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In vivo exposure of insect AMP resistant *Staphylococcus aureus* to an insect immune system

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ABSTRACT

Antimicrobial peptides (AMPs) are important immune effectors in insects. Bacteria have a limited number of ways to resist AMPs, and AMP-resistance is often costly. Recently, it has become clear that AMP activities *in vitro* and *in vivo* differ. Although some studies have followed the *in vivo* survival of AMP resistant pathogens, studying a pathogen resistant to the AMPs of that particular host has never been reported. Here, we infected the mealworm beetle *Tenebrio molitor* with *Staphylococcus aureus* strains that were evolved *in vitro* in the presence of one or two antimicrobial peptides from *T. molitor*. We found that the *Tenebrio* immune system could clear mutant Tenecin resistant strains at least as efficiently as sensitive controls. The bacterial load of Tenecin resistant *S. aureus* segregated by mutation. Strains with mutations in both the *pmt* and *rpo* operons showed the highest *in vivo* survival and therefore showed the lowest fitness cost amongst the evolved resistance mutations. In contrast, Tenecin resistant strains with mutations in the *nsa and rpo* operons showed much lower survival within the hosts. Our study shows that Tenecin resistant strains are phagocytosed at a lower rate. The *nsa/rpo* mutants were phagocytosed at a higher rate than other Tenecin resistant *S. aureus* strains. The differences in resistance against AMPs and phagocytosis did not translate into changes in virulence. AMP resistance, while a prerequisite for an infection in vertebrates, does not provide a survival advantage to *S. aureus* in a host environment that is dominated by AMPs.

1. Introduction

Antimicrobial peptides (AMPs) are important immune effectors against microbes in multicellular organisms (Zasloff, 2002) and are a main component of immune defenses in insects (Mylonakis et al., 2016). Upon infection insects deploy a nonspecific cocktail of AMPs to clear infections (De Gregorio et al., 2001, 2002; Vogel et al., 2011; Johnston et al., 2014). AMPs in combinations can give rise to synergistic antimicrobial effects (Pöppel et al., 2015; Yu et al., 2016; Zanchi et al., 2017), and is assumed to reduce the risk of bacterial resistance evolution (Lazzaro, 2008; Chernysh et al., 2015).

AMPs are also studied and developed as novel therapeutics against multidrug resistant microbial infections and biofilms (Ge et al., 1999; Zasloff, 2002; Giuliani et al., 2007; Mylonakis et al., 2016; Pfalzgraff et al., 2018). *In vitro* experimental studies, however, have demonstrated that bacteria can readily evolve resistance towards AMPs (Perron et al., 2006; Habets et al., 2012; Dobson et al., 2013; Makarova et al., 2018). Given the conserved resistance mechanisms of bacteria against AMPs (Joo et al., 2016b), cross resistance to human AMPs resulting from the medical use of AMPs, has been suggested to be a likely scenario and has been dubbed 'arming the enemy' (Bell and Gouyon, 2003). Also, *in vitro* experimentally evolved strains display cross-resistance to AMPs. For example, pexiganan-resistant *S. aureus* strains are cross-resistant to human neutrophil defensin-1, which highlights the potential risks of AMP therapy (Habets and Brockhurst, 2012). The 'arming the enemy' concept was previously tested (Dobson et al., 2014), however the *S. aureus* strains were selected against AMPs that were originally isolated from a frog (pexiganan), a pig (iseganan) and a bee (melittin) (Dobson et al., 2013).

Here, we report the results of a study where we infected the mealworm beetle *Tenebrio molitor* with *S. aureus* that were previously selected for resistance to two of *T. molitor* AMPs using *in vitro* experimental evolution (Makarova et al., 2018). We believe the present study to be the first that uses experimentally evolved bacteria and exposes them subsequently to the selective agent in the host environment. This is motivated by the observation that AMP resistance plays a key role in *S. aureus* infections in humans (Cheung and Otto, 2018). Early work has characterized the main AMPs of *T. molitor*. Tenecin 1 is an inducible anti-Gram positive defensin (Moon et al., 1994), Tenecin 2 is an inducible anti-Gram negative coleoptericin (Roh et al., 2009). Both of

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these AMPs, as well as six others, are up-regulated for at least 7 days in response to an experimental *S. aureus* infection (Johnston et al., 2014). They remain up-regulated for up to 21 days post infection (Makarova et al., 2016). Tenecin 1 is the only *Tenebrio* AMP for which high antimicrobial (Gram + ve) activity has been described (Johnston et al., 2014). We also recently showed that a knock-down of Tenecin 2 expression results in increased mortality of beetles after *S. aureus* infection (Zanchi et al., 2017), despite the fact that Tenecin 2 is not active against *S. aureus in vitro* (Johnston et al., 2014). This demonstrates that *in vitro* activities of AMPs have only limited power to predict the *in vivo* function.

A number of AMP resistance mechanisms have been described in bacteria (Nawrocki et al., 2014; Andersson et al., 2016; Joo et al., 2016b). As most AMPs are cationic peptides which attack the bacterial cell wall, a common resistance mechanisms is to alter the cell wall structure and net charge (Joo et al., 2016b). It was shown that *S. aureus* can evolve AMP-resistance with different mutations (Dobson et al., 2014; Makarova et al., 2018). These mutations did not segregate according to AMP (Johnston et al., 2016). In our previous *in vitro* evolution of AMP resistance experiment, all resulting resistant strains harbored a mutation in either the *pmt* or *nsa* operons (Makarova et al., 2018). Resistance to the beetle AMPs used was always associated with a reduction in growth rate and an increase in the duration of lag phase at 37° C.

We have shown before that nonsense mutation in PmtR (orthologous to the *Bacillus subtilis* YtrA (39% homology (Rigali et al., 2002)) is associated with AMP resistance (Johnston et al., 2016). Pmt is an ATP-binding cassette (ABC) transporter that regulates export of Phenol soluble modulin (PSM) peptides (Chatterjee et al., 2013) and facilitates the evasion of the defenses by destroying immune cells, including human neutrophils, erythrocytes, and osteoblasts (Wang et al., 2007; Cheung et al., 2012; Rasigade et al., 2013). PmtR encodes a GntR-type transcriptional regulator and is located upstream of the *pmtA-D* genes. Binding of PmtR to the operator site of the *pmt* promoter represses the *pmt* operon (Joo et al., 2016a). Moreover, Pmt A-D acts as an efflux pump disposing human AMPs along with PSM (Joo et al., 2016b), triggering AMP resistance as a putative AMP transport system (Li et al., 2007).

In *S. aureus*, mutations in the nisin susceptibility-associated two-component system NsaSR (also known as BceS/BceR (Yoshida et al., 2011) and BraS/BraR (Hiron et al., 2011)) provides resistance against nisin and bacitracin. The NsaR binding site is upstream of two ABC transporters BraDE and vraDE; the former involved in signal transduction by BraSR while the latter facilitated detoxification by efflux (Coates-Brown et al., 2018; Makarova et al., 2018). Probably due to increased level of phosphorylation, point mutations in the NsaSR operon lead to constitutive expression of VraDE. This explains how *S. aureus* is consequently capable of adapting high concentrations of nisin A (Arii et al., 2019). We suggest that this might be a common mechanism of AMP resistance.

Often mutations in either *pmt*RS or *nsa*RS are accompanied by mutations in *rpo*BC (Makarova et al., 2018). The double mutation in *nsa*RS and *rpoBC* where highly costly, as reflected in a significantly increased lag time. *E coli* with mutations in the *rpoBC* gene are rifampin resistant (Jin and Gross, 1988; Alifano et al., 2015). Mutations in *rpo*BC were observed in *S. aureus* resistant against AMPs (Kubicek-Sutherland et al., 2016; Makarova et al., 2018).

In the present study, we assessed the infection dynamics of a suite of strains of *S. aureus* previously selected for resistance to either the *T. molitor* AMP Tenecin 1 or to both Tenecin 1 & Tenecin 2 over 14 days (Makarova et al., 2018). We quantified bacterial load and host survival. Since resistance was associated with mutations in either the *pmt*RS or *nsa*RS operons (Makarova et al., 2018), we analyzed the effects of particular mutations on the survival of *S. aureus* within the host which allows us to interpret the *in vivo* fitness cost of AMP resistance. We also re-assessed the growth parameters of AMP resistant mutants *in vitro* at 25° C to better reflect the *in vivo* conditions. Finally, we assessed whether AMP resistant mutants were differentially recognized by host hemocytes.

2. Materials and methods

2.1. Culturing of Tenebrio molitor

Early instar *Tenebrio molitor* larvae were purchased from a commercial supplier. Larvae were reared in the dark at 25° C in groups of 500 individuals with *ad libitum* access to wheat bran. Freshly pealed apple pieces were supplied 3 times per week as a source of water. The cultures were checked on a daily basis for pupae and dead individuals were discarded. Pupae were examined under a binocular dissecting microscope to determine sex. Newly emerged adults were kept individually in grid boxes supplied with bran and piece of filter paper. Males and females were kept in separate grid boxes. Each beetle was provided with a 1 mm^3 piece of apple 3 times per week. All experiments were performed on 9–14 days old adults with a weight ranging between 0.120 g and 0.190 g.

2.2. Bacteria

2.2.1. Bacterial strains

AMP resistant *Staphylococcus aureus* strains as well as their respective sensitive controls and the ancestor strain (SH1000), were described previously (Makarova et al., 2018). The AMP resistant strains are either resistant to Tenecin 1 or to a combination of Tenecin 1 plus Tenecin 2. All strains were kept as glycerol stocks in −80° C.

2.2.2. Bacterial culture and injection

Streaks over Muller Hinton (MH) agar from the glycerol stocks were obtained and incubated at 30°C for 48 h. Then 3 separate colonies were touched with culture needle and transferred to MH broth. The liquid culture was incubated overnight at 25°C, the same temperature at which the beetles were reared. The optical density of the 16-h culture was adjusted to 0.95 at 600 nm.

The bacterial culture was centrifuged for 10 min at 7500 g. Then the supernatant was discarded, and the pellet was resuspended in Ringer solution. The last step was repeated twice. 5 μl of the prepared inoculum was injected into each beetle; a bacterial load which is equivalent to 5×10^6CFU. The injections were performed using a manual injector attached to a sterile disposable capillary needle. The bacterial load was injected between the fourth and fifth abdominal sternites parallel to the anterior posterior axis of the body. The needle was inserted on the peripheral lateral aspect of the haemocoel to avoid perforating the internal organs. If leakage was noticed the specimen was discarded and replaced.

2.3. Quantification of infection

2.3.1. Experimental design

15 *Staphylococcus aureus* resistant strains and 5 sensitive strains were nested in 5 lines in addition to the ancestral strain (21 strains in total (Makarova et al., 2018)). Each group injected with a particular strain consisted of 20 specimens. The same setup was considered for each sex. The negative control group was injected with 5 μl Ringer solution. The experiment was designed to monitor the survival of *Staphylococcus aureus* strains that are resistant to one or two of the beetles own immune effectors (AMP) versus those which lack such a defense mechanism through different phases of infection. Therefore, the CFU count was monitored at 1, 3, 7- and 14-days post infection.

2.3.2. Recovering Staphylococcus aureus

Haemolymph was collected by a perfusion bleed technique (Haine et al., 2008). Briefly, the beetles were immobilized by placing over ice

for 10 min. They were subsequently soaked in 70% ethyl alcohol and dried on a sterile tissue. A small incision was made with a sterile scalpel and 500 µl of phosphate buffer saline (PBS) was flushed into the haemocoel using a 22-gauge needle that was inserted into the plural membrane between the head and the thorax. The outflow was collected into a sterile 1.5 ml tube from the abdominal incision.

The freshly recovered haemolymph was vortexed and then 100 µl was plated on MH agar. Serial dilution was performed if necessary, according to the expected CFU count range at each time point. The plates were incubated at 30°C and CFUs were counted manually 2 days later.

2.4. Survival experiment

We also checked whether the survival of beetles would be affected if infected with AMP resistant versus AMP sensitive *Staphylococcus aureus* strains. Each group was injected with one of 21 *Staphylococcus aureus* strains. Each group consisted of 20 beetles. A treatment control was injected with Ringer solution and a full control was not injected at all. To exclude sex inferred differences if found, the experiment was performed on both sexes. This resulted into 46 groups in total in our experimental setup. Mortality was monitored for 60 days post injection because immunopathology costs of early life inflammation can manifest later in life in *Tenebrio molitor* (Khan et al., 2017).

2.5. In vitro phagocytosis assay

Individuals were washed with distilled water then anaesthetized for 7 min with CO_2 prior to haemolymph collection. Next, they were swabbed with 70% ethanol and distilled water respectively. Haemolymph samples were obtained by cutting off the tibia of the first pair of legs. Haemolymph was mixed with 20 µl of a suspension of ringer fluid (RF) (274 mM NaCl, 19 mM KCl, 9 mM $CaCl_2$) and an anticoagulation buffer (ACB) (4.5 mM citric acid and 9 mM sodium citrate, 5:1 v/v) (Control) and *S. aureus* (OD_{600} = 0.95) and incubated for 30 min at room temperature on clean microscopic slides covered with poly-L-lysine (Sigma-Aldrich P4707). Then samples were washed with RF and fixed with a 4% solution of paraformaldehyde in RF for 15 min at room temperature. Permeabilization was performed using 0.1% Triton-X 100 (Sigma-Aldrich T8532) for 30 min. Samples were incubated with 1% BSA, 22.52 mg/mL glycine in PBST (PBS + 0.1% Tween 20) for 30 min block nonspecific binding. The samples were incubated with the diluted *S. aureus* polyclonal antibody (1:800, Thermo FisherPA1-7246) in 1% BSA in PBST in a humidified chamber overnight at 4 °C. The samples were then incubated with the diluted Goat anti-Rabbit IgG (H + L) secondary antibody conjugated with Alexa Fluor 488 (Invitrogen A-11034) (1:500) in 1% BSA in PBS for 1 h at room temperature in the dark. A control was performed by incubating fixed haemocytes and bacteria in PBS with secondary antibodies. Three PBS washes were performed between each of the previously mentioned steps. Slides were examined under a fluorescence microscope with using Nikon Eclipse Ti2 microscope equipped with Nikon DS-Qi2 digital camera. Next, the F-actin cytoskeleton of haemocytes was stained with Texas Red-X® phalloidin (Invitrogen) for 20 min, in the dark, at room temperature. Five photos were captured at random fields in each sample then the haemocytes with and without bacteria were counted. The results were expressed as the mean percentage of cells with fully phagocytosed bacteria in the total number of haemocytes on the images. Photos were analyzed with ImageJ (version2) software. Minimal number of individuals used in the experiment was 9.

2.6. Data analysis

All the data were analyzed in R version 3.3.2 (RCoreTeam, 2013).

2.6.1. Quantification of infection

The entire ringer injected group showed no infection and therefore, it was excluded from further analysis. First, we analyzed whether the number of CFU of the recovered *S. aureus* strains could be explained by time post infection, the sensitivity of particular strains toward Tenecin1 and a cocktail of Tenecin1 plus Tenecin2 *in vitro*, and sex of the injected beetle. A mixed model was fitted to the data set to account for the nested ontogeny of the 21 strains used, bacteria nested in lines was implemented as random factor.

We performed two analyses of the CFU counts. First, the best fit to the data we could obtain was by Box Cox transforming the CFU data (power transform). Then the function “lme” from “nlme” package was used to run a linear mixed model (lmm) on the transformed data. Function “varIdent” implemented in the weight of the model help to fix the heteroscedasticity of the variances between each infection treatment. Moreover, to account for the zero-inflation of the data and the heteroscedasticity of the variances between each infection treatment, a generalized linear mixed model (glmm) with a negative binomial distribution was fitted. The function “glmmaTMB” from package “glmmTMB” was used to fit that model. For significant findings, post-hoc comparisons were performed using “lsmeans” function with a tukey adjustment from package “lsmeans”, using “Anova” function from package “car”. The two approaches revealed the same significant results.

We were also interested in whether the AMP used for selection would explain the CFU at different time point of infection. We focused on analyzing the most abundant mutations. So we analyzed the CFU data using lmm and glmm models as described before. Sex of the injected beetle and time post infection were also considered as explanatory variables in the tested model.

2.6.2. Survival analysis

We tested whether the AMP resistance of the infecting strains and the sex of the infected beetle, as well as the interaction between them, explained the mortality of the beetles over 60 days. We analyzed the data using the “survreg” function from “survival” package based on assumption of non-constant hazard using Weibull errors. This was compared to the simple model assuming a constant hazard with exponential distribution with “anova” function. The earlier proved to be better. Also, the analysis was double-checked using “WeibullReg” from “SurvRegCensCov” package which assumed an accelerated failure time survival model (AFT) with a Weibull distribution. The results were consistent, so we only show the results from the first model. The model was simplified using the function “step” from package “stats”, a selection based on AIC comparison. The minimal adequate model excluded the sex effect.

2.6.3. In vitro phagocytic activity analysis

We tested whether the AMP resistant *S. aureus* were recognized and internalized by *Tenebrio* ‘s hemocyte at a different ratio than that of the unselected control strains. We analyzed the data using general linear mixed model with a binomial error distribution. The line was considered as a random factor. Effects were assessed using “Anova” function from “car” package. Contrasts were calculated using “ghlt” function from “multcomp” package. Function “visreg” from package “visreg” was used to visualize the contrast plot of the treatment and the mutation effect.

3. Results

3.1. In vivo survival of AMP resistant Staphylococcus aureus

Throughout the course of infection, bacterial loads of AMP-sensitive *S. aureus* (unselected control) declined sharply 24 h post infection, from $5*10^6$ CFU–10^3 CFU. Bacterial loads of Tenecin resistant strains followed the same pattern (Fig. 1). Surprisingly, bacterial loads of Tenecin

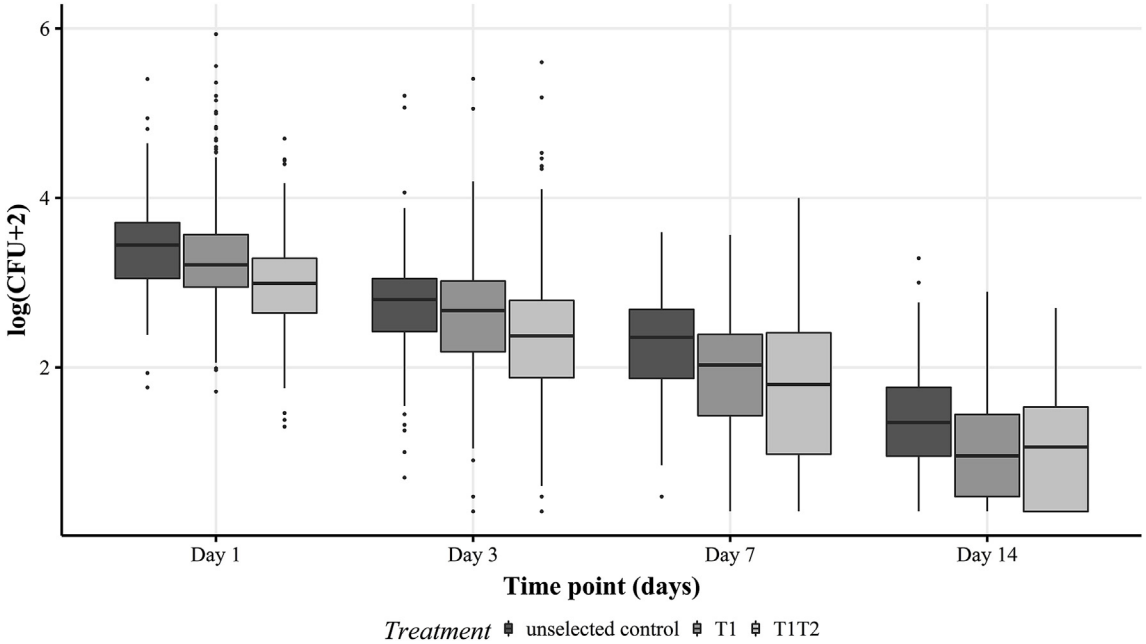

Fig. 1. Temporal infection dynamics of *S. aureus* strains selected in the presence of Tenecin 1 (defensin) and Tenecin 1 & 2 (defensin & coleoptercin), in *T. molitor* differed from unselected strains. Unselected strains survived better within the hosts than AMP-resistant strains. The CFU recovered from 100 µl of haemolymph are represented by box plots showing quartiles and medians. The bars represent the 1.5 interquartiles and the dots represent outliers. (Plots segregated by strain nested in lines for both sexes, see supplementary material: Fig. S1, S2 & S3).

Table 1
Standard deviations and Levene test for the homogeneity of variances in the CFU counts of unselected controls (AMP sensitive strains) and AMP resistant *S. aureus* at different time points of infection.

Time	AMP sensitive	AMP resistant	Levene's Test	
	SD	SD	F statistics	*P* value
Day 1	21112.18	40934.62	$F_{1,800} = 5.53$	**0.01**
Day 3	12928.71	20329.33	$F_{1,920} = 13.30$	**0.00028**
Day 7	621.78303	590.05	$F_{1,796} = 0.0015$	0.97
Day 14	163.26	71.45	$F_{1,867} = 0.071$	0.79

resistant strains were significantly lower than sensitive strains at all tested time points post infection (Anova (type I): time*treatment: $F_{6,3374} = 7.0839$; $p < .0001$). Tenecin 1 resistance did not confer any survival advantage to *S. aureus* in the host, and bacterial survival was lower than in infections with control strains (one day post infection: $T = -5.73309$, df = 3374, $p = < .0001$; 3 days post infection: $T = -5.77455$, df = 3374, $p = < .0001$, 7 days post infection: $T = -9.83414$, df = 3374, $p = < .0001$; 14 days post infection: $T = -6.78224$, df = 3374, $p = < .0001$). Bacterial loads of Tenecin 1 + 2 resistant strains showed significantly lower survival than Tenecin 1 selected lines one-week post infection ($T = -1.19618$, df = 3374, $p < .0001$). This suggests a higher fitness cost of evolving resistance to a cocktail of defensin and coleoptericin. The dispersion of the bacterial load of Tenecin selected lines is higher than the dispersion of unselected strains at days 1 and 3 post infection (Table 1).

3.2. Mutations conferring AMP resistance show in vivo reduced survival

The bacterial load of *pmt* mutants is lower than unselected control ($T_{day1} = -0.786$; $T_{day3} = -1.170$; $T_{day7} = -1.777$; $T_{day14} = -1.374$, df = 3515, $p < .0001$) (Fig. 2). The *rpo* mutants behaved similarly ($T_{day1} = -0.603$, df = 3515, $p = .039$; $T_{day3} = -0.757$, df = 3515, $p = .001$; $T_{day7} = -1.764$, df = 3515, $p < .0001$; $T_{day14} = -1.480$, df = 3515, $p < .0001$). However, an additional mutation in the *rpo* operon rescued the survival of *pmt* mutants to the unselected control level or even higher one week after infection ($T = 0.556$, df = 3515, $p = .01$). Therefore, strains possessing mutations in both *rpo* and *pmt* operons show the highest within host survival.

The bacterial loads of strains with a mutation in *nsa* operon fluctuate across infection time points (Fig. 2). At day 1 these mutants survive less well than the unselected control within the host ($T_{day1} = -4.081$, df = 3515, $p = < .0001$). This difference disappears at day 3 ($T_{day\ 3} = -1.418$, df = 3515, $p = .156$), but is present again one-week post infection ($T_{day\ 7} = -3.551$, df = 3515, $p = < .0001$). At day 14, *nsa* mutants showed the same loads as controls. ($T_{day\ 14} = 1.3651$, df = 3515, $p = .1$). An additional mutation in *rpo* operon did not influence the survival of *nsa* mutants until one-week post infection ($T_{day7} = 0.070$, df = 3515, $p = .801$). Additionally, 14 days post infection, the bacterial loads of *nsa*/rpo mutants survived less well in the host ($T = -1.044$, df = 3515, $p = .0001$).

Only one *S. aureus* strain of the 21 strains used in this study had a mutation in *rpo* operon without a second mutation in *pmt* or *nsa* operons (T1-1L). This strain had a lower bacterial load than the control across all time points ($T_{day1} = -2.178$, df = 3515, $p = .029$; $T_{day3} = -2.877$, df = 3515, $p = .004$; $T_{day7} = -6.724$, df = 3515, $p < .0001$; $T_{day14} = -6.424$, df = 3515, $p < .0001$).

3.3. Infection with AMP resistant S. aureus does not influence host survival

The survival of beetles infected with AMP resistant *S. aureus* compared with AMP sensitive unselected control strains did not segregate by treatment ($X^2 = 0.5483$, df = 1, $p = .459$, Fig. 3) or mutation ($X^2 = 1.888$, df = 5, $p = .8644$).

3.4. AMP resistant S. aureus are phagocytosed at a lower rate

The *in vitro* phagocytic activity of *Tenebrio* haemocytes segregated by treatment and sex (Analysis of deviance (type II Wald chi square test): treatment: $X^2 = 1117.09$, df = 2, $p < .0001$; sex: $X^2 = 147.05$, df = 1, $p < .0001$). The phagocytic ratios of Tenecin1 and Tenecin 1 + 2 selected lines were ca. 30% lower than sensitive unselected controls ($z = -29.83$, $p < .0001$, Fig. 4) (Fig. 5). Tenecin 1 selected

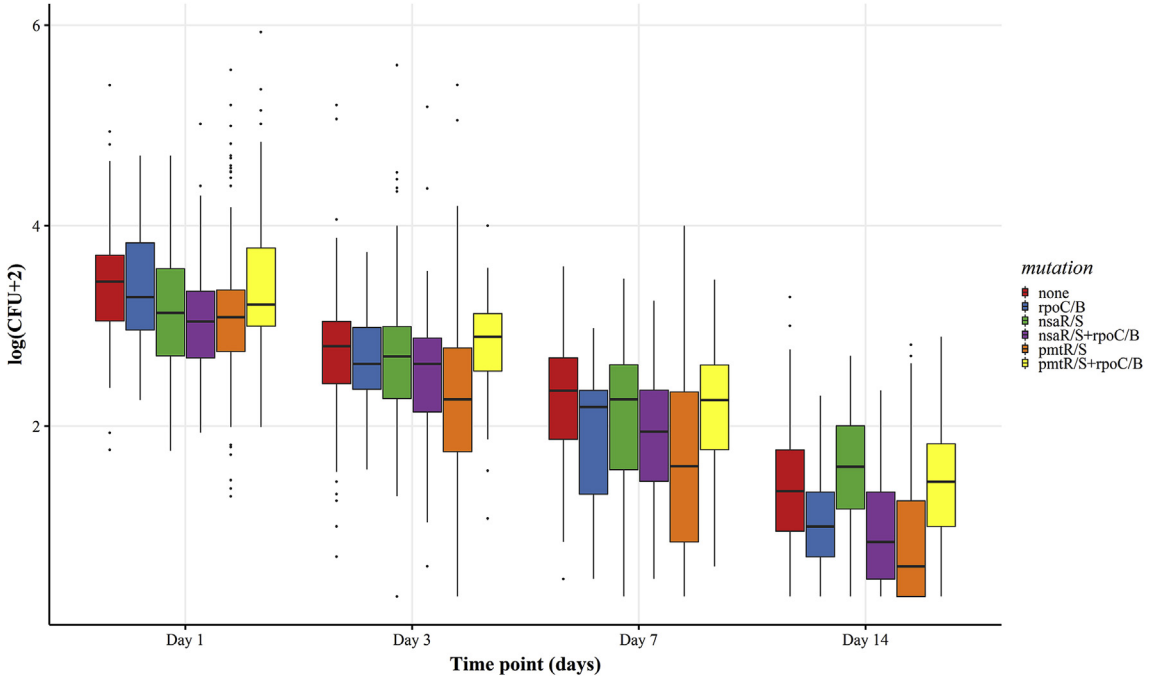

Fig. 2. *S. aureus* strains with mutations in *pmt* & *rpo* operons combined show increased survival in *T. molitor*. The bacterial loads of Tenecin selected mutants versus unselected control (none) is represented on a log scale of CFU recovered from 100 µl haemolymph at different infection time points.

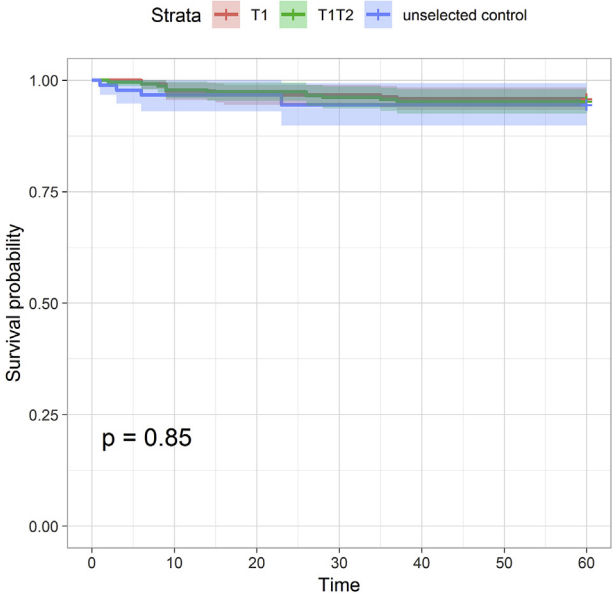

Fig. 3. AMP resistant *S. aureus* did not show increased virulence in *T. molitor*. The survival of *T. molitor* beetles infected with Tenecin 2 and/or Tenecin 1 resistant *S. aureus* strains did not different from the unselected control over 60 days of infection. For enlarged scale, see supplementary material: Fig. S4.

strains were phagocytosed at an odd ratio of 18% lower than the unselected control ($z = -18.60$, $p < .0001$). Strains that are selected against both Tenecin 1 and Tenecin 2 had been resistant to phagocytosis 33% more than the AMP sensitive strains ($z = -33.42$, $p < .0001$) and 17% to Tenecin 1 resistant strains ($z = -17.78$, $p < .0001$). The results above were averaged over sex and p values were adjusted for multiple testing (single step method: tukey).

3.5. All mutants except nsa&rpo show resistance against phagocytosis

Phagocytic activity segregates by mutations (Fig. 5 and Fig. 6, Analysis of deviance (type II Wald chis-quare test): mutation: $X^2 = 1042.89$, $df = 5$, $p < .0001$; sex: $X^2 = 139.89$, $df = 1$, $p < .0001$). Interestingly all mutants except *nsa/rpo* showed much lower phagocytotic ratios than unselected controls ($z = -30.79$, $p < .0001$). Tenecin 1 resistant as well as Tenecin 1 &Tenecin 2 resistant strains which have mutations in both *nsa* and *rpo* operons are phagocytosed only 3% less the unselected control ($z = -3.123$, $p = .02$). Phagocytic activity toward *pmt* mutants increased 3% when accompanied by a second mutation in *rpo* operon ($z = 3.281$, $p = .01$).

4. Discussion

Here, we studied the *in vivo* survival of *S. aureus* evolved to be resistant to the host's own AMPs using *Tenebrio molitor* as a host. We investigated different time points, corresponding to the acute and chronic phases of the infection. Even though the bacteria were resistant to one of the most abundant AMPs in the beetle haemolymph, we found that the bacterial load of AMP selected strains decreased notably segregating by both treatment and mutation. Host survival was not influenced by the resistance of the *S. aureus* strains, but Tenecin resistant strains were phagocytosed at a much lower rate. *In vitro* selected lines displayed lower fitness costs at the temperature of host infection (25°C) than in a previous study at 37°C (Makarova et al., 2018) (see supplementary material: Figs. S7 and S8).

Antimicrobial resistance evolves at a cost (Andersson and Hughes, 2010; Melnyk et al., 2015). The infection burden measured by bacterial load in the host is an important aspect of bacterial fitness (Howick and Lazzaro, 2014). Despite being resistant to the host's AMPs, Tenecin selected strains survived less well throughout the course of infection in *T. molitor* than the unselected controls. Strains that were selected against two AMPs showed lower survival than those selected against Tenecin 1. This lower bacterial survival can be interpreted as a fitness cost. From a host prospective, host resistance has a direct negative effect on bacterial fitness (Roy and Kirchner, 2000; Boots, 2008) as it interferes with within host pathogen survival and will reduce pathogen prevalence in a host population. Host investment in pathogen tolerance does not directly affect within-host pathogen survival, however the host evolves mortality or fecundity tolerance (Kutzer and Armitage, 2016).

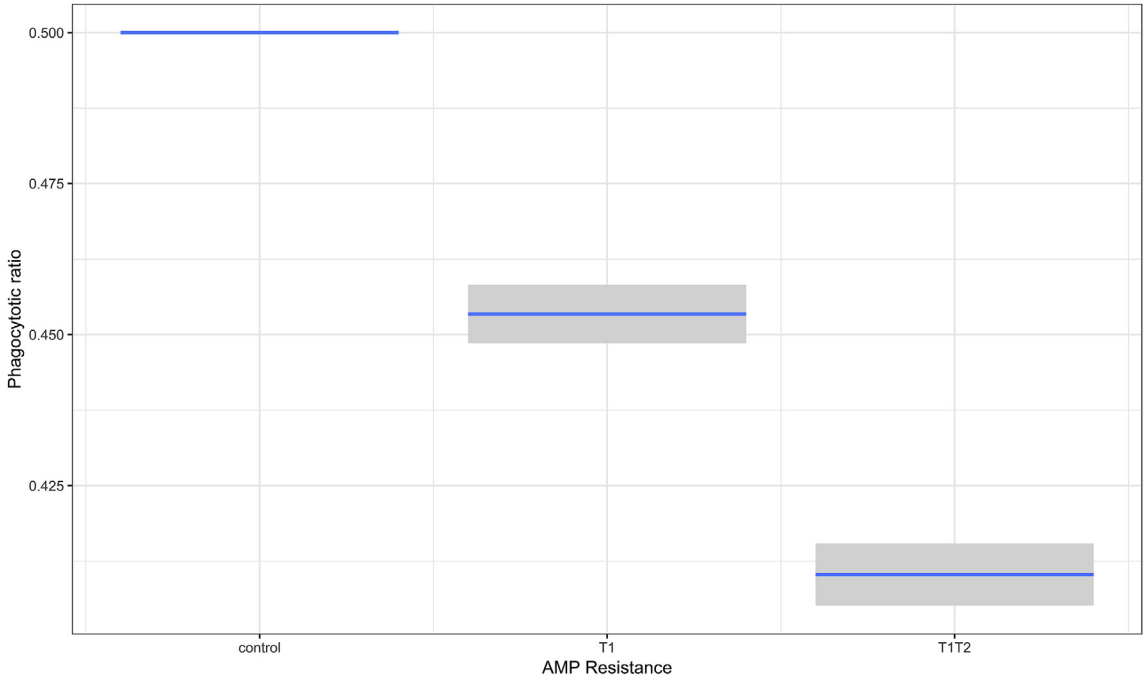

Fig. 4. Both Tenecin 1 resistant strains and Tenecin 1 &Tenecin 2 resistant *S. aureus* strains show elevated resistance against phagocytosis. The phagocytotic ratios of one droplet (1–2 μl) haemolymph after 30 min incubation with 20 μl of bacterial suspension (OD_{600} = 0.95) are represented by box plots showing the different quartiles and the marginal means extracted from a general linear mixed model with binomial error distribution using the visreg package. The bars represent the confidence interval retrieved for AMP resistant strains by a contrast plot. The results were grouped by treatment (Plots segregated by strain nested in lines for both sexes see supplementary material: Figs. S5 and S6).

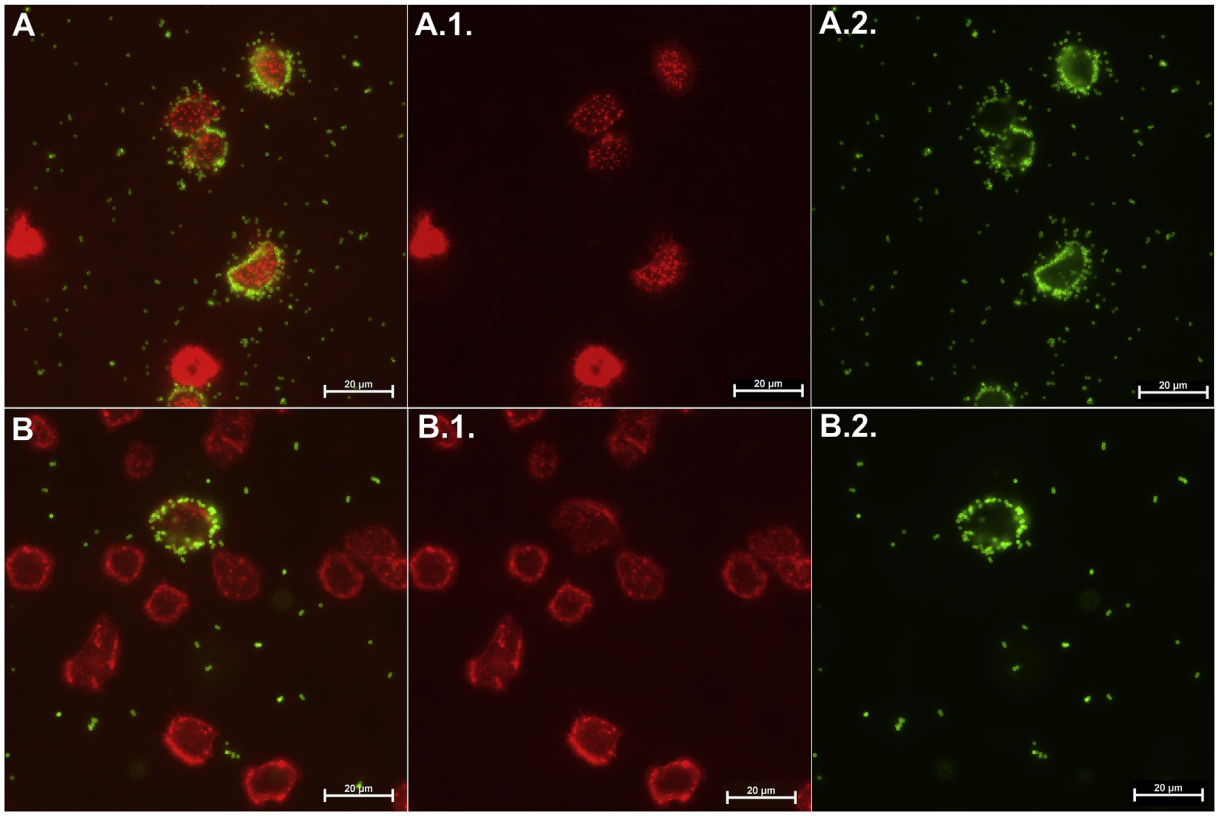

Fig. 5. Representative fluorescent photos of *Tenebrio molitor* haemocytes during phagocytosis of unselected control (A) and Tenecin resistant *S. aureus* (B) strains. A.1. and B.1. – F-actin cytoskeleton of haemocytes (red) stained with Texas Red-X phalloidin; A.2. and B.2. – bacteria (green) stained with using *S. aureus* polyclonal antibody (Thermo Fisher PA1-7246) and Goat anti-Rabbit IgG (H + L) secondary antibody conjugated with Alexa Fluor 488. A and B – merged photos. Photos were taken using Nikon Eclipse Ti2 microscope equipped with Nikon DS-Qi2 digital camera.

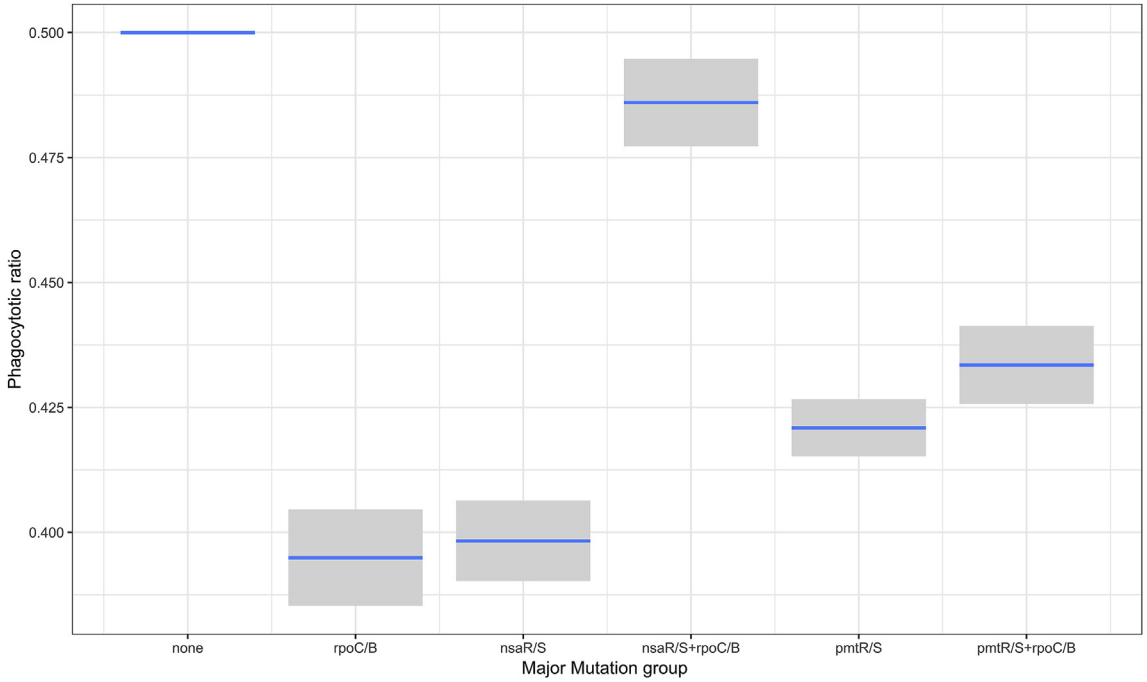

Fig. 6. Phagocytic ratio explained by mutations conferring AMP resistance in experimentally evolved *S. aureus*.

A host, harboring AMP resistant pathogens, should invest in reducing the pathogen load rather than tolerating it. We conclude that resistance against an abundant host AMP, with possible cross-resistance against other AMPs, did not result in increased bacterial load.

Resistance mutations are likely to cause a fitness cost because they target important biological functions in the cell (Melnyk et al., 2015). Our results showed that a mutation in *rpo*C incurred high fitness costs *in vivo* only, a situation that evolved with a low probability (1 strain out of 50 mutant carries a *rpo*C mutation that is not accompanied by *pmt* or *nsa* mutation (Makarova et al., 2018)). Other studies showed that *S. aureus* strains resistant to human AMP LL-37 with *rpo* mutations had reduced growth rates (Kubicek-Sutherland et al., 2016). It has been proposed that mutations in the genes *rpo*A and *rpo*C in *Mycobacterium tuberculosis* are compensatory mutations that alleviate the fitness cost incurred by rifampin resistance-conferring mutations in *rpo*B (Brandis et al., 2012). 25% of *S aureus* mutants in our study had *rpo*C mutation that was never accompanied by *rpo*B mutation in the same strain.

Tenecin resistance in *S. aureus* evolved with a mutation in either *pmt*RS or *nsa*RS which in a number of cases co-occurred with mutations in the *rpo*BC operon (Makarova et al., 2018). *S. aureus* strains having mutations in both *pmt*RS and *rpo*BC operons achieved a higher bacterial load *in vivo* than the unselected control. Moreover, *pmt/rpo* mutants survive better than strains having mutations in either *pmt* or *rpo* operons only. *pmt*RS mutants can continuously efflux host AMPs (Cheung et al., 2018) along with PSM cytotoxin bacterial secretion (Joo et al., 2016a). This is one of the few pumps that has been shown to accept AMPs as substrates (Cheung and Otto, 2018). PSM acts as a potent virulence factor against the host immune cells and affects key virulence-associated phenotypes (Chatterjee et al., 2013). Interestingly in our study, the advantage of having an additional *rpo*BC mutation was not found for *nsa*RS mutants. Although *S aureus* with a mutation in *nsa*RS operon achieved a bacterial load in the beetle equivalent to *pmt/rpo* mutants, *nsa/rpo*had a high fitness cost. It becomes clear that the second mutation is not always beneficial, yet, why the more costly double mutation evolves remains to be resolved.

A reduced growth rate *in vivo* (Majcherczyk et al., 2008) or *in vitro* (zur Wiesch et al., 2010) can reflect the fitness costs of a pathogen. We showed in an earlier study, at 37°C, that Tenecin 1 as well as Tenecin 1 + 2 selected lines showed slower growth rates in the exponential phase and extended lag phases (Makarova et al., 2018). In the present study, at 25°C, the temperature at which we rear *T. molitor*. *molitor*, we found that Tenecin 1 selected lines only showed extended lag phase compared to the unselected control. In contrary to 37°C (Makarova et al., 2018), the growth rates in the exponential phase did not segregate by treatment or mutation. Selected lines with the double mutation *nsa/rpo* showed the longest lag phase.

Neither the Tenecin selected *S. aureus* nor the ancestral strains decreased *T. molitor* survival. This finding could be explained by considering that host mortality due to infection is proportional to the within-host parasite replication rate (Read, 1994; Miller et al., 2006) and perhaps within-host bacterial load. In an earlier study (McGonigle et al., 2016), we found data supporting the idea that phagocytosed *S. aureus* in *T. molitor* re-enter the haemolymph. As we found, here, that AMP resistant lines are phagocytosed at a much lower rate, this could explain why the bacterial load is lower for most AMP resistant mutants and also possibly explain why the AMP resistant mutants did not exert increased virulence.

Bacterial AMP resistance is often conferred by changes in the cell wall structure and or of the net charge (Joo et al., 2016b). This can explain why Tenecin resistant strains were phagocytosed at a lower rate. NsaRS isan orthologous two-component system to the antimicrobial peptide sensing systemGraRS (Yang et al., 2012) which regulates the *Dlt* ABCD and the *MprF* expression in a VraFG dependent manner. The *dlt* operon regulates D-alanylation of the wall teichoic acids (Koprivnjak and Peschel, 2011) while *mprF* expression encodes proteins which positively charged lysine (Joo et al., 2016b). These evade electrostatic interaction mediated targeting of cationic AMPs (Falord et al., 2012). We suggest that this explains a reduced recognition by haemocytes and hence a lower phagocytic rate. In a study in *Drosophila* (Chung and Kocks, 2011), Eater-Fc was shown to facilitate Gram positive bacteria phagocytic recognition, and hence clearance. In presence of Cecropin A, a cationic antimicrobial peptide, Eater-Fc binding to live *E. coli* was promoted. Therefore, antimicrobial peptides might cooperate with phagocytic receptors to extend the range of microbes that can be targeted by a single, germ line-encoded receptor (Rahnamaeian et al., 2015). It is not clear why *nsa*RS/*rpo*BC mutants

are phagocytosed more than *nsa*RS mutants and *rpo*C mutants. The *pmt*RS capability of continuously pumping bacterial cytotoxin might also account for reduction in haemocytes number and hence phagocytic activity. PSM peptides (cytotoxin) were known to destroy human phagocytes, neutrophils (Cheung et al., 2012).

Bacterial AMP resistance mechanism leads to more pronounced pathogenicity *in vivo* (Cheung et al., 2018; Cheung and Otto, 2018). The *pmt/rpo* double mutants seem to come at no cost, and were as successful as the ancestral strain in establishing a persistent infection, and resistant to phagocytosis.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2019.04.017.

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