

Impact of daptomycin resistance on *Staphylococcus aureus* virulence

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Daptomycin resistance (DAP^R) in *Staphylococcus aureus* is associated with mutations in genes that are also implicated in staphylococcal pathogenesis. Using a laboratory-derived series of DAP exposed strains, we showed a relationship between increasing DAP MIC and reduced virulence in a *Galleria mellonella* infection model. Point mutations in *walK* and *rpoC* led to cumulative reductions in virulence and simultaneous increases in DAP MIC. A point mutation to *mprF* did not impact on *S. aureus* virulence; however deletion of *mprF* led to virulence attenuation and hyper-susceptibility to DAP. To validate our findings in *G. mellonella*, we confirmed the attenuated virulence of select isolates from the laboratory-derived series using a murine septicaemia model. As a corollary, we showed significant virulence reductions for clinically-derived DAP^R isolates compared to their isogenic, DAP-susceptible progenitors (DAP^S). Intriguingly, each clinical DAP^R isolate was persistent *in vivo*. Taken together, it appears the genetic correlates underlying daptomycin resistance in *S. aureus* also alter pathogenicity.

Introduction

Daptomycin (DAP) is a cyclic lipopeptide antibiotic that is increasingly being used to treat *Staphylococcus aureus* infections.¹ Unfortunately, therapeutic failures, albeit relatively uncommon, have been reported.^{1–3} Thus far, the mechanisms underlying daptomycin resistance (DAP^R) in *S. aureus* have focused on point mutations in genes involved in phospholipid biosynthesis, particularly *mprF*,⁴ which codes for lysyl-phosphatidylglycerol (L-PG) synthetase, *cls2*, which codes for cardiolipin synthase and *pgsA*, which codes for CDP-diacylglycerol-glycerol-3-phosphate-3-phosphatidyltransferase.⁵ It is hypothesized that these mutations lead to changes in phospholipid membrane composition, which may affect membrane charge causing electro-repulsion of calcium-complexed daptomycin or may directly affect daptomycin binding.⁵ Interestingly, daptomycin, when complexed with calcium, appears to act similar to cationic antimicrobial peptides, and therefore genetic mutations associated with daptomycin resistance have been shown to simultaneously confer resistance to host innate immune responses.⁴ Other genes associated with reduced susceptibility to daptomycin may also affect *S. aureus*-host interactions, particularly the sensor-histidine kinase, *walK* (previously *YycG*), which has an integral role in cell wall homeostasis but also regulates a number of genes important for virulence.^{6,7} The focus of this study was to assess for the first time the pathogenic consequences of DAP^R in *S. aureus*.

Methods

Bacterial strains used in this study are shown in Table 1. For the purpose of this study, daptomycin non-susceptible isolates (defined by an MIC of DAP >1 mg/L) are referred to as DAP^R. The laboratory-derived, DAP-exposed series was obtained from Friedman et al., and included a parent strain (CB1118) and 4 mutants isolated after serial *in vitro* DAP exposure over 20 days.⁸ These strains were previously genome sequenced and their cumulative mutations are shown in Table 1.⁸ An *mprF* deletion mutant (CB1118Δ*mprF*) was also included.⁹ In addition, 3 clinical *S. aureus* pairs were assessed, which included a daptomycin-susceptible (DAP^S) parent strain with its corresponding DAP^R daughter strain that developed after DAP therapy and clinical failure.⁵ Growth kinetic experiments were performed as described previously.¹⁰

We used *Galleria mellonella* as a substitute *in vivo* host to assess staphylococcal virulence as described previously.^{7,10,11} This model was chosen because *G. mellonella* not only have phagocytic cells in their hemolymph but they also rely on antimicrobial peptides for their immune defense, hence their utility for the study of DAP^R. Briefly, bacteria were injected into the hemocoel of each caterpillar (n = 16 / strain, 1 × 10⁵–1 × 10⁶ CFU/larvae) using a 10 μl Hamilton syringe.¹⁰ For the mammalian experiments, 10–15 C57BL/6 or CD-1 mice per strain were injected intraperitoneally with 2–4 × 10⁷ CFU of bacteria mixed with

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Table 1. Laboratory and clinically derived daptomycin-exposed *Staphylococcus aureus* strains used in this study

Strain	Description	DAP MIC (mg/L)
Laboratory-derived isolates ^a		
CB1118 ^b	Parent used for in vitro passage.	0.5
CB1618-d6	<i>mprF</i> (T345A)	2
CB1618-d9	<i>mprF</i> (T345A), <i>walK</i> (R263C)	4
CB1618-d13	<i>mprF</i> (T345A), <i>walK</i> (R263C), <i>rpoB</i> (A1086V)	4
CB1618-d20	<i>mprF</i> , (T345A), <i>walK</i> (R263C), <i>rpoB</i> (A1086V), <i>rpoC</i> (Q961K)	16
CB1118Δ <i>mprF</i>	<i>mprF</i> deletion mutant	0.125
Clinically-derived isolates ^c		
1) A8819 (S)	Osteomyelitis, septic arthritis	0.25
A8817 (R)		2
2) A8796 (S)	Vertebral osteomyelitis	0.5
A8799 (R)		2
3) A9754 (S)	Endocarditis	0.5
A9757 (R)		4

DAP, Daptomycin; S, Susceptible; MIC, Minimum Inhibitory Concentration; R, Resistant.

^aMutations described in ref.⁸

^bRefers to previously described strain MW2.

^cDescribed in ref.⁵

6% porcine gastric mucin (Sigma Aldrich) in 500 µl, and were monitored for 7 days.¹² To assess bacterial persistence, kidneys were harvested from surviving DAP^R infected animals 7-days post infection, and bacterial counts were performed. Survival curves were plotted using the Kaplan-Meier method with differences calculated using log-rank tests (GraphPad Prism v 6.0). Experiments were approved by the Institutional Animal Care and Use Committee at Cubist Pharmaceuticals, Inc. and Monash University prior to initiation of studies.

Results and discussion

To determine the impact of DAP^R on staphylococcal virulence we first infected *G. mellonella* with a genetically characterized *in vitro* derived series of *S. aureus* strains that had incremental increases in the MIC of DAP and a cumulative number of mutations (Table 1).⁸ Importantly, these strains grew similarly *in vitro* except for CB1618-d20, which was impaired for growth (Supplementary Fig. 1). As shown in Figure 1A, there was a significant trend of reducing virulence as the MIC of DAP increased ($P < 0.05$, Chi square for trend). However, when individual strains in the series were compared, significant reductions in virulence occurred only on 2 occasions. The first occurred due to a mutation (R263C) in the sensor histidine kinase known as *walK* (previously *ycgG*). Working together with its cognate response regulator (WalR), this 2-component regulatory system is indispensable for cell wall homeostasis in *S. aureus* but has also been reported to regulate virulence.⁶ This isolate (CB1618-d9) had an increase in DAP MIC from 2 mg/L to 4 mg/L and it was attenuated in killing *G. mellonella* ($P < 0.01$) compared to its progenitor (CB1618-d6) (Fig. 1A). The second occurred with the final isolate of the series (CB1618-d20), which acquired a mutation in *rpoC*, leading to a substantial rise in MIC of daptomycin to 16 mg/L and marked virulence attenuation (Fig. 1A).

DAP^R in *S. aureus* has most commonly been associated with ‘gain of function’ point mutations in *mprF*.¹³ This leads to greater L-PG (cationic) being produced and translocated to the outer layer of the membrane causing a reduction in the net-negative membrane charge. As a consequence, these mutations simultaneously lead to resistance to daptomycin and cationic antimicrobial peptides.¹⁴ It is not surprising then that we observed no decrease in virulence for the first isolate of the laboratory-exposed series harbouring an *mprF* T345A mutation (CB1618-d6) (Fig. 1A). Conversely, we would expect that deletion of *mprF* would create a strain not only hypersusceptible to daptomycin but also less virulent as it would be hypersusceptible to cationic antimicrobial peptides. To test this hypothesis, we assessed an *mprF* deletion mutant (CB1118Δ*mprF*) from the same wild-type strain.⁹ Not only was CB1118Δ*mprF* hypersusceptible to daptomycin (MIC 0.125 mg/L, Table 1), it was also significantly less virulent compared to its parent strain ($P < 0.001$, Fig. 1B). Taken together, these data further highlight MprF as an attractive drug target, as inhibition of the protein may render *S. aureus* simultaneously susceptible to antimicrobials and the host innate immune system.¹⁵

To validate our findings from *G. mellonella* and to assess the impact of DAP^R on mammalian disease, we assessed the virulence of each attenuated mutant strain from the laboratory-derived series using an established murine septicaemia model.¹² Consistent with that observed in *G. mellonella*, CB1618-d9 (DAP MIC = 4 mg/L) and CB1618-d20 (DAP MIC = 16 mg/L) were significantly attenuated for virulence when compared to their susceptible parent strain (CB1118) ($P < 0.001$ for each) (Fig. 1C). We were intrigued by the survival of the DAP^R infected mice and wanted to assess whether the bacteria were being cleared or were persisting within the host. To test this, we assessed the bacterial burden in the kidneys of mice infected with each DAP^R strain at 7-days post infection. Despite the surviving mice, the kidneys of mice infected with CB1618-d9 showed high

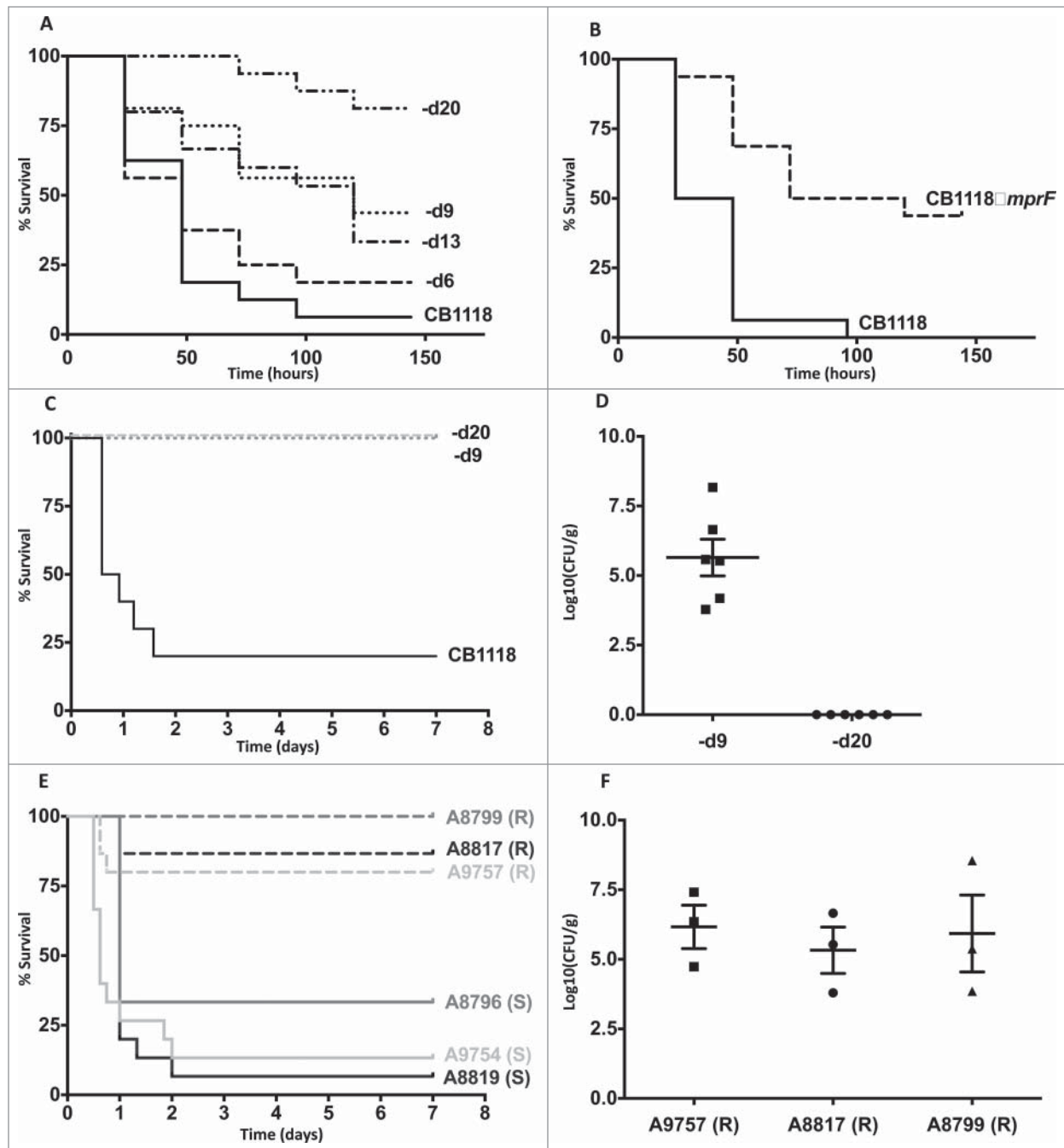


Figure 1. Daptomycin resistance in *S. aureus* correlates with altered virulence and *in vivo* persistence. **(A)** *G. mellonella* infection of a laboratory-derived series of isolates with incremental increases in daptomycin MIC was performed ($n = 16$ for each strain). For clarity, CB1618-d6, CB1618-d9, CB1618-d13, CB1618-d20 are represented by d6, d9, d13 and d20 respectively. Virulence attenuation was observed for CB1618-d9 when compared to CB1618-d6 ($P < 0.01$) and CB1618-d20 when compared with CB1618-d13 ($P < 0.01$). No significant virulence attenuation was observed for CB1618-d6 ($P = 0.44$) and CB1618-d13 ($P = 0.70$) when compared to their respective progenitor strains. **(B)** An *mprF* deletion strain (CB1118 Δ *mprF*) produced significantly less killing of *G. mellonella* when compared to its progenitor ($P < 0.001$, $n = 16$ for each strain). **(C)** CB1618-d9 and CB1618-d20 were attenuated for virulence in a murine septicaemia model ($P < 0.001$, $n = 10$ for each strain). **(D)** CB1618-d9 was capable of *in vivo* persistence as determined by bacterial densities in the kidneys of mice 7-days post infection. In contrast, bacterial burden was not observed in the kidneys of mice infected with CB1618-d20. **(E)** Virulence of 3 DAP-exposed clinical pairs was assessed using a murine septicaemia model. The daptomycin-resistant (R) isolates were significantly attenuated for virulence compared to their susceptible progenitors (S) ($P < 0.001$, $n = 15$ for each strain) and **(F)** were persistent in the kidneys of infected mice out to 7-days post-infection.

bacterial burdens (average of 2.5×10^7 CFU/g of tissue) indicating that this strain was persistent *in vivo* (Fig. 1D). In contrast, no bacteria were recovered from mice infected with CB1618-d20 suggesting that mutations in this strain were associated with *in vitro* and *in vivo* fitness costs (Fig. 1D and Supp. Fig. 1A).

As a corollary to what was observed for the laboratory-derived series, we next infected mice with 3 clinical *S. aureus* pairs consisting of a DAP^S parent strain with its corresponding DAP^R daughter strain that arose after DAP therapy and clinical failure. Each of the pairs were associated with severe infections including osteomyelitis, septic arthritis and endocarditis and were confirmed to be isogenic by whole genome sequencing as described previously (Table 1).⁵ As shown in Figure 1E, each of the clinical DAP^R strains were significantly attenuated for virulence when compared to their DAP^S progenitor strain ($P < 0.001$). Furthermore, as shown with the laboratory derived DAP^R strain (CB1618-d9), despite surviving animals out to 7 days post-infection, high bacterial loads were recovered from the kidneys of mice infected with each DAP^R strain (Fig. 1F). These data lend further support that clinical DAP^R *S. aureus* strains have the capacity to persist *in vivo*.

To our knowledge, this is the first study to describe an association between the development of DAP^R and altered *S. aureus* pathogenicity. Subtle genetic changes associated with DAP^R, such as a point mutation in WlkK (R263C), can have significant impact on the ability of the bacteria to cause mammalian disease. Intriguingly, despite DAP^R *S. aureus* strains causing less lethal disease, they have the capacity to persist *in vivo*, a finding consistent with that observed in humans.¹⁶⁻¹⁸ Developing a deeper understanding of the genetic determinants impacting antibiotic resistance and virulence in *S. aureus* will provide critical insights

into novel therapeutic strategies for this aggressive human pathogen.

Conflicts of Interest

A.Y.P has been to one advisory board meeting for Ortho-McNeil-Janssen and AstraZeneca, and has received a speaker's honorarium from AstraZeneca for one presentation. G.M.E. has served on Scientific Advisory Boards for Cubist, Bayer Schering, Johnson & Johnson Pharmaceutical Research and development, Novartis, Pfizer, Shionogi, Theravance; he has had research training support from Cubist, research contracts from Novoxel, Pfizer and Theravance, and speaking honoraria from Novartis. He serves on the Board of Directors of the National Foundation for Infectious Diseases. R.C.M. served as a consultant to Cubist, Forest, Merck, Novartis, Ortho, Johnston and Johnston, Pfizer, Theravance, and Wyeth. E. M received grant support and served on the advisory board for Astellas Pharma and received grant support from T2 Biosystems. L.I.M and A.R. are scientists working for Cubist Pharmaceuticals. All other authors have no conflicts of interest.

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