Effect of clpP and clpC deletion on persister cell number in Staphylococcus aureus

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Staphylococcus aureus is responsible for a wide variety of infections that include superficial skin and soft tissue infections, septicemia, central nervous system infections, endocarditis, osteomyelitis and pneumonia. Others have demonstrated the importance of toxin–antitoxin (TA) modules in the formation of persisters and the role of the Clp proteolytic system in the regulation of these TA modules. This study was conducted to determine the effect of clpP and clpC deletion on S. aureus persister cell numbers following antibiotic treatment. Deletion of clpP resulted in a significant decrease in persister cells following treatment with oxacillin and erythromycin but not with levofloxacin and daptomycin. Deletion of clpC resulted in a decrease in persister cells following treatment with oxacillin. These differences were dependent on the antibiotic class and the CFU ml⁻¹ in which the cells were treated. Persistor revival assays for all the bacterial strains in these studies demonstrated a significant delay in resumption of growth characteristic of persister cells, indicating that the surviving organisms in this study were not likely due to spontaneous antibiotic resistance. Based on our results, ClpP and possibly ClpC play a role in persister cell formation or maintenance, and this effect is dependent on antibiotic class and the CFU ml⁻¹ or the growth phase of the cells.

INTRODUCTION

The opportunistic pathogen, Staphylococcus aureus, is one of the most frequent causes of a wide variety of hospital- and community-acquired infections. S. aureus infections range from superficial skin and soft tissue infections to life-threatening illnesses such as toxic shock, pneumonia, endocarditis and septicemia (Lowy, 1998; Shoji et al., 2011). It has been demonstrated in S. aureus and in several other bacterial species that treatment with high concentrations of antibiotics results in rapid death with a small surviving fraction of antibiotic-sensitive cells (Bigger, 1944; Keren et al., 2004a, b; Lewis, 2007; Möker et al., 2010). These surviving cells are bacterial cells that are in a dormant slow-growing or non-growing state protecting them from the toxic effects of antibiotics and other environmental hazards (Maisonneuve et al., 2011; Keren et al., 2004a, b; Lewis 2007). These antibiotic-sensitive surviving cells are called persister cells. Persister cells are not genetically different from antibiotic-sensitive cells, but they differ phenotypically (Levin, 2004).

Persistor cell studies in Escherichia coli have shown that few persisters are present during the early exponential growth phase. However, there is a sudden distinct increase in persisters during the mid-exponential growth phase (Lechner et al., 2012). This has also been demonstrated in S. aureus (Keren et al., 2004a). The number of persisters depends on the growth stage, and by maintaining a culture of S. aureus at the early exponential phase via reinoculation, persisters are eliminated. Therefore, persister cells do not appear to occur in response to antibiotics but are rather specialized survivor cells (Keren et al., 2004a).

The genetically identical nature of persister cells to normal non-tolerant cells suggests that persister formation may be attributed to epigenetic changes (Dhar & McKinney, 2007). The apparent lack of persister cells in early exponential phase may be due, in part, to low concentrations of persister proteins that may act as quorum-sensing molecules (Lewis, 2007). Others have shown that two processes may influence persister formation: a stochastic variation in the level of specific persister proteins and a controlled, regulated threshold.

Abbreviations: ADEP, acyldepsipeptide; CFU, colony-forming unit; TA, toxin–antitoxin; TSA, trypticase soy agar; TSB, trypticase soy broth.
level of these proteins which may be dependent on population density (Lewis, 2007; Tashiro et al., 2012).

Gene expression in persisters has been investigated through RNA profiling from isolated persister cells (Keren et al., 2004a). They found ~300 genes in E. coli that may function during persister formation (Keren et al., 2004b). These genes include the error prone global response to DNA damage also known as the SOS response genes (such as recA, sulA, umuDCA and umuDC), phage shock operon genes and several heat and cold shock genes (cspH, htrA, ilpAB, htpX and clpB) (Keren et al., 2004b). Additionally, among the ~300 genes identified that could possibly contribute to persister formation, a small set of approximately 2% of the genes belong to toxin–antitoxin (TA) modules (Keren et al., 2004b).

TA loci code for two components: a toxin that inhibits cell growth and an antitoxin that reversibly inactivates the toxin (Maisonneuve et al., 2011). In most cases, the toxins have longer half-lives than do the antitoxins. Reduction of the antitoxin concentration frees its toxin to interfere with or degrade a cellular target such as mRNA, DNA gyrase or DNA helicase (Donegan et al., 2010). A variety of phenotypes can be found through the activation of a toxin; however, phenotypes related to growth, stress response, starvation and persistence are often observed (Donegan et al., 2010). It has been shown that deletion of five or more of the 10 TA loci in exponentially growing E. coli cells resulted in a 100–200-fold decrease in persisters (Maisonneuve et al., 2011). Similar results were obtained when the deletions were made in the TA loci in the reverse order, suggesting that the functions they perform in E. coli are highly redundant (Maisonneuve et al., 2011).

At least three TA modules exist in S. aureus including mazEF, axe1-txe1 and axe2-txe2 (Donegan & Cheung, 2009; Yoshizumi et al., 2009). Donegan et al. (2010) examined the proteolytic regulation of TA systems by ClpPC in S. aureus and found that ClpP is essential for the degradation of all known S. aureus antitoxins.

Others have shown that the Clp proteolytic system plays a role in both virulence and environmental adaptation in S. aureus (Frees et al., 2003, 2005, 2014). Clp proteases are well conserved among most bacterial species and are composed of a core proteolytic chamber. In S. aureus, ClpP can associate with the ATPases ClpC or ClpX to form the ClpPC or ClpPX proteases (Frees et al., 2004). A new class of antibiots, acyldepsipeptides (ADEPs), targets the Clp proteolytic system. The ClpP proteolytic chamber is tightly regulated requiring either ClpC or ClpX for proteolytic function. Binding of ADEPs alleviates this regulation. In turn, the ADEP-activated ClpP core is capable of proteolytic degradation in the absence of the regulatory Clp-ATPases. This uncontrolled proteolysis leads to inhibition of bacterial cell division and eventually cell death (Brötz-Oesterhelt et al., 2005). Even more interesting was the discovery that ADEPs were effective in killing persister cells. When ADEPs bind to the ClpP complex, the protease is activated resulting in the uncontrolled degradation of over 400 proteins and in essence causing the cell to self-digest. Furthermore, combined treatment of ADEP with rifampicin resulted in complete eradication of S. aureus persister-rich biofilms in vitro and in two mouse models of chronic infection (Brötz-Oesterhelt et al., 2005; Conlon et al., 2013).

While it is clear that activation of the ClpP proteolysis is effective in eliminating persister cells, no studies have examined the effect of clpP or clpC deletion on persister numbers in S. aureus. Our studies suggest that deletion of clpP and clpC leads to a reduction in persister cell number following antibiotic treatment.

**METHODS**

**Bacterial strains and growth culture conditions.** Table 1 includes the list, description and source of S. aureus strains used in this study. S. aureus strains were suspended in trypticase soy broth (TSB; Becton Dickenson) containing 10% glycerol for long-term storage and kept at −70°C (Shapiro et al., 2011). Frozen cultures were thawed at room temperature. Fifty microlitres of each S. aureus strain was inoculated in 5 ml of TSB and incubated overnight (16–18 h) at 37°C.

Growth experiments were performed using a 1:1000 dilution of S. aureus overnight cultures in 50 ml TSB in 250 ml Erlenmeyer flasks. Cultures were placed in an incubator with shaking at 200 r.p.m. at 37°C for the duration of all growth experiments (Controlled Environment Incubator Shaker; New Brunswick Scientific). All samples from various S. aureus cultures were diluted by serial 10-fold dilutions and plated on

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus RN4220 8325-4</td>
<td>A restriction minus derivative of S. aureus strain 8325-4</td>
<td>Kreiswirth et al. (1983)</td>
</tr>
<tr>
<td>SH1000 8325-4</td>
<td>with a repaired ribU gene</td>
<td>Horshburgh et al. (2002)</td>
</tr>
<tr>
<td>ALC4977 SH1000</td>
<td>with a selectionless deletion of clpC</td>
<td>Donegan et al. (2010)</td>
</tr>
<tr>
<td>ALC6490 SH1000 clpC complement</td>
<td></td>
<td>Donegan et al. (2010)</td>
</tr>
<tr>
<td>ALC5105 SaclpP with a repaired ribU gene</td>
<td></td>
<td>Donegan et al. (2010)</td>
</tr>
<tr>
<td>ALC5105-pCU1 :: clpP ALC5105 with the pCU1 plasmid containing clpP</td>
<td></td>
<td>This study</td>
</tr>
<tr>
<td>VKS1011 SH1000 mazA3-lacZ integration (Ern*)</td>
<td></td>
<td>Singh &amp; Singh (2012)</td>
</tr>
</tbody>
</table>

*S. aureus strains were suspended in TSB containing 10% glycerol for long-term storage and kept at −70°C. Frozen cultures were thawed at room temperature. Fifty microlitres of each S. aureus strain was inoculated in 5 ml of TSB and incubated overnight (16–18 h) at 37°C.

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trypticase soy agar (TSA; Becton Dickinson). TSA plates were placed in an incubator at 37°C for 48 h, and the colony-forming units (CFU) were counted.

Antibiotic concentrations and exposure time used in this study were 80 times the MIC for 16–17 h to minimize the survival of spontaneous antibiotic-resistant mutants and ensure eradication of tolerant but killable cells (Yang et al., 2015). The antibiotic concentrations used in this study were as follows: oxacillin, 30 µg ml⁻¹; erythromycin, 30 µg ml⁻¹; levofloxacin, 15.2 µg ml⁻¹; daptomycin, 400 µg ml⁻¹.

**DNA manipulations.** Plasmid DNA was isolated using the Qiagen Miniprep kit (Qiagen). Chromosomal DNA was isolated using a DNAzol kit (Molecular Research Center) from lysostaphin-treated *S. aureus* cells according to the manufacturer’s instructions. All restriction and modification enzymes were purchased from Promega. PCR was performed using a Peltier Thermal Cycler–200 system (MJ Research). DNA manipulations were carried out using standard procedures. The oligonucleotide primers were obtained from Eurofins.

**Complementation of the clpP mutant strain ALC5105.** For complementation of the *clpP* mutant, a DNA fragment starting 310 nt upstream and terminating 393 nt downstream of the *clpP* gene was PCR amplified (an approximately 1.3 kb amplicon) using 5′-GGATCCGA-CATTGGCGGGATCTCT-3′ and backward primer 5′-AAGCTTAC-CAAGTCTTGGATCTGTC-3′ and *S. aureus* SH1000 genomic DNA as the template. This amplicon was cloned in vector pGEM®-T from where it was subcloned in vector pCU1 (Augustin et al., 1992) at BamHI and HindIII sites. The resulting construct pCU1::*clpP* was transferred into *S. aureus* RN4220 by electroporation and subsequently transduced into the *clpP* mutant ALC5101 using the phage 80–α transduction procedure as described previously by Singh et al. (2007).

**SH1000 and ALC5105 growth cultures.** Others have shown that mutation of *clpP* in *S. aureus* causes reduction in the bacterial cell size (Michel et al., 2006). Therefore, at any particular OD₆₀₀ the number of a *clpP* mutant would be higher than the wild-type. To determine the average cell number at each OD of this *clpP* mutant (ALC5105), a growth curve was generated and compared to a growth curve generated using wild-type *S. aureus* strain SH1000. Standard growth conditions, as described above, were used to prepare *S. aureus* SH1000 and *clpP* mutant ALC5105 cultures. Fifty millilitres of TSB was inoculated with 50 µl overnight culture and incubated at 37°C with shaking at 200 r.p.m. (Shapiro et al., 2011). One millilitre aliquots of wild-type SH1000 and *clpP* mutant ALC5105 were harvested at an OD of 600 nm of light (OD₆₀₀: 0.25, 0.50, 0.75, 1.0, 1.5 and 2.0 (±0.05) using a spectrophotometer (BioMate 3®; ThermoScientific). Additional samples of wild-type SH1000 were harvested at OD₆₀₀ of 3.0 and 4.0. Each 1 ml aliquot underwent a serial 10-fold dilution before plating on TSA. All samples were plated in triplicate (Keren et al., 2004a). Plates were incubated at 37°C and grown for 48 h.

Following incubation, plates with countable numbers of isolated colonies (30–300 CFU) were recorded and graphed. A best-fit line was calculated, and the OD₆₀₀ of *clpP* mutant ALC5105 that resulted in the same cell number of the wild-type SH1000 at OD₆₀₀ of 0.25, 0.50, 0.75, 1.0, 1.5 and 2.0 was determined (Fig. 1). This experiment was performed three different times to generate the average cell counts for each bacterial strain.

**Persistase assay.** The number of persistase cells was determined based on cell viability following antibiotic treatment. Overnight cultures of *S. aureus* strains [SH1000 (wild-type), ALC5105 (*clpP* mutant), ALC5105-pCU1::*clpP* (*clpP* complemented in trans), ALC4977 (*clpC* mutant) and ALC6490 (*clpP* complemented)] were prepared using standard growth conditions. Fifty microlitres of overnight culture was inoculated into 50 ml TSB in 250 ml flasks. Cultures were then placed in an incubator at 37°C with shaking at 100 r.p.m. At the appropriate OD₆₀₀ (0.25, 0.50, 0.75, 1.0, 1.5 and 2.0 for SH1000, ALC5105-pCU1::*clpP*, ALC4977 and ALC6490, or 0.11, 0.21, 0.32, 0.58, 0.87 and 1.2 for ALC5105), a 1 ml aliquot was removed and placed in a 1.5 ml Eppendorf tube containing antibiotic. An additional 100 µl sample was removed and placed into a 1.5 ml Eppendorf tube containing 900 µl sterilized TSB to provide live counts before antibiotic treatment.

The 100 µl samples were diluted using serial 10-fold dilutions and plated in triplicate onto TSA plates and placed in the incubator at 37°C for 48 h. Following incubation, plates with countable numbers of isolated colonies were recorded, and the CFU ml⁻¹ was determined as the average between replicates.

Each 1 ml aliquot at the respective OD₆₀₀ was placed in a 1.5 ml sterile Eppendorf tube. Thirty microlitres of the appropriate antibiotic was added (oxacillin, 30 µg ml⁻¹; erythromycin, 30 µg ml⁻¹; levofloxacin, 15.2 µg ml⁻¹; daptomycin, 400 µg ml⁻¹); the tube was vortexed and placed in an incubator at 37°C with rotation overnight (16–17 h). Following antibiotic treatment, the cells were centrifuged and washed with fresh sterilized cold TSB. This process was repeated three times. Following wash, cultures were plated on ice. Each sample was then subjected to serial 10-fold dilution and plated in triplicate onto TSA plates. Plates were incubated at 37°C for 48 h. Plates containing countable colonies were counted, and the CFU ml⁻¹ was determined.

The per cent survival was then calculated by dividing the antibiotic-treated CFU ml⁻¹ by the untreated CFU ml⁻¹ and multiplying by 100. The data for the mean per cent survivals were represented as mean per cent survival ±sd and compared using the Student’s t-test. Differences in mean per cent survivals were considered significant at P<0.05. These experiments were replicated at least three times for each strain, OD₆₀₀ and antibiotic.

**Persistase assay.** The time required for revival of persistase cells was determined based on growth curves of antibiotic-treated and antibiotic-untreated cultures. Overnight cultures of SH1000, ALC5105, ALC5105-pCU1::*clpP*, ALC4977 and ALC6490 were diluted 1:1000 in 50 ml of fresh TSB and grown to mid-exponential phase (OD₆₀₀, 0.750). Erythromycin-resistant *S. aureus* strain VKS1011 was used during erythromycin persistase revival assay as a positive control. At mid-exponential phase, a 100 µl aliquot was used to inoculate 10 ml of fresh TSB and incubated at 37°C with rotation overnight to serve as an untreated control. An additional 35 ml of culture was transferred to a clean 125 ml Erlenmeyer flask. The appropriate concentration of each antibiotic (oxacillin, 30 µg ml⁻¹, or erythromycin, 30 µg ml⁻¹) was added to each flask. Flasks were incubated at 37°C with shaking overnight.

Following overnight incubation (16–17 h), 1 ml aliquots of untreated control cultures were removed and placed in 1.5 ml Eppendorf tubes. Antibiotic-treated cultures were removed and placed in 250 ml plastic centrifuge bottles. Treated cultures were centrifuged (Sorval Legend XTR Centrifuge; ThermoScientific) at 6000 g at 4°C for 10 min. Following centrifugation, the supernatant was removed, and the pellet was suspended in 1 ml fresh TSB. After suspension, the 1 ml was transferred to a 1.5 ml Eppendorf tube. This process was performed for each antibiotic-treated culture. After transfer to 1.5 ml Eppendorf tubes, treated and untreated samples were centrifuged and washed with fresh cold TSB three times.

Treated and untreated samples were diluted to a turbidity of 0.10 at an OD₆₀₀ after oxacillin treatment or to match cell viability after erythromycin treatment. When erythromycin was used, viable cell number after overnight antibiotic exposure was determined. Washed treated and untreated samples were diluted and plated on TSA plates, then incubated at 37°C overnight. Plates containing countable colonies were counted, and the CFU ml⁻¹ was determined. Untreated samples were serially diluted to achieve the same viable cell number as in treated samples (1×10⁶ CFU ml⁻¹).
Using a Honeycomb 2 100-well plate, 200 µl of each culture, at 1 turbidity of 0.10 at 600 nm.

The point of persister revival was determined as a threefold increase in OD at each OD. The time required for each culture to reach this point was averaged and recorded. The revival time was measured by taking the difference between untreated and treated samples. The revival time was determined by taking the difference between untreated and treated growth times.

**RESULTS AND DISCUSSION**

**Growth curve analysis of the clpP mutant (ALC5105) and the wild-type strain (SH1000)**

Initial experiments using samples obtained at OD$_{600}$ of 0.25, 0.50, 0.75, 1.0, 1.5 and 2.0 for the clpP mutant (ALC5105) and the wild-type strain (SH1000) resulted in no differences in per cent survival after oxacillin treatment (data not shown). Others have demonstrated that mutation of clpP in *S. aureus* resulted in a reduction in cell size (Michel et al., 2006). If the clpP mutant was smaller in size than the wild-type strain, then the cell density or CFU ml$^{-1}$ at each OD$_{600}$ would be higher for the mutant. This would change the amount of antibiotic that the mutant cells were exposed to when compared to the wild-type cells and could explain the lack of differences in per cent survival after oxacillin treatment.

To determine if cell densities or CFU ml$^{-1}$ were higher in the clpP mutant (ALC5105) than the wild-type strain (SH1000), growth curve experiments were conducted for all antibiotics and each concentration. SH1000 and mutant ALC5105 were diluted 1 : 1000 in TSB and shaken at 37 °C. Samples were taken at indicated OD$_{600}$ of 0.25, 0.50, 0.75, 1.0, 1.5 and 2.0. Samples were serially diluted and plated on TSA. Data are average CFU ml$^{-1}$ of three independent experiments obtained in each sample at each OD$_{600}$.

**Fig. 1.** Growth curve analysis of *S. aureus* wild-type and clpP mutant cells. To normalize cell density due to the smaller cell size of clpP mutant cells, a growth curve of wild-type and clpP mutant cells was obtained. Overnight cultures of SH1000 and clpP mutant ALC5105 were diluted 1 : 1000 in TSB and shaken at 37 °C. Samples were taken at indicated OD$_{600}$ of 0.25, 0.50, 0.75, 1.0, 1.5 and 2.0. Samples were serially diluted and plated on TSA. Data are average CFU ml$^{-1}$ of three independent experiments obtained in each sample at each OD$_{600}$.
the clpP mutant and the wild-type strain (Fig. 1). The CFU ml\(^{-1}\) for the clpP mutant was higher than the CFU ml\(^{-1}\) for the wild-type strain when both strains were sampled, diluted and counted at the same OD\(_{600}\). The clpP mutant CFU ml\(^{-1}\) at the following OD\(_{600}\) of 0.11, 0.21, 0.32, 0.58, 0.87 and 1.19 was approximately the same CFU ml\(^{-1}\) as the wild-type strain at OD\(_{600}\) of 0.25, 0.50, 0.75, 1.0, 1.5 and 2.0, respectively. It appears that the clpP mutant used in this study may have also been reduced in cell size following mutation of the clpP gene and resulted in higher CFU ml\(^{-1}\) for all the OD\(_{600}\) used in this study (Fig. 1). When the clpP mutant and the wild-type strain were harvested at the OD\(_{600}\) listed above, no significant differences in CFU ml\(^{-1}\) were observed (data not shown). The CFU ml\(^{-1}\) of both strains at the OD\(_{600}\) listed above were 2.6\(\times\)10\(^7\) (±3.3\(\times\)10\(^6\)), 6.8\(\times\)10\(^7\) (±1.3\(\times\)10\(^7\)), 1.3\(\times\)10\(^8\) (±3.5\(\times\)10\(^7\)), 2.3\(\times\)10\(^8\) (±6.8\(\times\)10\(^7\)), 5.3\(\times\)10\(^8\) (±9.1\(\times\)10\(^7\)) and 8.8\(\times\)10\(^8\) (±1.2\(\times\)10\(^8\)).

No changes in CFU ml\(^{-1}\) were observed in the clpC mutant (ALC4977) or in the clpC complement (ALC6490; data not shown). As observed by others, complementation of the clpP mutant (ALCS105-pCU1::clpP) restored CFU ml\(^{-1}\) to wild-type levels (data not shown) (Michel et al., 2006).

Effect of clpP deletion on persister cell number after antibiotic exposure

Using standard growth conditions, three S. aureus strains, wild-type (SH1000), clpP mutant (ALC5105) and clpP complement (ALC5105-pCU1::clpP), were sampled at six different CFU ml\(^{-1}\) (2.6\(\times\)10\(^7\), 6.8\(\times\)10\(^7\), 1.3\(\times\)10\(^8\), 2.3\(\times\)10\(^8\), 5.3\(\times\)10\(^8\) and 8.8\(\times\)10\(^8\) CFU ml\(^{-1}\)). The samples were then treated with: 30 µg ml\(^{-1}\) oxacillin, 30 µg ml\(^{-1}\) erythromycin, 15.2 µg ml\(^{-1}\) levofloxacin or 400 µg ml\(^{-1}\) daptomycin. Differences in persister cell number were recorded as per cent survival.

Oxacillin treatment resulted in significant decreases in per cent survival when the clpP mutant was compared to wild-type at CFU ml\(^{-1}\) of 6.8\(\times\)10\(^7\) (P=0.013) and 1.3\(\times\)10\(^8\) (P=0.002) (Table 2). The clpP complemented strain’s per cent survivals were as high as or higher than wild-type at all CFU ml\(^{-1}\) except for the 6.8\(\times\)10\(^7\) CFU ml\(^{-1}\) sample.

Erythromycin treatment resulted in significant decreases in per cent survival when the clpP mutant was compared to wild-type at CFU ml\(^{-1}\) of 1.3\(\times\)10\(^8\) (P<0.0001), 2.3\(\times\)10\(^8\) (P<0.0001) and 5.3\(\times\)10\(^8\) (P=0.0075) (Table 3). The clpP complemented strain (ALC5105-pCU1::clpP) showed partial restoration to wild-type per cent survival levels and was significantly higher than the clpP mutant at 1.3\(\times\)10\(^8\) (P=0.0009), 2.3\(\times\)10\(^8\) (P=0.0002) and 5.3\(\times\)10\(^8\) (P=0.0002).

Levofloxacin and daptomycin treatment resulted in no statistical difference in per cent survival between clpP mutant and wild-type cells.

The decrease in persister cell number observed in clpP mutant ALC5105 could be attributed to several possible mechanisms. The role of TA modules in the formation of persister cells has been implicated through transcriptional profiling of isolated persister cells (Keren et al., 2004b). Additionally, deletion of five or more TA loci in E. coli results in a significant decrease in persister cell numbers (Maisonpierre et al., 2011). Donegan et al. (2010) have demonstrated that ClpP is required for the degradation of all known antitoxins in S. aureus. Our results suggest that deletion of clpP function may be related to the cell’s inability to free toxin and consequent failure to enter the persistent state resulting in a decreased tolerance to antibiotics.

Interestingly, it has been shown that the mazEF TA operon is transcriptionally expressed in normal growth conditions; however, expression is reduced during late stationary phase. Moreover, expression of mazEF was increased in the presence of penicillin, tetracycline and erythromycin (Donegan & Cheung, 2009). This observation may provide one reason for the low level of persisters observed in the clpP mutant during early and mid-exponential phases after oxacillin or erythromycin exposure, as clpP mutants would lack the ability to degrade antitoxin and, in particular, MazE.

Table 2. Per cent survival of wild-type (SH1000), clpP mutant (ALC5101) and clpP complemented (ALC5105-pCU1::clpP) strains following treatment with oxacillin

<table>
<thead>
<tr>
<th>CFU ml(^{-1})</th>
<th>SH1000</th>
<th>ALC5101</th>
<th>ALC5105-pCU1::clpP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>per cent survival (±sd)</td>
<td>per cent survival (±sd)</td>
<td>per cent survival (±sd)</td>
</tr>
<tr>
<td>2.6(\times)10(^7)</td>
<td>0.0074 (0.0052)</td>
<td>0.0047 (0.0013)</td>
<td>0.0068 (0.0081)</td>
</tr>
<tr>
<td>6.8(\times)10(^7)</td>
<td>2.43 (1.76)</td>
<td>0.020* (0.010)</td>
<td>0.027 (0.010)</td>
</tr>
<tr>
<td>1.3(\times)10(^8)</td>
<td>12.9 (8.02)</td>
<td>0.023* (0.0021)</td>
<td>20.4 (19.4)**</td>
</tr>
<tr>
<td>2.3(\times)10(^8)</td>
<td>35.3 (14.0)</td>
<td>29.7 (17.2)</td>
<td>74 (9.5)**</td>
</tr>
<tr>
<td>5.3(\times)10(^8)</td>
<td>60.0 (22.1)</td>
<td>69.1 (30.9)</td>
<td><strong>ND</strong></td>
</tr>
<tr>
<td>8.8(\times)10(^8)</td>
<td>99.8 (29.5)</td>
<td>46.1 (25.5)</td>
<td><strong>ND</strong></td>
</tr>
</tbody>
</table>

*P<0.05; ALC5101 vs SH1000.
**P<0.05; ALC5101 vs ALC5105-pCU1::clpP.
Table 3. Per cent survival of wild-type (SH1000), clpP mutant (ALC5101) and clpP complemented (ALC5105-pCU1::clpP) strains following treatment with erythromycin

<table>
<thead>
<tr>
<th>CFU ml⁻¹</th>
<th>SH1000 per cent survival (±SD)</th>
<th>ALC5101 per cent survival (±SD)</th>
<th>ALC5105-pCU1::clpP per cent survival (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.6×10⁷</td>
<td>0.76 (0.66)</td>
<td>0.27 (0.13)</td>
<td>0.092 (0.14)</td>
</tr>
<tr>
<td>6.8×10⁷</td>
<td>0.34 (0.21)</td>
<td>0.068 (0.080)</td>
<td>4.8 (2.9)</td>
</tr>
<tr>
<td>1.3×10⁸</td>
<td>44.1 (3.0)</td>
<td>0.24 (0.031)*</td>
<td>23.7 (4.6)**</td>
</tr>
<tr>
<td>2.3×10⁸</td>
<td>100 (12)</td>
<td>0.92 (0.92)*</td>
<td>77.7 (9.8)**</td>
</tr>
<tr>
<td>5.3×10⁸</td>
<td>84.0 (27.8)</td>
<td>3.81 (0.79)*</td>
<td>118 (14.6)**</td>
</tr>
<tr>
<td>8.8×10⁸</td>
<td>143 (75.1)</td>
<td>111 (17.1)</td>
<td>114 (19.8)</td>
</tr>
</tbody>
</table>

*P<0.05; ALC5101 vs SH1000.
**P<0.05; ALC5101 vs ALC5105-pCU1::clpP.

The decrease in persister cell number in the clpP mutant was not observed throughout the entire growth cycle and differed between antibiotic classes. Oxacillin exposure resulted in significantly lower per cent survival during early to mid-exponential growth phase (2.6×10⁷ to 6.8×10⁷ CFU ml⁻¹); however, an increase in per cent survival was observed between mid-log to early stationary phase (6.8×10⁷ to 5.3×10⁸ CFU ml⁻¹; Table 2). Others have shown that antitoxins in S. aureus have half-lives that are about one-half as long as the antitoxins in E. coli (Donegan & Cheung, 2009). It is possible that the labile staphylococcal antitoxins eventually degrade, allowing the freed accumulated toxins to exert their effect on the cell and allow an increase in persistence during the later phases of growth. However, TA expression throughout the growth cycle would have to be examined alongside antibiotic treatments. The increase in persister survival may also be attributed to a persistence mechanism independent of ClpP.

A low number of persister cells was detected in clpP mutants after erythromycin exposure. However, the effect of the clpP mutation on persister number was observed during the later phases of growth (1.3×10⁸ to 5.3×10⁸ CFU ml⁻¹; Table 3). While TA systems may still play a role with little TA being made in both wild-type and clpP mutant cells due to inhibited protein synthesis, the difference may also be due to an increase in non-functional proteins in the clpP mutant when compared to wild-type (Shoji et al., 2011).

Low levels of persisters (less than 3%) were detected in both wild-type and clpP mutant cells following levofloxacin or daptomycin exposure at all CFU ml⁻¹ tested. Unlike oxacillin which kills growing cells, levofloxacin can kill both growing and non-growing cells (Booun et al., 2000). The S. aureus strains used in these studies were quite sensitive to levofloxacin (MIC, 0.375 µg ml⁻¹). So much killing occurred at all stages of the growth cycle that no significant differences could be observed between the wild-type and the clpP mutant (data not shown). Additionally, no significant differences were seen between wild-type and clpP mutant cells following daptomycin exposure. This may be due to the ability of daptomycin to function as a membrane depolarizer (Kohanski et al., 2010). Additionally, clpP has been shown to play a role in cell wall metabolism and cell division; as daptomycin targets cell wall synthesis, it is possible that the added stress contributes to the low level of persisters (Feng et al., 2013).

Table 4. Per cent survival of wild-type (SH1000), clpC mutant (ALC4977) and clpC complemented (ALC6490) strains following treatment with oxacillin

<table>
<thead>
<tr>
<th>CFU ml⁻¹</th>
<th>SH1000 per cent survival (±SD)</th>
<th>ALC4977 per cent survival (±SD)</th>
<th>ALC6490 per cent survival (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.6×10⁷</td>
<td>0.0050 (0.0019)</td>
<td>0.0070 (0.0076)</td>
<td>0.0038 (0.00028)</td>
</tr>
<tr>
<td>6.8×10⁷</td>
<td>4.01 (4.23)</td>
<td>1.55 (1.99)</td>
<td>1.90 (0.34)</td>
</tr>
<tr>
<td>1.3×10⁸</td>
<td>14.2 (9.84)</td>
<td>5.78 (3.45)</td>
<td>7.45 (4.98)</td>
</tr>
<tr>
<td>2.3×10⁸</td>
<td>28.4 (12.2)</td>
<td>10.3 (3.71)*</td>
<td>31.3 (20.2)**</td>
</tr>
<tr>
<td>5.3×10⁸</td>
<td>53.1 (11.4)</td>
<td>55.1 (17.2)</td>
<td>54.3 (50.8)</td>
</tr>
<tr>
<td>8.8×10⁸</td>
<td>87.3 (10.9)</td>
<td>81.5 (8.76)</td>
<td>94.9 (54.8)</td>
</tr>
</tbody>
</table>

*P<0.05 ALC4977 vs SH1000.
**P<0.05 ALC4977 vs ALC6490.
Lastly, the differences in persister numbers between the wild-type strain and the mutant could be due to the strains being in different growth phases when they were sampled. Others have shown that persister numbers increase in late log phase and are highest in stationary phase of growth (Keren et al., 2004a). Since the mutant was smaller than the wild-type strain, we chose to sample these strains at the same CFU ml⁻¹ rather than at the same OD₆₀₀. If the wild-type strain was in late stationary phase or stationary phase while the mutant was in early to mid-log phase, the wild-type strain would have more sisters present in the culture than the mutant strain.

While it is evident that clpP deletion results in a decrease in persister cells, much remains unclear. The number of persisters varies between antibiotic and the growth stage in which the cells are treated, suggesting that the complete mechanism for bacterial persistence is multifaceted. Others have demonstrated that differences in per cent survival depend on the antibiotic (Hofsteenge et al., 2013).

Table 5. Persister revival regrowth times following antibiotic treatment

<table>
<thead>
<tr>
<th>Strain*</th>
<th>SH1000 wild-type</th>
<th>clpP mutant ALC5105</th>
<th>clpP complement ALC5101-pCU1 :: clpP</th>
<th>clpC mutant ALC4977</th>
<th>clpC complemented ALC6490</th>
<th>Erythromycin-resistant VKS1011</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxacillin Untreated</td>
<td>90±0</td>
<td>140±17.3</td>
<td>210±30</td>
<td>100±3.66</td>
<td>100±3.66</td>
<td>NA</td>
</tr>
<tr>
<td>Oxacillin Treated</td>
<td>505±37.5</td>
<td>500±31.2</td>
<td>495±65</td>
<td>605±56.7</td>
<td>465±108.1</td>
<td>NA</td>
</tr>
<tr>
<td>Revival time (h)</td>
<td>-7</td>
<td>-6</td>
<td>-5</td>
<td>-6</td>
<td>-6</td>
<td>NA</td>
</tr>
<tr>
<td>Erythromycin Untreated (min)</td>
<td>90±0</td>
<td>220±60.6</td>
<td>210±30</td>
<td>175±15</td>
<td>170±31.2</td>
<td>90±15</td>
</tr>
<tr>
<td>Erythromycin Treated (min)</td>
<td>565±85.2</td>
<td>700±150.2</td>
<td>700±38</td>
<td>555±117.1</td>
<td>535±17.3</td>
<td>105±0</td>
</tr>
<tr>
<td>Revival time (h)</td>
<td>-8</td>
<td>-8</td>
<td>-8</td>
<td>-6</td>
<td>-6</td>
<td>-0.25</td>
</tr>
</tbody>
</table>

NA, Not applicable.

*Exponential phase (OD₆₀₀ 0.750) cultures of S. aureus strains (SH1000, ALC5105, ALC5101-pCU1 :: clpP, ALC6490 and ALC6491) were treated with antibiotic (oxacillin, 30 µg ml⁻¹, or erythromycin) or untreated overnight (16-17 h). Treated and untreated cultures were washed and suspended to matching cell viability. Resumption of growth was subsequently measured at 15 min intervals using OD₆₀₀ for 16 h. Data are represented as the average of three independent experiments.

identified as a protein that was digested by ClpP (Feng et al., 2013). Cellular levels of Spx are regulated by proteolytic control (Feng et al., 2013). Under non-stress conditions, Spx is kept at a low concentration through degradation by the ClpPX or ClpPC. Under disulfide stress, Spx is stabilized and transcriptionally activates a number of genes required for oxidative stress tolerance. In Bacillus subtilis, clpP mutants showed an accumulation of Spx, which negatively affected competence and sporulation (Nakano et al., 2002; You et al., 2008). Furthermore, the CtsR regulon in staphylococci comprises the clp genes as well as other chaperone genes such as dnaK and groESL (Chastanet et al., 2003). ClpP-mediated degradation of CtsR may allow for de-repression of the ctsR and transcription of stress genes (Chastanet et al., 2003; Elsholz et al., 2010; Frees et al., 2004). Interestingly, deletion of dnaK resulted in a decrease in persister cells and stress response (Singh et al., 2007).

Additionally, DNA damage repair proteins have been implicated as targets for ClpP proteolysis (Feng et al., 2013). In S. aureus, the ClpPX as well as the ClpPC proteases contribute to degradation of LexA auto cleavage products (Cohn et al., 2011). Therefore, the low level of persisters observed in clpP mutant cells may be attributed to a decrease in overall stress response, and the differences among antibiotics may be explained through the antibiotics, specific target and degree of stressors (Kim et al., 2011).

Lastly, the differences in persister numbers between the clpP mutant and the wild-type strain could be due to the strains being in different growth phases when they were sampled for the persister assay. Others have shown that persister numbers increase in late log phase and are highest in stationary phase of growth (Keren et al., 2004a). Since the mutant was smaller than the wild-type strain, we chose to sample these strains at the same CFU ml⁻¹ rather than at the same OD₆₀₀. If the wild-type strain was in late stationary phase or stationary phase while the mutant was in early to mid-log phase, the wild-type strain would have more sisters present in the culture than the mutant strain.

Effect of clpC deletion on persister cell number after antibiotic exposure

Using standard growth conditions, wild-type (SH1000), clpC mutant (ALC4977) and clpC complemented (ALC6490) strains were sampled at six different CFU ml⁻¹ (2.6×10⁷, 6.8×10⁷, 1.3×10⁸, 2.3×10⁸, 5.3×10⁸ and 8.8×10⁸ CFU ml⁻¹). The samples were then treated with 30 µg ml⁻¹ oxacillin, 30 µg ml⁻¹ erythromycin, 15.2 µg ml⁻¹ levofloxacin or 400 µg ml⁻¹ daptomycin. Differences in persister cell number were recorded as per cent survival.

A statistical difference between wild-type and clpC mutant per cent survival was observed at only one CFU ml⁻¹ of 2.3×10⁸ (P=0.013) after treatment with oxacillin (Table 4). Complementation of clpC resulted in restoration to wild-type per cent survival. There was no statistical difference observed at any CFU ml⁻¹ between wild-type and the clpC mutant following treatment with erythromycin, levofloxacin or daptomycin.
Our results suggest that *clpC* may play a minor role, if any, in persister formation or maintenance. Donegan & Cheung (2009) have demonstrated that along with ClpP, ClpC function is required for degradation of *S. aureus* antitoxins. To facilitate ClpP degradation, substrates interact with the Clp ATPase that powers unfolding and translocation of substrate into the proteolytic chamber (Frees *et al.* 2007). In *S. aureus*, ClpC and ClpX ATPases can perform this function. The deletion of *clpC* appears to only decrease persister cell numbers after oxacillin exposure at 1 CFU ml$^{-1}$, and it does not appear to have a role in persister formation following erythromycin, levofloxacin or daptomycin treatment.

ClpX may serve as the chaperone to enable ClpP breakdown of antitoxin. Donegan *et al.* (2010) demonstrated that the antitoxin MazE in a *clpC* mutant was still broken down at one-fourth the rate of wild-type. However, breakdown of all the antitoxins was still observed when the ClpX ATPase gene was mutated. They propose that ClpP may break down MazE without the aid of ClpC. Therefore, it is unlikely that ClpX was facilitating antitoxin breakdown, and if ClpC is involved in persister formation or maintenance, its role, if any, is minor.

In low-GC Gram-positive bacteria, the negative heat shock regulator CtsR controls the expression of *clp* genes.
(Chastanet et al., 2003; Frees et al., 2014). Interestingly, in S. aureus, the CtsR regulon also contains the chaperone genes dnaK and groESL. The synthesis of classical chaperones is directly connected with that of Clp proteins in S. aureus resulting in the possible enhancement and adaptability during stress and infection (Chastanet et al., 2003). Therefore, the loss of ClpC may also be compensated for by other chaperones enabling antibiotic tolerance.

**Persister revival**

To ensure that the per cent survival in each experiment was due to persister cells and not to spontaneous antibiotic-resistant mutants, a persister revival assay was performed (Table 5). Following antibiotic treatment, survivors that are persisters take significantly longer to start growing again when compared to untreated cells or resistant cells (Balaban et al., 2004; Kwan et al., 2013). Exponential phase (OD600, 0.750) cultures of S. aureus strains (SH1000, ALC5105, ALC5105-pCU1::clpP, ALC6490 and ALC6491) were treated with an antibiotic (oxacillin, 30 µg ml⁻¹ or erythromycin, 30 µg ml⁻¹) or grown in TSB (untreated) overnight (16–17 h). Treated and untreated cultures were washed and suspended in TSB to viable cell counts of 1×10⁶ CFU ml⁻¹ when treated with erythromycin and to an OD600 of 0.10 when treated with oxacillin. Resumption of growth was measured every 15 min at OD600 for 16 h. The revival time was defined as the time in minutes that the OD600 got to three times the starting OD600. Data are represented as the average of three independent experiments (Table 5). The revival times after treatment with each antibiotic resulted in a significant lag time compared to untreated controls, therefore, verifying that the survivors of antibiotic treatment were persister cells and spontaneous resistance was not selected in these studies. No significant differences in revival times of SH1000, ALC5101, ALC5101-pCU1::clpP, ALC4977 and ALC6490 were observed. To further validate the results, an erythromycin-resistant strain was used to illustrate the lack of lag time that would be expected in resistant organisms. Following erythromycin treatment, wild-type SH1000 cells displayed a 7 h lag time, while the erythromycin-resistant VKS1011 cells started growing again in 15 min (Fig. 2). Our results demonstrate that persister cells measured after antibiotic treatment with oxacillin and erythromycin were persister cells and most likely not due to selection for spontaneous antibiotic-resistant mutants.

**GENERAL SUMMARY AND CONCLUSIONS**

* S. aureus is one of the most frequent causes of a wide variety of hospital- and community-acquired infections. *S. aureus* infections range from superficial skin and soft tissue infections to life-threatening illnesses such as toxic shock syndrome, pneumonia, endocarditis and septicemia (Lowy, 1998; Shoji et al., 2011). Treatment failure following *S. aureus* infections is frequent and may, in certain infections, be due to antibiotic-tolerant cells called persisters (Conlon et al., 2013). In *E. coli*, TA loci have been shown to be important in the development of persisters (Maisononneuve et al., 2011). Others have demonstrated the role of clpP and clpC in the regulation of these TA loci in *S. aureus* (Donegan & Cheung, 2009). This study was conducted to determine the effect, if any, of clpP and clpC deletion on persister number in *S. aureus* following antibiotic treatment.

Cultures of *S. aureus* strains SH1000 (WT), ALC5105 (clpP mutant), ALC5105-pCU1::clpP (clpP complement), ALC4977 (clpC mutant) and ALC6490 (clpC complement) were grown in TSB at 37°C. The bacterial cultures were grown to six different CFU ml⁻¹ and then treated with antibiotic at concentrations that were 80 times the MIC: oxacillin, 30 µg ml⁻¹; erythromycin, 30 µg ml⁻¹; levofloxacin, 15.2 µg ml⁻¹; daptomycin, 400 µg ml⁻¹. Per cent survival was calculated for each bacterial strain treated by each of the antibiotics grown to the 6 CFU ml⁻¹.

Exposure of the clpP mutant ALC5105 to oxacillin treatment resulted in a significant decrease in per cent survival at CFU ml⁻¹ of 6.8×10⁷ and 1.3×10⁸ when compared to wild-type. Differences were also observed after erythromycin treatment at CFU ml⁻¹ of 1.3×10⁸, 2.3×10⁸ and 5.3×10⁸. No differences in per cent survival were observed between the clpP mutant and wild-type following levofloxacin or daptomycin treatment. Exposure of the clpC mutant ALC4977 to oxacillin treatment resulted in a significant decrease in per cent survival at CFU ml⁻¹ of 2.3×10⁸ when compared to wild-type. There were no differences in per cent survival observed between the clpC mutant and wild-type following erythromycin, levofloxacin or daptomycin treatment.

Overall, our results demonstrated an antibiotic and CFU ml⁻¹ or growth-phase-dependent role for ClpP and possibly for ClpC in *S. aureus* persistence. Persister revival experiments demonstrated that the survivors in the persister assay did not survive antibiotic treatment because of antibiotic resistance but survived because they were persisters.

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**REFERENCES**


