

Fig. S1. Acetate production and pH profile of *S. aureus*. The extracellular acetate concentrations of culture supernatants (solid circles) from the ***(A)*** WT (unbuffered media), ***(B)*** WT grown in MOPS buffered media and ***(C)*** *ackA* mutant (unbuffered media) were determined over 120 h using a commercially available kit (Roche). The pH (solid squares) was determined using a pH probe (n=3, Mean ± SD). ***(D)*** Cell viability of the WT and *ackA* mutant were measured in stationary phase following growth in TSB-G (n=3, Mean ± SD).

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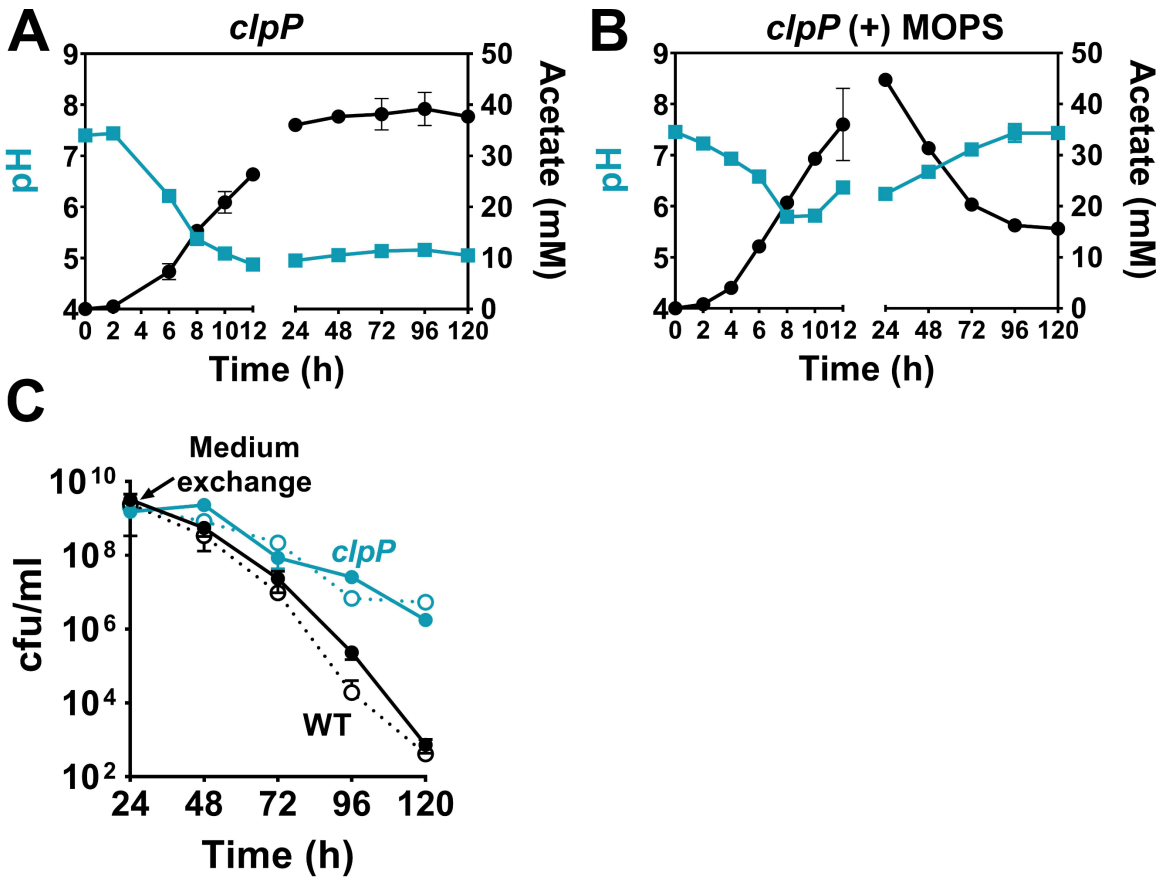

Fig. S2. Extracellular factors do not contribute to the survival of the *clpP* mutant in stationary phase. Acetate production (solid circles) and pH profile (solid squares) of the *clpP* mutant following growth in ***(A)*** TSB-G (unbuffered media) and ***(B)*** TSB-G supplemented with MOPS, pH 7.4 (n=3, Mean ± SD). ***(C)*** Stationary phase viability of the WT and *clpP* mutant. Following the growth of WT and *clpP* mutant in TSB-G for 24 h (closed circles), cell-free culture supernatants of both strains were cross-substituted. Cells were washed with sterile saline to remove any traces of original growth media before media exchange. Cell viability traces of *clpP* and WT controls (open circles) that did not undergo media exchange are shown in dotted lines (n=3, Mean ± SD).

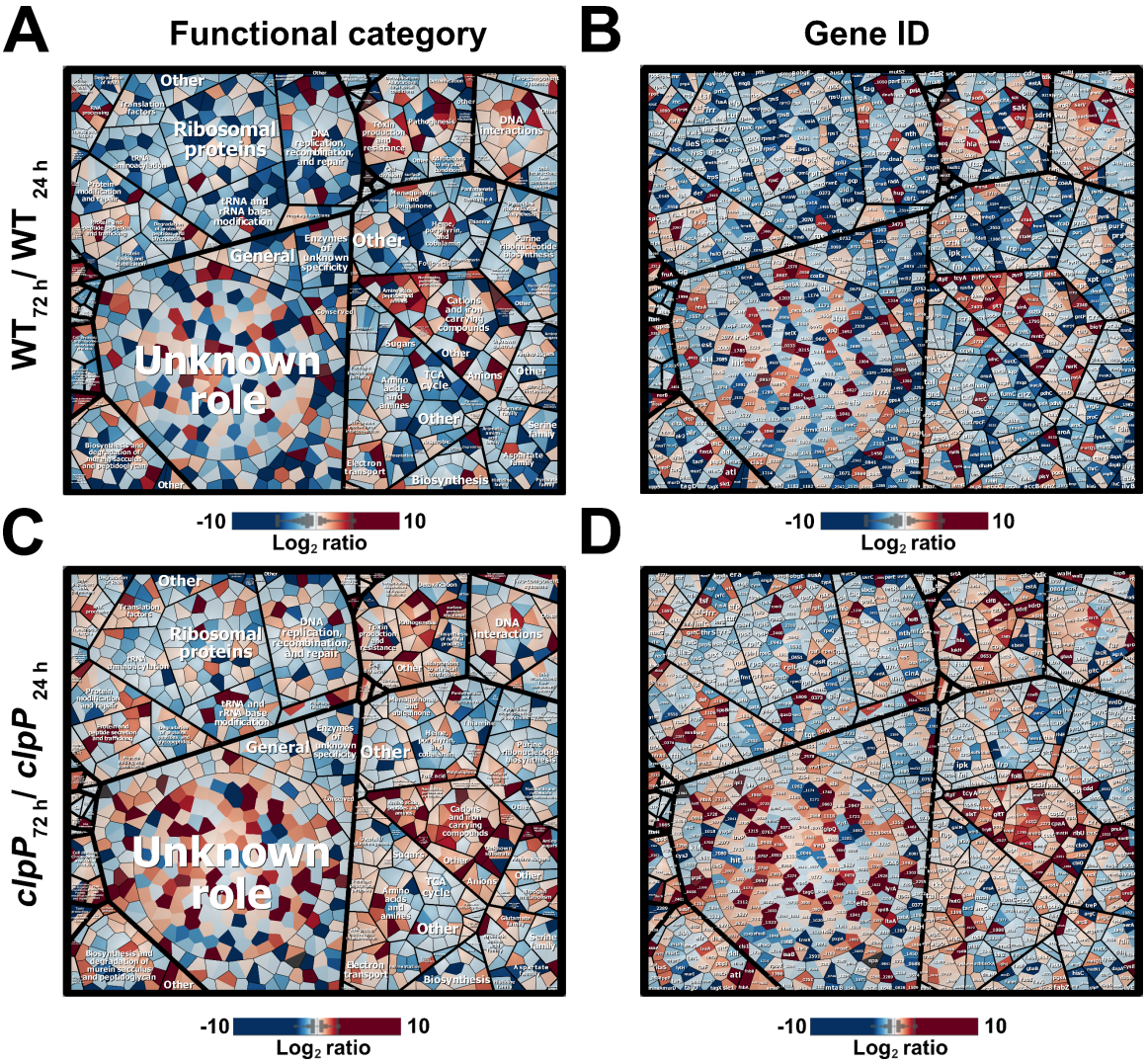

Fig. S3. Proteomic profiling of stationary phase cells following acetate stress. ***(A, B)*** Voronoi Treemaps of WT and ***(C, D)*** *clpP* mutant were generated from Log_2 ratios of their respective intracellular proteins at 72 h and 24 h of growth in TSB-G. Proteins with altered ratios were clustered based on the TIGRFAM annotations and depicted as functional categories. ***(A, C)*** represent functional sub-categories and ***(B, D)*** represent the corresponding gene IDs/ protein names. SAUSA300_XXXX locus tags are annotated as _XXXX in Voronoi Treemaps.

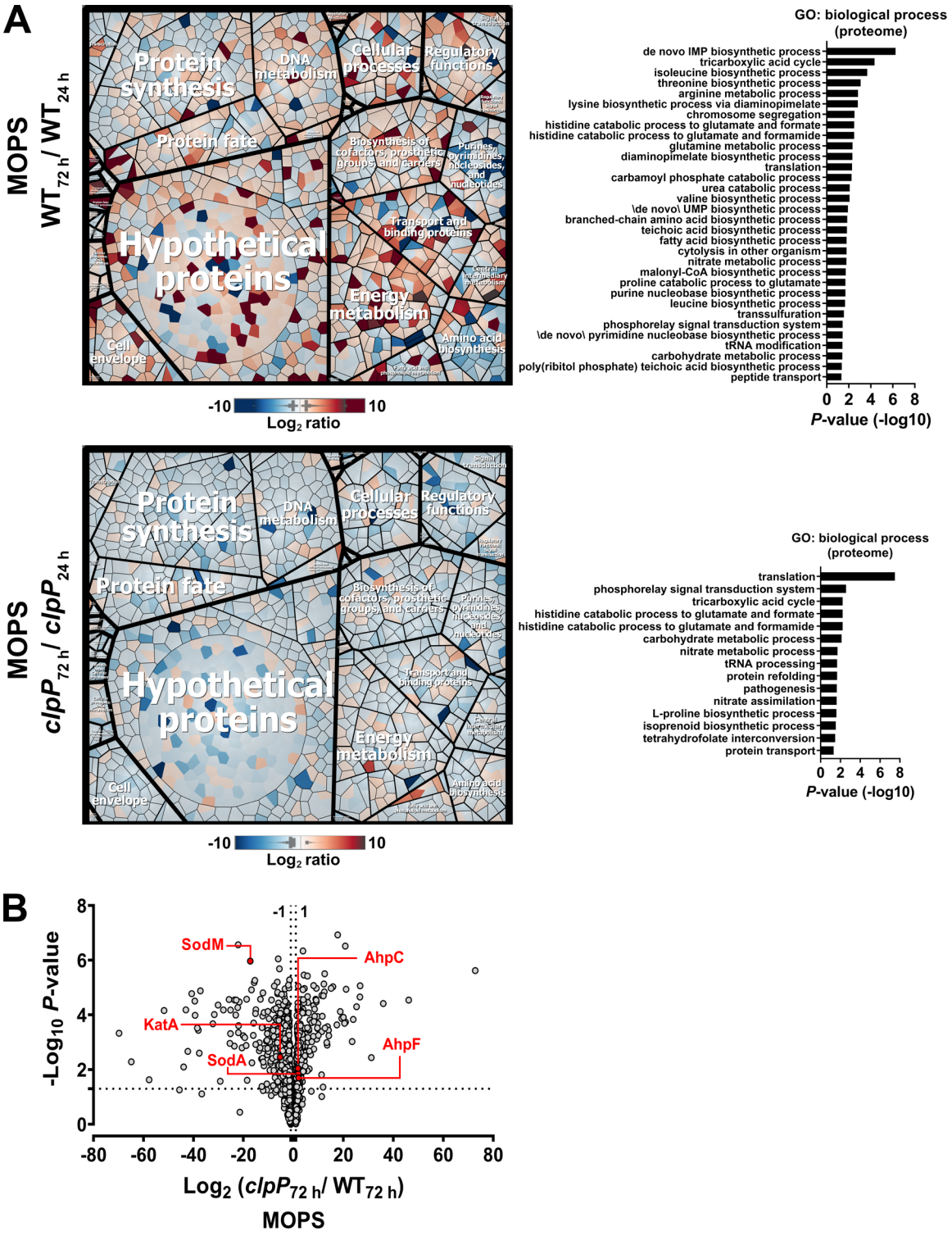

Fig. S4. Altered proteome of stationary phase cells grown in MOPS buffered TSB-G media, pH 7.4 ***(A)*** Voronoi Treemaps and GO-term enrichment analysis of WT and *clpP* mutant. The Voronoi Treemaps were generated from Log_2 ratios of intracellular proteins at 72 h and 24 h of growth in TSB-G. Proteins with altered ratios were clustered based on the TIGRFAM annotations

and depicted as functional categories. Additional subcategories and gene ID annotations are indicated in *SI Appendix*, Fig. S5 ***(B)*** Volcano plot of the intracellular proteins in stationary phase cells. Each data point represents individual proteins organized according to their mean *clpP*/ WT Log_2 fold-change ratios (y-axis) following 72 h of growth in TSB-G supplemented with MOPS, pH 7.4. The horizontal dotted line indicates the cut-off for proteins that showed significantly altered abundance ($P \leq 0.05$).

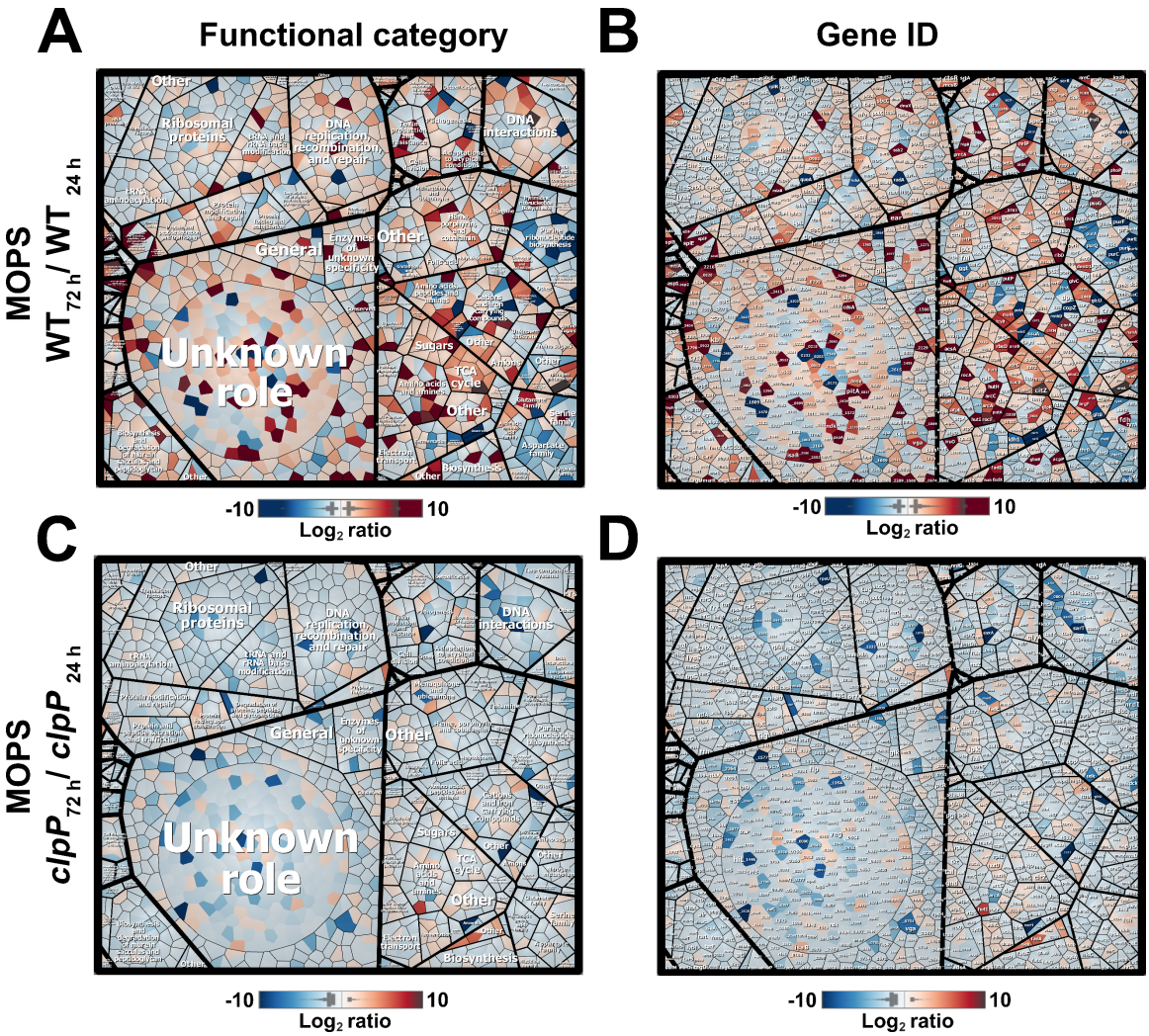

Fig. S5. Proteomic profile of stationary phase cells grown in MOPS buffered TSB-G media, pH 7.4 ***(A, B)*** Voronoi Treemaps of WT and ***(C, D)*** *clpP* mutant were generated from Log_2 ratios of their respective intracellular proteins at 72 h and 24 h of growth in TSB-G supplemented with MOPS, pH 7.4. Proteins with altered ratios were clustered based on the TIGRFAM annotations and depicted as functional categories. ***(A, C)*** represent functional sub-categories and ***(B, D)*** represent the corresponding gene IDs/ protein names. SAUSA300_XXXX locus tags are annotated as _XXXX in Voronoi Treemaps.

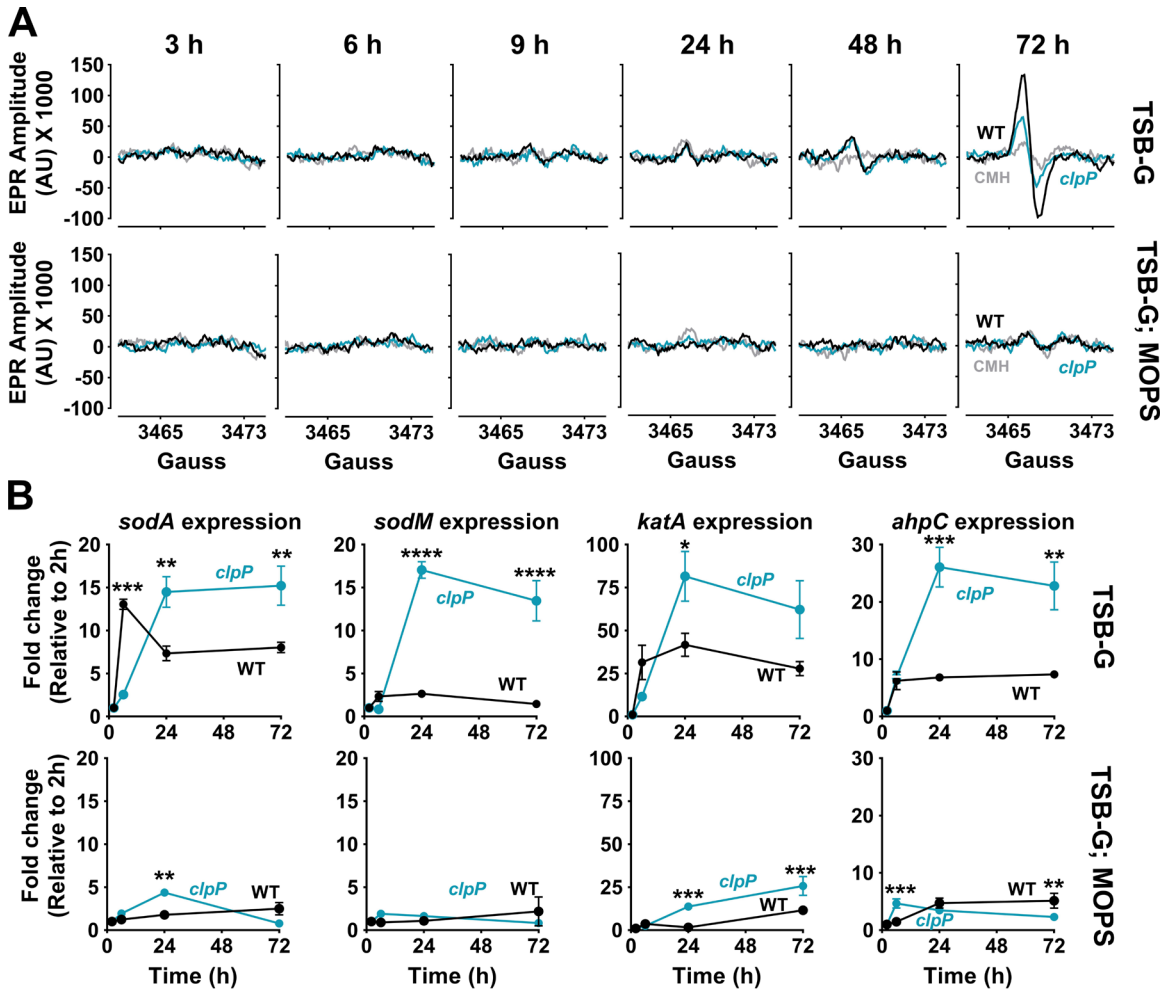

Fig. S6. Temporal patterns of endogenous ROS production and antioxidant gene expression of *S. aureus* (A) Representative EPR spectroscopic traces of *S. aureus* in different growth stages of batch culture. ROS was detected using the spin probe, CMH. (n=3) ***(B)*** The fold-change in the expression of *sodA, sodM, ahpC* and *katA* were determined over time following growth in TSBG and TSBG buffered with MOPS at pH 7.4. The fold-change expression values were determined relative to the expression of these target genes in WT strain at 2h of growth (n=3, Mean ± SEM).

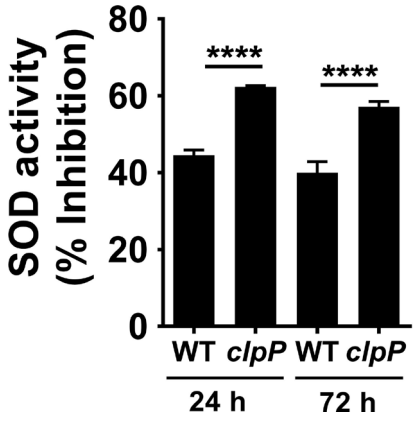

Fig. S7. SOD activity following growth of *S. aureus* in MOPS buffered TSB-G media, pH 7.4. SOD activity of cell extracts at 24 h and 72 h were determined as the percent inhibition of WST-1 reduction by superoxide using a commercially available kit (Sigma). WST-1, water-soluble tetrazolium salt (n=3, Mean ± SD).

Table S1. Stationary phase death rates ($k_{max}^{\ddagger}$)

Figure	Strain	k_{max}	Significance	Regression model
Fig 1C	WT in TSB-G	0.193 ± 0.015	****	Log-linear regression
	WT in TSB-G; MOPS	0.002 ± 0.0001		
Fig 2C	WT	0.159 ± 0.017	ns	Log-linear regression
	clpP	0.066 ± 0.001		
	clpP comp	0.175 ± 0.027		
Fig 2D	WT	0.183 ± 0.015	****	Log-linear regression
	clpP	0.066 ± 0.005		
	clpC	0.146 ± 0.005	**	
	clpX	0.076 ± 0.005	****	
Fig 2E	WT	0.173 ± 0.230	**	Log-linear regression
	clpX$_{I265E}$	0.083 ± 0.005	ns	
	clpX$_{I265E}$ comp	0.166 ± 0.025		
Fig 4C	WT	0.217 ± 0.028	**	Log-linear regression
	clpP	0.125 ± 0.023	ns	
	sodA	0.235 ± 0.023	ns	
	clpPsodA	0.240 ± 0.031		
Fig 4D	WT	0.183 ± 0.025	****	Log-linear regression
	clpP	0.066 ± 0.003	*	
	sodM	0.140 ± 0.010	****	
	clpPsodM	0.070 ± 0.010		
Fig 4G	WT	0.185 ± 0.010	****	Log-linear regression
	clpP	0.079 ± 0.010	ns	
	ahpC	0.207 ± 0.023	**	
	clpPahpC	0.135 ± 0.016		
Fig 4H	WT	0.185 ± 0.010	****	Log-linear regression
	clpP	0.079 ± 0.010	ns	
	katA	0.178 ± 0.008	ns	
	clpPkatA	0.072 ± 0.013		

‡ k_{max} values were calculated using GInaFiT (9)
Statistical significance for Fig 1C was determined using two-tailed Student's *t*-test.
Statistical significance for Fig 2C-4H was determined using one-way ANOVA and Tukey's multiple comparisons test (* p≤0.05, ** p≤0.01, *** p≤0.001, **** p<0.0001).

Table S2. Stationary phase death rates (k_{max} ‡) following antibiotic treatment

Antibiotic	Strain	k_{max}	Significance	Regression model
Kanamycin	WT	0.223 ± 0.011	**	Log-linear + shoulder
	clpP	0.175 ± 0.013		
Gentamicin	WT	0.223 ± 0.011	*	Log-linear + shoulder
	clpP	0.176 ± 0.015		
Erythromycin	WT	0.049 ± 0.017	ns	Log-linear regression
	clpP	0.028 ± 0.002		
Trimethoprim	WT	0.032 ± 0.001	*	Log-linear regression
	clpP	0.019 ± 0.007		
Tetracycline	WT	0.025 ± 0.005	ns	Log-linear regression
	clpP	0.027 ± 0.001		
Chloramphenicol	WT	0.086 ± 0.015	*	Log-linear + shoulder
	clpP	0.120 ± 0.010		
Vancomycin	WT	0.040 ± 0.0003	ns	Log-linear regression
	clpP	0.040 ± 0.004		
Oxacillin	WT	0.096 ± 0.015	ns	Log-linear + shoulder
	clpP	0.083 ± 0.005		

‡ k_{max} values were calculated using GInaFiT (9)
Statistical significance for Fig 1C was determined using two-tailed Student's *t*-test.
Statistical significance for Fig 2C-4H was determined using one-way ANOVA and Tukey's multiple comparisons test (* p≤0.05, ** p≤0.01, *** p≤0.001, **** p<0.0001).

Table S3. Strains and plasmid used in this study

Strain	Description	Source
***S. aureus* JE2**	*S. aureus* USA300 LAC 13C cured of endogenous plasmids	(1)
JE2 *clpP*	*bursa aurealis* transposon mutant, Erm^R	(1)
JE2 *clpC*	*bursa aurealis* transposon mutant, Erm^R	(1)
JE2 Δ*clpX*	*clpX* in-frame deletion, Erm^R	(10)
JE2 *clpP*::*clpP* (*clpP* comp)	Chromosomal complementation (SaPI site) of *clpP* mutant, $CdCl_2$ and Erm^R	(11)
JE2 $clpX_{I265E}$	JE2 expressing $ClpX_{I265E}$ variant, Erm^R	(2)
JE2 $clpX_{I265E}$::*clpX* (*clpX* comp)	Chromosomal complementation of JE2 $ClpX_{I265E}$ variant, $CdCl_2^R$, Erm^R	This study
JE2 *sodA*	*bursa aurealis* transposon mutant, Erm^R	(1)
JE2 *sodM*	*bursa aurealis* transposon mutant, Erm^R	(1)
JE2 *katA*	*bursa aurealis* transposon mutant, Erm^R	(1)
JE2 *ahpC*	*bursa aurealis* transposon mutant, Erm^R	(1)
JE2 *clpPsodA*	*bursa aurealis* transposon mutant, Tet^R and Erm^R	This study
JE2 *clpPsodM*	*bursa aurealis* transposon mutant, Tet^R and Erm^R	This study
JE2 *clpPkatA*	*bursa aurealis* transposon mutant, Tet^R and Erm^R	This study
JE2 *clpPahpC*	*bursa aurealis* transposon mutant, Tet^R and Erm^R	This study

Plasmid	Description	Source
pAQ21	SapI integrating vector carrying *clpX* gene driven by its native promoter	(2)

Table S4. Primers used in this study

Primer name	Sequence 5'-3'	Source
SaPI1_fwd	gtgcttcaccagcaccacatgctg	(11)
pJC1111_fwd2	ggtattagtttgagctgtcttggttcattgattgc	
ahpC_fwd	tgcttgcaggaaacttgacg	This study
ahpC_rev	aaccaaggctggcaacgaat	
kat_fwd	gtcccaattgcaccacctat	This study
kat_rev	atcgccacattctgtgcatg	
sodA_fwd	gttgaaccagtcactgcttg	This study
sodA_rev	acctcttggcacagactcat	
sodM_fwd	acaatgtacgctacgctgct	This study
sodM_rev	acaccttgtagatgctccac	
clpP_F	ttcctacagttattgaaacaac	This study
clpP_R	tgcagttaagaagttatcacg	
clpC_F	ggtagattaactgagcgtgc	This study
clpC_R2	cgctcttcaaattcaccacg	
RTPCR spx_F	cctggcttattacgtcgtcca	This study
RTPCR spx_R	accatacgttgtgcttcttgt	
RTPCR clpP_F	acaagcgcaagactcagaga	This study
RTPCR clpP_R	ccattgatgcagccataccg	
RTPCR clpX_F	ttggtggtgcctttgatggt	This study
RTPCR clpX_R	ggcttgcaaatcttctgggc	
RTPCR sodA_F	gttcaggttgggcttggtta	This study
RTPCR sodA_R	gcgtgttcccatacgtcttaaa	
RTPCR ahpC_F	attaacgctgacggaattgg	This study
RTPCR ahpC_R	tcccatttagctgggcatac	
JE2 RT sigA_F	aactgaatccaagtgatcttagtg	This study
JE2 RT sigA_R	tcatcaccttgttcaatacgtttg	

Dataset S1 (separate file). Proteomic dataset of USA300 JE2 (WT) at 72 h relative to 24 h of growth in TSB-G.

Dataset S2 (separate file). Proteomic dataset of *clpP* mutant at 72 h relative to 24 h of growth in TSB-G.

Dataset S3 (separate file). Proteomic dataset of *clpP* mutant at 72 h relative to WT at 72 h of growth in TSB-G.

Dataset S4 (separate file). Proteomic dataset of USA300 JE2 (WT) at 72 h relative to 24 h of growth in TSB-G supplemented with 100mM MOPS, pH7.4.

Dataset S5 (separate file). Proteomic dataset of *clpP* mutant at 72 h relative to 24 h of growth in TSB-G supplemented with 100 mM MOPS, pH, 7.4.

Dataset S6 (separate file). Proteomic dataset of *clpP* mutant at 72 h relative to WT at 72 h of growth in TSB-G supplemented with 100mM MOPS, pH, 7.4.

Dataset S7 (separate file). Reaction level differences in cell metabolism for WT and *clpP* mutant.

SI References

1. Fey PD*, et al.* (2013) A genetic resource for rapid and comprehensive phenotype screening of nonessential *Staphylococcus aureus* genes. *mBio* 4(1):e00537-00512.
2. Stahlhut SG*, et al.* (2017) The ClpXP protease is dispensable for degradation of unfolded proteins in *Staphylococcus aureus*. *Sci Rep* 7(1):11739.
3. Zalis EA*, et al.* (2019) Stochastic variation in expression of the tricarboxylic acid cycle produces persister cells. *mBio* 10(5).
4. Engman J & von Wachenfeldt C (2015) Regulated protein aggregation: a mechanism to control the activity of the ClpXP adaptor protein YjbH. *Mol Microbiol* 95(1):51-63.
5. Levine RL*, et al.* (1990) Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* 186:464-478.
6. Schmittgen TD & Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc* 3(6):1101-1108.
7. Schagger H (2006) Tricine-SDS-PAGE. *Nat Protoc* 1(1):16-22.
8. Nakano MM, Hajarizadeh F, Zhu Y, & Zuber P (2001) Loss-of-function mutations in *yjbD* result in ClpX- and ClpP-independent competence development of *Bacillus subtilis*. *Mol Microbiol* 42(2):383-394.
9. Geeraerd AH, Valdramidis VP, & Van Impe JF (2005) GInaFiT, a freeware tool to assess non-log-linear microbial survivor curves. *Int J Food Microbiol* 102(1):95-105.
10. Baek KT*, et al.* (2014) beta-Lactam resistance in methicillin-resistant *Staphylococcus aureus* USA300 is increased by inactivation of the ClpXP protease. *Antimicrob Agents Chemother* 58(8):4593-4603.
11. Chen J, Yoong P, Ram G, Torres VJ, & Novick RP (2014) Single-copy vectors for integration at the SaPI1 attachment site for *Staphylococcus aureus*. *Plasmid* 76:1-7.