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β-Lactam Resistance in Methicillin-Resistant *Staphylococcus aureus* USA300 Is Increased by Inactivation of the ClpXP Protease

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Methicillin-resistant *Staphylococcus aureus* (MRSA) has acquired the *mecA* gene encoding a peptidoglycan transpeptidase, penicillin binding protein 2a (PBP2a), which has decreased affinity for β-lactams. Quickly spreading and highly virulent community-acquired (CA) MRSA strains recently emerged as a frequent cause of infection in individuals without exposure to the health care system. In this study, we found that the inactivation of the components of the ClpXP protease substantially increased the β-lactam resistance level of a CA-MRSA USA300 strain, suggesting that the proteolytic activity of ClpXP controls one or more pathways modulating β-lactam resistance. These pathways do not involve the control of *mecA* expression, as the cellular levels of PBP2a were unaltered in the *clp* mutants. An analysis of the cell envelope properties of the *clpX* and *clpP* mutants revealed a number of distinct phenotypes that may contribute to the enhanced β-lactam tolerance. Both mutants displayed significantly thicker cell walls, increased peptidoglycan cross-linking, and altered composition of monomeric muropeptide species compared to those of the wild types. Moreover, changes in Sle1-mediated peptidoglycan hydrolysis and altered processing of the major autolysin Atl were observed in the *clp* mutants. In conclusion, the results presented here point to an important role for the ClpXP protease in controlling cell wall metabolism and add novel insights into the molecular factors that determine strain-dependent β-lactam resistance.

The rapid spread of methicillin-resistant *Staphylococcus aureus* (MRSA) has made the treatment of staphylococcal infections increasingly difficult (1, 2). Community-acquired (CA) MRSA strains of the USA300 type cause particular concern because of their frequent isolation and the severity of infection they cause (3). Methicillin and other β-lactam antibiotics inhibit the growth of *S. aureus* by covalently binding to the transpeptidase domain of penicillin binding proteins (PBPs), which cross-link the polypeptide chains of the cell wall component peptidoglycan. The resistance of MRSA strains is caused by the acquisition of the *mecA* gene encoding the alternative transpeptidase penicillin binding protein 2a (PBP2a), with very low affinity for almost all β-lactam antibiotics (4–7). Recently, anti-MRSA β-lactams, such as ceftaroline and ceftobiprole, with stronger binding to PBP2a, have been discovered.

Clinical MRSA isolates exhibit highly variable resistance levels toward methicillin, with MICs ranging from <3 μg/ml (comparable to those of susceptible strains) to 1,600 μg/ml in highly resistant strains (8). The mechanisms underlying this variation remain poorly understood, but the lack of correlation between the resistance level and the level of *mecA* expression suggests that factors other than PBP2a modulate the strain-specific level of β-lactam resistance (8–11). Indeed, genetic screens have identified a number of auxiliary factors in addition to PBP2a that are critical for resistance to β-lactam antibiotics (12, 13). Examples include cell division proteins, endogenous PBPs, and enzymes involved in the synthesis of teichoic acids and peptidoglycan precursors (5, 14–21). Intriguingly, the realization that β-lactam resistance depends on auxiliary factors opens up new possibilities for the treatment of MRSA infections, as drugs that inhibit the functions of auxiliary factors are predicted and have been shown to work synergistically with β-lactams to kill MRSA (22, 23).

Intracellular proteolysis carried out by energy-dependent proteases is one of the most conserved biological processes. During the infection process, bacterial pathogens depend on energy-dependent proteases for both the general turnover of damaged and nonfunctional proteins and the degradation of short-lived regulatory proteins (24). Accordingly, the highly conserved ClpXP protease is essential for the virulence of *S. aureus* in both systemic and abscess models of infection (25, 26), and it has also been implicated in the virulence of other pathogens, such as *Listeria monocytogenes*, *Salmonella*, and *Bacillus anthracis* (24, 27). The ClpXP protease is composed of proteolytic and ATPase subunits. Fourteen ClpP subunits constitute a proteolytic chamber that is accessible only through a narrow pore, which prevents the entrance of native folded proteins (reviewed in reference 24). ClpX serves to specifically recognize, unfold, and translocate substrates into the ClpP proteolytic chamber. ClpX belongs to the family of closely related Clp ATPases and can also function independently of ClpP as a molecular chaperone (28). The treatment of MRSA infections with daptomycin *in vivo* or vancomycin *in vitro* has been shown to select for mutants that carry loss-of-function mutations in *clpX* or *clpP*, suggesting that ClpX and ClpP are involved in the response of *S. aureus* to antibiotics that are active against the cell wall (29–31). This finding prompted us to investigate if inactivating the components of the ClpXP protease modulates the susceptibility of *S. aureus* to antibiotics targeting the cell wall. We

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TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Genotype/description	Source or reference
S. aureus strains		
8325-4	MSSA strain cured of prophages	S. Foster, University of Sheffield, 73
HI2209	8325-4Δ*clpX*	25
HI2300	8325-4Δ*clpP*	25
HI2304	8325-4Δ*clpC*	42
SA564	Low-passage clinical isolate	74
HI2781	SA564Δ*clpX*	36
HI2726	SA564Δ*clpP*	64
JE2	CA-MRSA strain USA300 LAC cured of plasmids	NARSA (http://www.narsa.net) (75)
HI3393	JE2 Δ*clpX*	This study
NE912	JE2 *clpP*::ΦNΣ	Nebraska Transposon Mutant Library (http://www.narsa.net)
KB1399	JE2 *clpP*::ΦNΣ, transduced from NE912	This study
NE699	JE2 *clpC*::ΦNΣ	Nebraska Transposon Mutant Library (http://www.narsa.net)
COL	Hospital-acquired MRSA strain	76
HI3469	COLΔ*clpX*	This study
HI2570	COLΔ*clpP*	64
RN4220	Restriction-deficient derivative of strain 8325-4	R. Novick, New York University
KB1257	8325-4Δ*clpX ermB*	This study
ANG406	SEJ1Δ*atl*	39
SEJ1	RN4220Δ*spa*	77
ATCC 29213	Control strain for MIC testing	
Plasmids		
pBT2	Allelic exchange vector	33
pAM367	Plasmid containing *ermB* marker on BamHI fragment	W. L. Kelley and A. Monod (University of Geneva), unpublished data
pKB1221	Plasmid to insert *ermB* near *clpX*	This study

found that inactivating *clpX* or *clpP* increased the level of resistance to β-lactam antibiotics in a CA-MRSA USA300 strain and at the same time affected a number of cell envelope properties associated with resistance.

MATERIALS AND METHODS

Strains and culture conditions. *S. aureus* strains (Table 1) were cultured in tryptic soy broth (TSB) (Oxoid) at 37°C with aeration. *S. aureus* strain JE2-derived strains were obtained from the Network of Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) program (supported under NIAID/NIH contract HHSN272200700055C).

Construction of *S. aureus* mutants. (i) USA300 JE2 and COL Δ*clpX* mutants. The *clpX* gene was deleted in *S. aureus* strains JE2 and COL by transduction with bacteriophage Φ11, using an *ermB*-tagged *S. aureus* 8325-4Δ*clpX* mutant (KB1257) as the donor strain for the transduction. KB1257 was constructed by inserting an *ermB* marker 8 kb from *clpX* between the convergently transcribed genes with locus tags SAOUHSC_1768 and SAOUHSC_1769. The *ermB* marker was inserted into the chromosome as follows: first, two PCR fragments were amplified from strain 8325-4 using the primer pairs KB65F (5′-CATTTCCATTTGCGGTACCTCC-3′) and KB65R (5′-GCATTAACTTGGATCCTTAACTTATG-3′), and KB66F (5′-CATAAGTTAAGGATCCAAGTTAATGC-3′) and KB67R (5′-TTAGGTCTGCAGATCGGATCAAC-3′) for the upstream and downstream fragments, respectively. The primers KB65R and KB66F are overlapping and contain substitutions that introduce a BamHI restriction site. The PCR fragments were joined by the splicing-by-overlap-extension PCR method (32) using KB65F, introducing a KpnI site, and KB67R, introducing a PstI site, and the resulting fragment was cloned into the KpnI and PstI sites of the allelic exchange vector pBT2 (33), resulting in plasmid pKB1217. A BamHI fragment containing *ermB* was excised from the plasmid pAM367 (W. L. Kelley, personal communication) and cloned into the BamHI site of pKB1217. The resulting plasmid, pKB1221, was then transformed into *S. aureus* strain RN4220 and subsequently introduced into 8325-4. The resulting strain was grown in the presence of erythromycin at the nonpermissive temperature for plasmid replication to select for double crossover and insertion of the *ermB* marker into the chromosome, as well as loss of the plasmid, as described previously (34). A plasmid-cured resistant mutant was selected (KB1239), and the presence of the *ermB* marker was verified by PCR with the primer pair KB76F (5′-GTTTCATTTCCATTTGCTATACCTCC-3′) and KB76R (5′-TATTCATCGCACGTATTACTTCCA-3′). The *ermB* marker was introduced into strain 8325-4Δ*clpX* by transduction using bacteriophage Φ11, yielding strain KB1244 containing *ermB* and the *clpX* deletion. From this strain, Δ*clpX* and the *ermB* marker were cotransduced into wild-type 8325-4, giving rise to KB1257, the donor strain for the transduction of the Δ*clpX-ermB* region into strains JE2 and COL.

(ii) JE2 *clpP*::ΦNΣ. The *clpP* gene disrupted by the erythromycin resistance-coding transposon, ΦNΣ, was moved by transduction with bacteriophage Φ11 from NE912 into a clean JE2 wild-type background, yielding strain *S. aureus* KB1399.

Susceptibility testing. Etest strips (bioMérieux) were used to determine the MICs of ertapenem, daptomycin, and vancomycin for all strains, as well as those of other β-lactams (except ceftaroline and ceftobiprole) for the 8325-4, SA564, and JE2 strains. Broth microdilution assays were used according to the guidelines of the Clinical and Laboratory Standards Institute (35) to determine the MICs of oxacillin, imipenem, cefoxitin, and cefuroxime (all from Sigma) for the COL strains. The Trek Sensititre Vizion broth microdilution system (Thermo Fisher Scientific) was used to determine the MICs of linezolid, ceftaroline, and ceftobiprole for all strains. *S. aureus* strain ATCC 29213 was included as a reference in all MIC determinations.

Western blotting. *S. aureus* cultures were grown in TSB at 37°C. At an optical density at 600 nm (OD_{600}) of 0.5, the cultures were split, and 16 μg/ml oxacillin was added to one of the culture aliquots. All cultures were grown for an additional 30 min. The cells were harvested by centrifugation at 5,000 × *g*, the cellular extracts were prepared by lysostaphin treatment, and the volumes normalized to the optical density of the initial culture

TABLE 2 Antibiotic susceptibility of *clpX*, *clpP*, and *clpC* mutants in different *S. aureus* strain backgrounds

	MIC (mg/liter) for indicated *S. aureus* strain or variant:													
	8325-4				SA564			JE2				COL		
Antibiotic	WT[a]	Δ*clpX*	Δ*clpP*	Δ*clpC*	WT	Δ*clpX*	Δ*clpP*	WT	Δ*clpX*	*clpP*::ΦNΣ	*clpC*::ΦNΣ	WT	Δ*clpX*	Δ*clpP*
Oxacillin	0.19–0.25	0.38–0.5	1	0.25	0.38–0.5	1.5–4	0.75	32–48	192–256	>256	48	256	256	256
Ertapenem	0.19	0.19	0.19	0.125	0.19	0.25	0.19	0.5–0.75	2–3	16–24	0.5–0.75	>32	>32	>32
Imipenem	0.047	0.047	0.047	0.064	0.064	0.064	0.094	0.125	0.5–0.75	3–4	0.13–0.19	32	16	16
Cefoxitin	3	3	3	3	4	4	4	24	32–256	>256	24–32	256	256	256
Cefuroxime	1.5	1.5	1.5	1.5	3	3	1.5	24	>256	>256	24–32	2,048	2,048	2,048
Ceftaroline	0.25	0.25	0.25	0.25	0.25	0.25	0.5	0.5	1	1	0.5	2	2	2
Ceftobiprole	0.25	0.5	0.5	0.5	0.5	0.5	0.5	1	2	2	1	2	2	2
Linezolid	2	2	<1	2	4	4	2	2	2	2	2	2	2	2
Vancomycin	1.5	1.5	1.5	1.5	1.5	1	1.5	1	1.5	1.5	1.5	1.5	3	1.5
Daptomycin	0.38–0.75	0.75–1	0.38–0.5	0.38	0.125–0.25	0.38	0.25–0.38	0.19–0.38	0.38	0.5–0.75	0.25	1	1.5	1

[a] WT, wild type.

were loaded onto 4 to 12% SDS-PAGE gels (NuPAGE). Western blotting was performed as previously described (36) using an antibody against PBP2a (37). The representative blots from three independent experiments are shown (see Fig. 2). In the first experiment, a range of different oxacillin concentrations, from 4 to 256 μg/ml, was tested without any difference in the observed PBP2a levels (data not shown), and 16 μg/ml was chosen as the concentration for all further experiments.

Population analysis profiles. Antibiotic resistance profiles were determined by plating appropriate dilutions of an overnight culture on tryptic soy agar plates containing increasing concentrations of oxacillin, meropenem, or cefoxitin (all from Sigma), or on brain heart infusion agar (Oxoid) plates containing 50 μg/ml $CaCl_2$ and increasing daptomycin (Sigma) concentrations, as described previously (38). The plates were incubated at 37°C, and the number of CFU per ml was determined after 48 h.

Autolytic activity. An analysis of Triton X-100-induced autolysis was performed as described previously (39). In short, overnight cultures were diluted into fresh TSB medium and grown to an OD_{600} of 1. The cells were harvested at 8,000 × *g* and washed twice in 10 mM sodium phosphate buffer (pH 7.0) and twice in ice-cold double-distilled water (ddH_2O), before being suspended in 10 mM sodium phosphate buffer (pH 7.0) containing 0.05% (vol/vol) Triton X-100. The suspensions were incubated at 37°C and autolysis was monitored by measuring the OD_{600} values at the indicated time points.

Zymographic analyses were performed essentially as described previously (39). Cultures were grown and harvested as described above, and the pellet was suspended in SDS sample buffer and boiled for 20 min. The boiled samples were centrifuged at 17,000 × *g*, and volumes of supernatant normalized to the optical density of the initial culture were loaded onto 7.5% SDS-PAGE gels containing 3% (wt/vol) heat-killed *S. aureus* SA564 cells. The gels were washed twice in ddH_2O before overnight incubation in 0.2 M phosphate buffer (pH 7.0) at 37°C. The gels were subsequently stained with 0.1% methylene blue.

Electron microscopy and image analysis. Overnight cultures of *S. aureus* strains SA564, SA564Δ*clpX*, SA564Δ*clpP*, JE2, JE2Δ*clpX*, and JE2*clpP*::ΦNΣ were diluted 1:200 into 40 ml of fresh TSB and grown at 37°C to an OD_{600} of 1.0. Bacteria from a 10-ml culture aliquot were collected by centrifugation at 8,000 × *g*, and the cell pellets were suspended in fixation solution (2.5% glutaraldehyde in 0.1 M cacodylate buffer [pH 7.4]) and incubated overnight at 4°C. The fixed cells were further treated with 2% osmium tetroxide, followed by 0.25% uranyl acetate for contrast enhancement. The pellets were dehydrated in increasing concentrations of ethanol, followed by pure propylene oxide, and then embedded in Epon resin. Thin sections for electron microscopy were stained with lead citrate and observed in a Philips CM100 BioTWIN transmission electron microscope fitted with an Olympus Veleta camera with a resolution of 2,048 by 2,048 pixels. For quantitative analysis, the images were acquired in an unbiased fashion by using the multiple image alignment function in the ITEM software (Olympus). Sample processing and microscopy were performed at the Center for Integrated Microscopy, Faculty of Health and Medical Sciences, University of Copenhagen.

An analysis of cell size was performed in Fiji (ImageJ) by running a script that automatically measures the area of the particles that fit predefined criteria on circularity and minimum size. The lower cutoff for size was chosen to be 40% of the average of the 10 largest particles in each frame. At least 300 cells were measured in each strain. Transmission electron microscopy (TEM) shows only a thin two-dimensional slice of the cells, and the measured areas will therefore show considerable variability in size, and the mean area size will be an underestimate of the true cell size. Cell wall thickness was measured in the ITEM software (Olympus). At least 25 cells distributed on ≥3 image frames were measured, and 3 to 5 measurements of cell wall thickness were performed on each cell. Student's two-sample *t* test was used to calculate statistical significance between the mutants and their cognate wild types.

Muropeptide analysis by HPLC. One liter TSB medium was inoculated with overnight cultures of *S. aureus* strains SA564, SA564Δ*clpX*, SA564Δ*clpP*, JE2, JE2Δ*clpX*, and JE2*clpP*::ΦNΣ to an OD_{600} of 0.06 and grown at 37°C to late-log phase (OD_{600}, approximately 1.5). The cultures were then cooled on ice and the bacteria were subsequently collected by centrifugation. Peptidoglycan was purified, digested with mutanolysin, and analyzed by high-performance liquid chromatography (HPLC), as described previously (39). Muropeptide profiles were determined for each strain in duplicate, and HPLC chromatograms from one representative experiment are shown (see Fig. 4). Data analysis was carried out in the software environment R, using the function package zoo for calculations of the area under the elution curve (40, 41).

RESULTS

Loss of ClpX or ClpP increases β-lactam resistance in CA-MRSA. To investigate if the ClpXP protease has an impact on the resistance to cell wall-active antibiotics, we inactivated *clpX* or *clpP* in four different *S. aureus* strains: 8325-4 (methicillin-sensitive *S. aureus* [MSSA], standard laboratory strain), SA564 (MSSA, low-passage clinical isolate), COL (MRSA, early clinical isolate), and USA300 JE2 (a plasmid-cured derivative of the CA-MRSA strain USA300 LAC). Susceptibilities to antibiotics acting on the cell wall (vancomycin, daptomycin, and β-lactam antibiotics) were tested by Etest or broth microdilution assays, and for comparison, the protein synthesis inhibitor linezolid was included. We found that while inactivation of *clpX* or *clpP* did not alter the susceptibility to linezolid or vancomycin, inactivation of *clpX* caused slightly in-

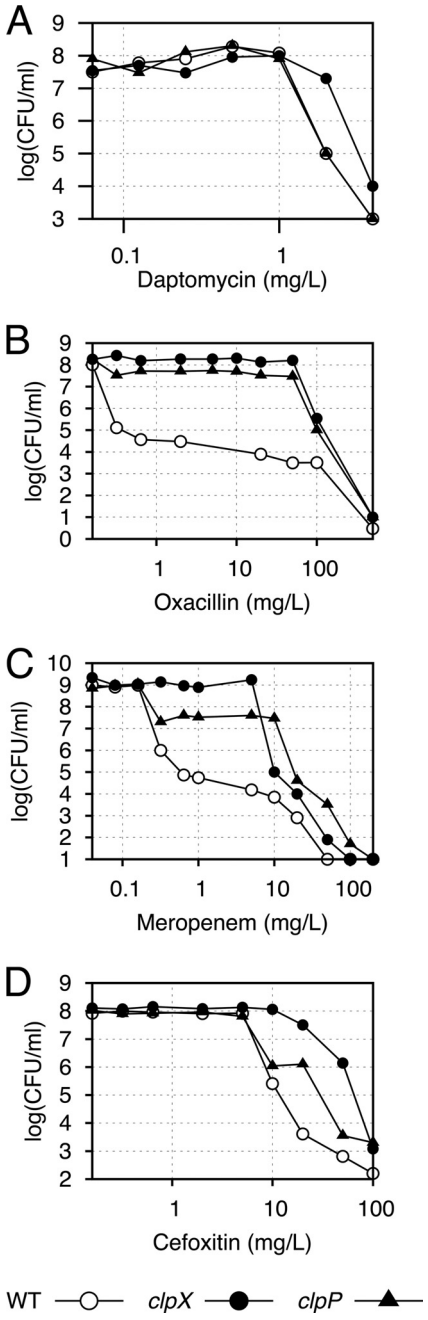


FIG 1 Population analysis profiles. JE2 wild type (WT), JE2Δ*clpX*, and JE2 *clpP*::ΦNΣ were plated on increasing concentrations of daptomycin (A), oxacillin (B), meropenem (C), and cefoxitin (D), as indicated. Representative data from three individual experiments are shown.

creased daptomycin MICs in all four strain backgrounds (Table 2). The putative role of ClpX in controlling daptomycin susceptibility was supported by the finding that the *clpX* (but not the *clpP*) deletion caused a susceptibility shift in population analyses (Fig. 1A). However, the most striking result was that the inactivation of *clpP* or *clpX* had a significant strain-dependent effect on the susceptibility to β-lactams: in the MSSA strains 8325-4 and SA564, disruption of *clpX* and *clpP* slightly decreased susceptibility to oxacillin but not to other β-lactams tested (Table 2). In the MRSA strain USA300 JE2, however, the disruption of *clpX* or *clpP* resulted in a drastic increase in the MICs of all tested β-lactams, except ceftaroline and ceftobiprole, which have high affinity for PBP2a (Table 2). In particular, the deletion of *clpP* had a substantial effect on β-lactam resistance, with a 32-fold increase in the MICs of ertapenem and imipenem and a >10-fold increase in the MICs of cefoxitin and cefuroxime. The effect of deleting *clpX* was less pronounced, with around 5-fold increases in the MICs of oxacillin, ertapenem, imipenem, and cefoxitin, and a >10-fold increase in the MIC of cefuroxime. In *S. aureus*, ClpP can associate with an alternative substrate recognition factor, ClpC (42). The observation that the lack of ClpP had a greater effect on the resistance level than did the lack of ClpX might indicate that ClpP controls resistance partly via ClpC. However, this does not seem to be the case, as the inactivation of *clpC* did not change the MICs of any of the tested antibiotics (Table 2). USA300 strains exhibit a relatively low level of resistance compared to that of other MRSA strains. COL is an example of a highly resistant MRSA strain, and in this background, we did not see an effect of the *clp* mutations on the resistance level to any of the tested antibiotics (Table 2). We conclude that ClpX and ClpP control one or more pathways that determine the β-lactam resistance level of the clinically important CA-MRSA clone USA300 but not in the highly resistant COL strain.

Inactivation of *clpX* or *clpP* converts USA300 from a heterogeneously resistant to a homogeneously resistant MRSA strain without affecting PBP2a expression. Cultures of CA-MRSA strains often display heterogeneity with respect to β-lactam susceptibility, meaning that the majority of cells exhibit a low level of antibiotic resistance, while a minority of cells are highly resistant (43). To assess if an MRSA strain is hetero- or homogeneously resistant, a population analysis profile (PAP) can be obtained by plating dilutions of a stationary culture on plates containing a wide range of antibiotic concentrations to determine the colony count at each concentration (38). In this analysis, JE2 exhibited a typical heterogeneous profile, with the majority of cells being killed by low concentrations of oxacillin, meropenem, and cefoxitin, while a small subpopulation survived much higher concentrations (Fig. 1). Strikingly, the deletion of *clpX* or *clpP* transformed JE2 into a homogeneously highly resistant strain. This effect of ClpXP inactivation was seen for all three β-lactam antibiotics but was most pronounced for oxacillin (Fig. 1). Although the β-lactam MIC values determined for the *clpP* mutant were higher than those for the *clpX* mutant (Table 2), the *clpP* mutant population showed some sensitivity to sub-MICs of meropenem and cefoxitin, compared to the *clpX* mutant, which showed a more homogeneous resistance pattern toward these antibiotics (Fig. 1). A simple explanation for the high homogenous β-lactam resistance in JE2 upon inactivation of ClpX or ClpP is that the cellular amount of PBP2a is increased. However, no differences in PBP2a levels were observed between the wild-type JE2 strain and the *clp* mutants (Fig. 2), and we concluded that ClpXP influences β-lactam resistance independently of the level of PBP2a.

Loss of ClpX or ClpP causes substantial changes in the cell wall structure. The decreased susceptibilities of the *clp* mutants prompted us to investigate if the lack of ClpX or ClpP causes changes in the cell envelope structure. To this end, wild-type and mutant SA564 and JE2 strains were analyzed by transmission electron microscopy (TEM) (Fig. 3). Measurements of cell wall thickness revealed that the cell walls of *clpX* and in particular *clpP* mu-

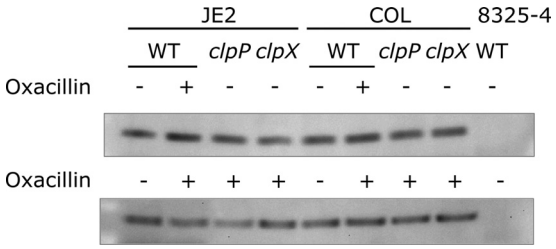
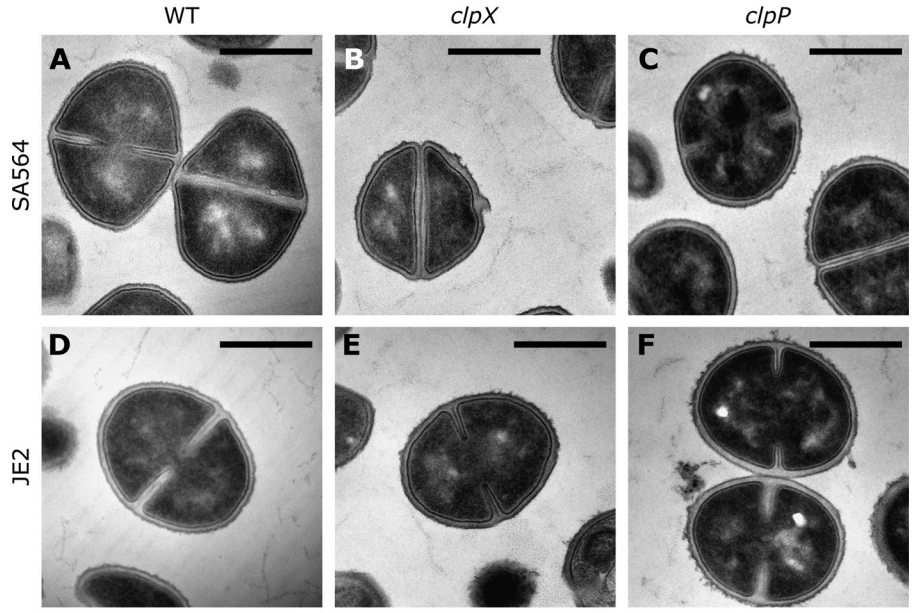


FIG 2 Cellular concentration of PBP2a in the presence (+) or absence (−) of inducer (16 mg/liter oxacillin) determined by Western blotting. PBP2a levels were similar in the wild type and *clp* mutants and in JE2 and COL. Strain 8325-4 is included as a strain not expressing PBP2a. Representative blots from three independent experiments are shown.

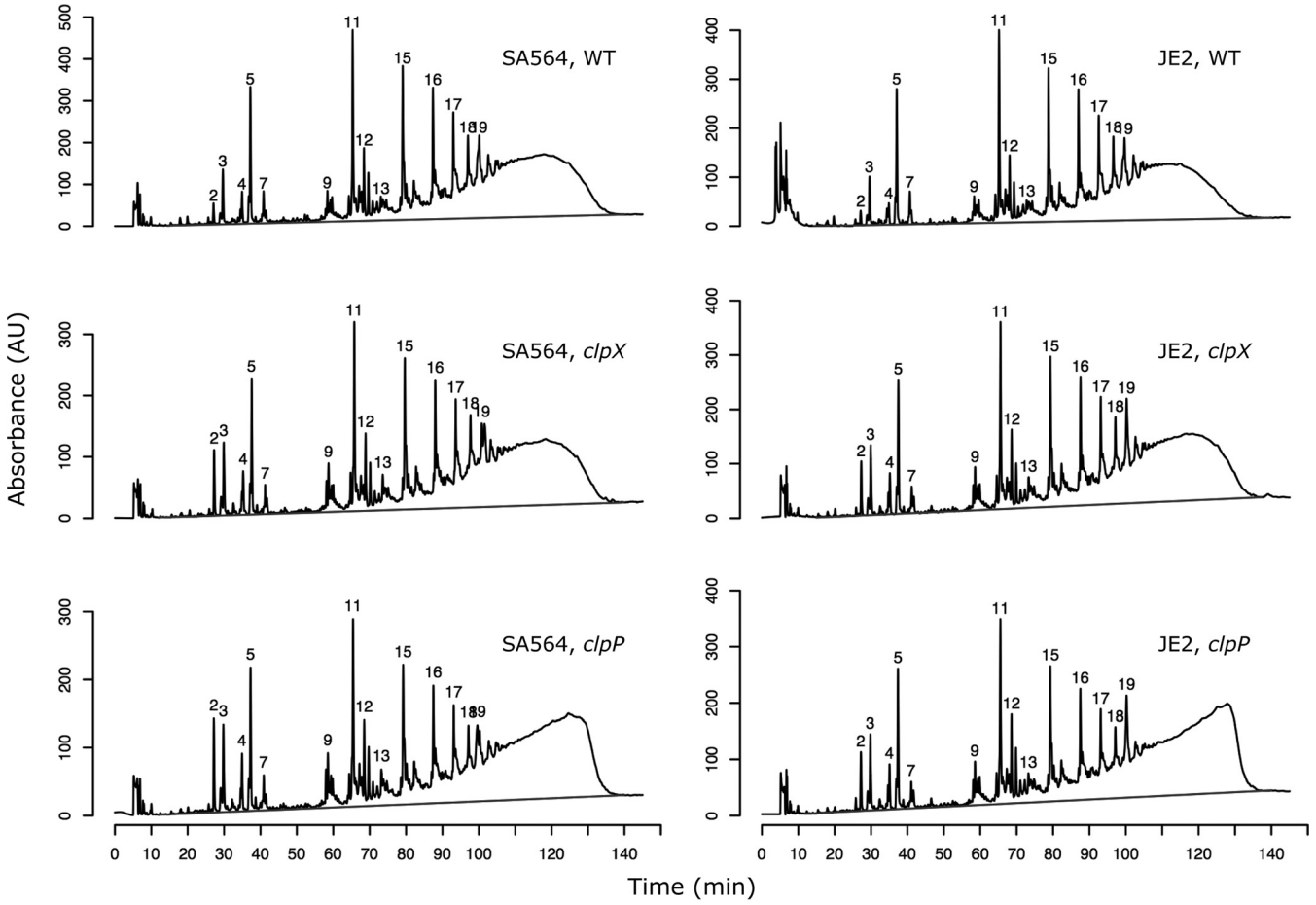
tant cells were thicker than the cell walls of the respective wild-type cells. We also noted that the outer edge of the cell wall appeared less distinct and fuzzier in the *clpX* and *clpP* mutants than in the wild-type cells (Fig. 3). Furthermore, quantitative analysis of the electron microscopy images revealed that the SA564 *clpX* and *clpP* cells were significantly smaller than the wild-type cells (Fig. 3). The smaller diameter of the SA564 *clpX* and *clpP* cells corresponds to a calculated average cell volume that is approximately half the volume of the wild-type cells. In the JE2 background, however, wild-type and *clpP* mutant cells were of approximately the same size, whereas only *clpX* mutants were slightly smaller (Fig. 3). In conclusion, the TEM analysis showed that the disruption of *clpX* or *clpP* led to increased cell wall thickness and changes in the appearance of the cell wall, regardless of the strain background.

To gain a deeper insight into the cell wall properties of the *clp* mutants, we purified the peptidoglycan from SA564, JE2, and their cognate *clpX* and *clpP* mutants, and determined the muropeptide composition by HPLC following mutanolysin digestion. A comparison of the HPLC profiles showed that the deletion of *clpP* caused a drastic change in the peptidoglycan compositions of both the MSSA and the MRSA strains (Fig. 4). A substantial increase in the abundance of muropeptides with long retention times was observed, indicating a hyper-cross-linked peptidoglycan layer for the *clpP* mutants in both the SA564 and JE2 strain backgrounds. As shown in Fig. 4, oligomeric muropeptides with a retention time of >104 min, corresponding to ≥9-mers, constituted approximately 57% of the peptidoglycan in the *clpP* mutants compared to approximately 48% in the wild-type strains. Even more highly cross-linked muropeptides with retention times of >120 min constituted up to three times more of the peptidoglycan in the cells that lacked ClpP. In contrast, there was a decrease in the abundance of trimeric to octameric muropeptides (retention times, 78 to 104 min) in the *clpP* mutants in both strain backgrounds. No effect was observed on the abundance of mono-



	Mean cell diameter (μm) ± SD	Mean cell wall thickness (nm) ± SD
SA564 wt	0.79 ± 0.09	33 ± 2.3
SA564 Δ*clpX*	0.64 ± 0.06 ($p < 0.001$)	38 ± 3.3 ($p < 0.001$)
SA564 Δ*clpP*	0.64 ± 0.08 ($p < 0.001$)	40 ± 3.5 ($p < 0.001$)
JE2 wt	0.75 ± 0.10	36 ± 2.5
JE2 Δ*clpX*	0.71 ± 0.08 ($p < 0.001$)	39 ± 4.0 ($p < 0.01$)
JE2 *clpP*::ϕNΣ	0.74 ± 0.08 ($p = 0.07$)	43 ± 3.4 ($p < 0.001$)

FIG 3 Representative TEM images of SA564 (A), SA564Δ*clpX* (B), SA564Δ*clpP* (C), JE2 (D), JE2Δ*clpX* (E), and JE2 *clpP*::ΦNΣ (F). The scale bar corresponds to 500 nm. The table shows the results of the measurements of cell diameter and cell wall thickness for various *S. aureus* strains and variants. *P* values show the results of Student's *t* tests between mutants and their cognate wild types.



	% Muropeptides				
	Monomers 19' - 54'	Dimers 54' - 78'	Trimers - 8mers 78' - 104'	9mers and above 104' - end	Highly crosslinked 120' - end
SA564 wt	5.9	10.9	31.2	51.5	19.3
SA564 Δ*clpX*	6.1	11.3	33.3	49.3	18.9
SA564 Δ*clpP*	6.6	10.6	27.0	55.8	29.1
JE2 wt	5.3	11.6	36.3	46.9	11.5
JE2 Δ*clpX*	5.3	10.7	31.7	52.3	18.9
JE2 *clpP*::ϕNΣ	6.0	10.7	26.8	56.6	30.7

FIG 4 Altered peptidoglycan structure in *clpX* and *clpP* mutants. HPLC chromatograms of mutanolysin-digested peptidoglycan purified from the indicated strains. The peak numbering is according to de Jonge et al. (44). The table shows the muropeptide composition expressed as a percentage of the total area under the curve for each strain. The dark grey lines indicate baselines used for calculations of area under the curves. Representative results of two individual experiments are shown.

meric (retention times, 19 to 54 min) or dimeric (retention times, 54 to 78 min) muropeptides. The deletion of *clpX* resulted in only slight alterations in the peptidoglycan composition; while no change in the level of cross-linking was observed in the SA564 strain background, a small increase in the concentrations of highly cross-linked muropeptides was seen in the *clpX* mutant in the JE2 background (Fig. 4).

In addition to the observed changes in the abundances of late eluting muropeptides, we noted differences in the relative abundances of the monomeric peaks. Thus, in both strain back-

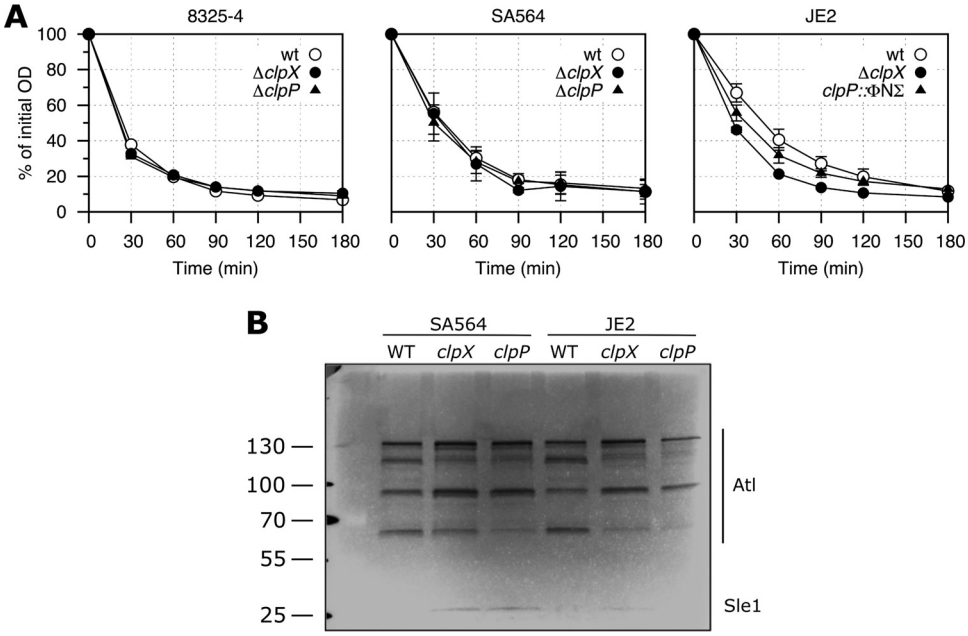


FIG 5 Effect of lack of ClpX or ClpP on autolytic activity. (A) Triton X-100-induced autolysis. Mean and standard deviation (SD) values of three independent experiments are shown for the various strains. (B) Zymogram showing cell wall-associated proteins run on an SDS gel containing heat-killed *S. aureus* SA564 wild-type cells. Representative results of three independent experiments are shown. The positions of the molecular mass standards are indicated on the left (in kilodaltons).

grounds, the peak eluting at 27.1 min was notably higher in the *clpX* and *clpP* mutants than in the wild-type strains, and the integration of the monomeric peaks showed that this peak was approximately three times more abundant relative to the total abundance of monomeric muropeptides in the *clpX* and *clpP* mutants than in the parental strains. Based on the relative retention times of the monomeric peaks, we suggest that this peak most likely corresponds to peak 2, using the numbering of de Jonge et al. (44), and to our knowledge, no chemical structure has been assigned to this peak.

Altered autolysin activity in the *clpX* and *clpP* mutants. Increased autolytic activity has been shown to correlate with increased β-lactam resistance (39, 45, 46); hence, we measured the rate of Triton X-100-induced autolysis in the *clpX* and *clpP* mutants. The deletion of *clpX* or *clpP* in the MSSA strain backgrounds had no effect on the autolytic rate (Fig. 5A), whereas a slight increase was observed for the *clpX* or *clpP* mutants in the JE2 strain background. We also examined if the *clp* mutations altered the amounts or activities of the autolytic enzymes by analyzing cell wall hydrolytic enzymes from wild-type and mutant cells on zymograms. As can be seen in Fig. 5B, the disruption of *clpX* or *clpP* in both SA564 and JE2 altered the intensities of several bands. The low-molecular-weight band was reported to be caused by the activity of the 32-kDa peptidoglycan hydrolase Sle1 (47). Interestingly, increased Sle1 activity was observed in extracts derived from the *clp* mutants, which is consistent with our recent finding that Sle1 is a substrate of ClpXP (48). The high-molecular-weight bands are due to the activity of the major autolysin Atl, as these bands are absent in extracts derived from an *atl* mutant strain (data not shown). Atl is a bifunctional murein hydrolase that is produced as a 138-kDa precursor protein, sequentially cleaved to generate 115- and 85-kDa intermediate products, and further processed to generate a 62-kDa *N*-acetylmuramyl-L-alanine amidase and a 51-kDa *N*-acetylglucosaminidase (49, 50). In both the wild type and the *clp* mutants, we identified Atl-specific bands of all the expected sizes, except the 51-kDa band. Notably, the 85-kDa Atl intermediate accumulated in extracts from the *clp* mutants, indicating that the processing of the 85-kDa product is slowed down in the mutants. ClpXP is an intracellular protease and therefore is likely to impact Atl processing indirectly via its effect on the expression of extracellular proteases (25). Alternatively, the association of the 85-kDa product with the cell wall is stronger in the *clp* mutants than in the wild type.

DISCUSSION

The ClpXP protease is essential for virulence of *S. aureus* (25, 26). The inhibition of ClpXP proteolytic activity has therefore been suggested as a potential antimicrobial therapeutic strategy, and compounds have recently been identified that target the activity of ClpXP (51, 52). Several findings, however, indicate that the role of ClpXP during infection may be complex in *S. aureus*. In one study, MRSA was isolated from a patient before and at different time points after the start of treatment with daptomycin. One of the mutations acquired in these clinical isolates was a loss-of-function mutation in *clpX* (30). Similarly, *S. aureus* strains selected for daptomycin or vancomycin resistance in the laboratory were shown to harbor mutations in *clpP* together with mutations in other genes, such as *walK* and *agrA* (29, 31). Thus, it seems that *S. aureus* may under some circumstances benefit from shutting down Clp proteolytic activity during treatment with antibiotics that target the cell wall.

The results of the present study emphasize the existence of a molecular link between the activity of the ClpXP protease and the susceptibility to antibiotics acting on the cell wall. First, we show that *S. aureus* strains belonging to the prevalent MRSA clone

USA300 can become highly β-lactam resistant if the ClpXP protease is inactivated. Second, the *clpX* mutants examined in our study were less sensitive to daptomycin in all tested strain backgrounds, suggesting that the ClpX chaperone controls pathways involved in daptomycin susceptibility independently of ClpP. The development of daptomycin nonsusceptibility is often accompanied by a concomitant fall in β-lactam resistance, a phenomenon designated the daptomycin/β-lactam "seesaw effect" (53, 54). Our results indicate, however, that *clpX* decreases daptomycin susceptibility by a mechanism not leading to the seesaw effect.

The increased resistance to β-lactam antibiotics in USA300 caused by the loss of ClpX or ClpP underscores the complexity of the expression of β-lactam resistance in MRSA. Only a few other genes have been reported to increase β-lactam resistance when inactivated (39, 55–57). Most notably, the disruption of *gdpP* increased resistance to penicillin as much as 32-fold in the USA300 strain LAC*, and it was also reported that the *gdpP* mutant strain shows an increase in cross-linked peptidoglycan and a decrease in cell size (39, 46, 58). *gdpP* encodes a phosphodiesterase that specifically cleaves cyclic diadenylate monophosphate (c-di-AMP), a newly discovered essential secondary messenger in *S. aureus* (39). In several studies, c-di-AMP has now been linked to cell wall properties and β-lactam resistance (59). Another nucleotide messenger, ppGpp, the effector molecule of the stringent response, was also shown to greatly influence the level of β-lactam resistance exhibited by MRSA strains (46, 60, 61). It may be speculated that ClpXP exerts its effect on β-lactam resistance, at least partly, via c-di-AMP or ppGpp; notably, we show that *clpX* and *clpP* mutants share some phenotypes with a *gdpP* mutant, namely, a decrease in cell size and an increase in peptidoglycan cross-linking, although the cross-linking is much more pronounced in the *clpP* mutants than in a *gdpP* mutant (39). Increased levels of c-di-AMP and ppGpp have been implicated in the conversion from low-level heterogeneous resistance to high-level homogeneous resistance in MRSA (46, 61). For ppGpp, the shift to homogeneous resistance was shown to be mediated via the increased expression of PBP2a (61). However, as we show here, this was not the case in the *clp* mutants. Furthermore, similar levels of PBP2a were observed in the JE2 and COL strains, consistent with previous observations that the strain-specific level of resistance is not associated with the cellular amount of PBP2a (8).

Both the structure and thickness of the cell wall were altered substantially by the loss of ClpX or ClpP, showing that the activities of ClpX and ClpP take part in controlling cell wall synthesis in *S. aureus*. In support of this conclusion, we recently identified several enzymes in the peptidoglycan-synthesis pathway, including GlmS, MurI, FemA, FemB, MurE, MurC, and PBP2, as potential substrates of the ClpXP or ClpCP protease (48). Interestingly, all of these factors have been identified as auxiliary factors that are required for β-lactam resistance (13), and it is conceivable that ClpP-mediated proteolysis may influence a number of enzymatic steps in this pathway. As β-lactams inhibit PBP activity by competitively binding to the active site, an increase in the substrate concentrations in *clpX* or *clpP* mutants would in theory increase the concentration of β-lactams required for growth inhibition and thereby increase the β-lactam resistance level.

The largest increase in β-lactam MICs was observed in the JE2Δ*clpP* mutant for imipenem and ertapenem preferentially binding to PBP1 (62). Interestingly, the inhibition of PBP1 can turn on the expression of major virulence factors, like the Panton-Valentine leukocidin (63). The underlying mechanism is unknown, but it has been hypothesized that alterations in the composition of peptidoglycan caused by PBP1 dysfunction may be compensated for by repressing the expression of autolytic genes, and this signal transduction pathway involves global transcriptional regulators, like SarA and Rot, which also control the expression of virulence regulation (63). We now speculate that there may be a causal link between the altered cell wall metabolism and the changed expression of a number of global regulators (including Agr, Rot, and MgrA) observed in cells devoid of ClpXP (36, 64).

Although the loss of ClpX or ClpP gave rise to higher β-lactam resistance, increased cell wall thickness, and an altered peptidoglycan structure, the phenotypes were much more pronounced in the *clpP* mutants than in the *clpX* mutants. Consequently, ClpX or ClpP must have additional roles independent of the other in modulating β-lactam resistance and cell wall properties in *S. aureus*. We ruled out the involvement of the ClpCP protease, as a *clpC* mutant did not show an increase in β-lactam resistance. However, it is possible that ClpCP and ClpXP have partially redundant functions such that the loss of both, which is the case in the *clpP* mutant, gives rise to more severe phenotypes than those caused by a loss of the ClpXP protease alone. Alternatively, the chaperone activity of ClpX may affect β-lactam resistance and cell wall structure properties in an opposite manner from the activity of ClpXP, resulting in an intermediate phenotype in the absence of ClpX. Consistent with this hypothesis is the ClpP-independent role of ClpX in stress response and gene regulation of *S. aureus* (25, 36).

The degree of cell wall changes in the *clpX* and *clpP* mutants, respectively, correlated with the level of β-lactam resistance in the USA300 background, indicating that the underlying pathways are linked in this strain. The increase in higher-order cross-linking combined with the concomitant decrease in lower-order cross-linking and the unchanged abundance of monomeric muropeptides in the *clp* mutants are in accordance with the random addition model for *S. aureus* peptidoglycan synthesis, in which oligomers are formed by random linkage between less-cross-linked muropeptides rather than by sequential addition of monomers (65). Higher-order peptidoglycan cross-linking in *S. aureus* is catalyzed by the monofunctional nonessential transpeptidase PBP4 (14, 66, 67), which in MRSA functions in concert with PBP2 and PBP2a to build highly cross-linked peptidoglycan (14). PBP4 has been shown to affect the β-lactam resistance levels in MRSA strains (19); however, the requirement for PBP4 in the expression of β-lactam resistance is again strain dependent, as PBP4 is required in the CA-MRSA strains USA300 and MW2 (19) but not in the hospital-acquired (HA)-MRSA strain COL (68). It may be hypothesized that ClpXP affects β-lactam resistance levels through the modulation of PBP4 activity, which correlates with the absence of an effect of deleting *clpXP* in COL on β-lactam resistance. Alternatively, the increased peptidoglycan cross-linking or altered muropeptide composition of the *clpXP* mutants may affect the activity or localization of PBP2a in MRSA such that it becomes less sensitive to β-lactams. This possibility is especially intriguing, given that PBP2a has an allosteric domain (69, 70) with which peptidoglycan can interact to change the affinity of β-lactams to the active site (69–71). It is therefore plausible that the ability of β-lactams to inhibit PBP2a is highly influenced by the altered peptidoglycan structure observed in the absence of ClpXP, whereas the β-lactam sensitivities of the MSSA PBPs are less af-

fected due to their lack of allosteric regulation (71, 72). The virtually unchanged resistance to ceftaroline and ceftobiprole that we found for the *clp* mutants may reflect the ability of these antibiotics to bind to the allosteric domain of PBP2a and thereby predispose it to β-lactam inhibition (71, 72).

In summary, we have shown that a lack of the ClpXP protease converts the quickly spreading heterogeneously resistant CA-MRSA clone USA300 into a homogeneously highly resistant strain, and this change is unrelated to the amount of PBP2a. We investigated the effect of inactivating *clpX* and *clpP* on cell wall appearance, cell size, peptidoglycan composition, and autolytic activity in both USA300 and an MSSA strain. Two major conclusions can be drawn: first, while β-lactam susceptibility is only very minimally decreased in *clp* mutants in MSSA strains, cell wall thickness, peptidoglycan composition, and autolysin activity are affected in both MRSA and MSSA strains. Second, since deleting *clpP* has a larger effect on β-lactam resistance, cell wall thickness, and peptidoglycan cross-linking than does deleting *clpX*, ClpX and ClpP have additional cellular functions, which contribute to the modulation of the cell wall properties in *S. aureus*.

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