

Staphylococcus aureus Persisters Tolerant to Bactericidal Antibiotics

Sabrina Lechner^a Kim Lewis^b Ralph Bertram^a

^aInterfakultäres Institut für Mikrobiologie und Infektionsmedizin, Lehrbereich Mikrobielle Genetik, Eberhard Karls Universität Tübingen, Tübingen, Germany; ^bAntimicrobial Discovery Center, Department of Biology, Northeastern University, Boston, Mass., USA

Key Words

Persister cells • Multidrug tolerance • Antibiotics • Antimicrobials • *Staphylococcus aureus*

Abstract

Bacterial persister cells are non- or slow-growing reversible phenotypic variants of the wild type, tolerant to bactericidal antibiotics. We analyzed here *Staphylococcus aureus* persister levels by monitoring colony-forming unit counts of planktonically grown cells treated with six different antimicrobials over time. The model laboratory strains HG001–HG003, SA113 and the small colony variant (SCV) strains *hemB* and *menD* were challenged by the compounds at different logs of minimal inhibitory concentration (MIC) in exponential or stationary growth phase. Antibiotic tolerance was usually elevated in SCV strains compared to normally growing cells and in stationary versus exponential phase cultures. Biphasic killing kinetics, typical for persister cell enrichment, were observed in both growth phases under different selective conditions. Treatment of exponential phase cultures of HG001–HG003 with 10-fold MIC of tobramycin resulted in the isolation of persisters which upon cultivation on plates formed either normal or phenotypically stable small colonies. Trajectories of different killing curves indicated physiological heterogeneity within persister subpopulations. Daptomycin added at 100-fold MIC to stationary phase SA113 cells rap-

idly isolated very robust persisters. Fractions of antibiotic-tolerant cells were observed with all *S. aureus* strains and mutants tested. Our results refute the hypothesis that *S. aureus* stationary phase cells are equivalent to persisters, as not all of these cells showed antibiotic tolerance. Isolation of *S. aureus* persisters of different robustness seems to depend on the kind and concentration of the antibiotic, as well as on the strain used.

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Introduction

Bacterial persister cells were described by Joseph W. Bigger [1944] who studied the action of penicillin against staphylococci. Addition of the antibiotic to liquid cultures led to the sorting out of a small number of survivors he termed persisters. Upon reinoculation, these cells gave rise to a culture that again was susceptible to the antibiotic, forming a new subpopulation of persisters. Decades later, comparable observations were made [Gunnison et al., 1964; Mayhall et al., 1976; Newsom, 1970], confirming that a fraction of *Staphylococcus aureus* cells within a common culture survived antibiotic treatment although the original strains were susceptible to it. These cells were neither spheroplasts nor L-forms, they did not arise in response to the antibiotic and tolerance was not trans-

ferred to the progeny. Recently staphylococcal persisters were revisited, providing insights into the distribution of *S. aureus* and *S. epidermidis* persisters in biofilms and planktonically grown cells [Keren et al., 2004a; Möker et al., 2010; Shapiro et al., 2011]. Persisters have been identified in an increasing number of microorganisms, including *Pseudomonas aeruginosa* [Brooun et al., 2000; Harrison et al., 2005; Möker et al., 2010; Spoering and Lewis, 2001], *Escherichia coli* [Keren et al., 2004a, b; Shah et al., 2006], *Mycobacterium tuberculosis* [Keren et al., 2011] and the unicellular fungus *Candida albicans* [Lafleur et al., 2010; reviewed in Lewis, 2010a]. To identify genetic mechanisms underlying the persister state, Moyed and colleagues [Moyed and Bertrand, 1983; Moyed and Broderick, 1986; Scherrer and Moyed, 1988] carried out a selection for *E. coli* mutants with increased survival to ampicillin, which resulted in the identification of a mutant carrying the *hipA7* allele producing elevated amounts of persisters. Persisters are presumably formed due to stochastic variation in the levels of persister gene expression irrespective of antibiotic action [Balaban et al., 2004]. At the same time, the probability of persister formation can be strongly increased by stress. It was reported that the majority of *E. coli* persisters under DNA-damaging conditions are produced by expression of the TisB toxin, which is under the control of the SOS response [Dörr et al., 2009]. By definition, persisters are not mutants, but are regarded as phenotypic variants that are isogenic to normally growing cells within a common culture [Lewis, 2007, 2010a, b; Wiuff et al., 2005]. Persister levels in most species examined increase sharply during late exponential growth, reaching ~1% at the stationary phase [Keren et al., 2004a]. Unraveling the molecular mechanisms of persister formation in species other than *E. coli* has only recently begun, reflected by recent studies in *P. aeruginosa* or *M. tuberculosis* [Keren et al., 2011; Möker et al., 2010]. Regarding *S. aureus*, the observation that a stationary culture of strain ATCC 55585 was completely tolerant to the fluoroquinolone ciprofloxacin had suggested that stationary cells may be equivalent to persisters in this organism [Keren et al., 2004a]. We examined this possibility in the present study and found that stationary cultures of *S. aureus*, similarly to other species examined, represent a mixture of regular cells and persisters. We also considered persister formation by *S. aureus* small colony variant (SCV) strains that are intrinsically tolerant to selected antibiotics. SCVs are often isolated in hospitals and contribute to antibiotic recalcitrance of infections [von Eiff, 2008]. SCVs from both clinical isolates and genetically manipulated mutants are frequently impaired in the

respiratory chain. They may carry mutations in *hemB* that is involved in hemin biosynthesis [von Eiff et al., 1997], or *menD* required for menaquinone production [Bates et al., 2003], or may be auxotrophic for thymidine. Notably, their smaller size pertains to the colony but not on the single-cell level [Proctor et al., 2006]. Whether SCVs are also affected in persister production is an important question which we examined in this study.

Results

Examination of Persister Levels of Exponential Phase S. aureus Cells

To isolate *S. aureus* persister cells, we wanted to identify conditions under which (1) the bulk of cells of a culture is rapidly eradicated after the addition of an antimicrobial compound; (2) a low percentage of cells within this culture is still capable of forming colonies on nonselective rich media after up to 48 h, and (3) the colony-forming unit (CFU) count of the liquid culture does not increase to a sizeable extent after the antibiotic challenge, but exhibits a plateau or a slow decrease during the course of the experiment. Such outcomes are reflected by biphasic killing curves when CFU counts are plotted against time [Balaban et al., 2004; Dhar and McKinney, 2007]. During extensive in vitro antibiotic challenge experiments, we tested model laboratory *S. aureus* strains (table 1) exhibiting either normal growth or an SCV phenotype in liquid rich medium by five different antibiotics and one disinfectant applied at 1-, 10-, and 100-fold the minimal inhibitory concentration (MIC). These compounds and respective MIC values determined at the onset of this study are summarized in table 2. In a first approach, time-dependent eradication of exponentially grown cells was monitored. Results explicated in the following are restricted to conditions under which the trajectories of resulting killing curves indicated either persister isolation or other interesting phenomena. When ciprofloxacin was added to the cultures at 10-fold MIC, a rapid decrease in CFU counts was observed ranging from 94% (*menD*) to 99% (SA113) reduction after 2 h, followed by rather stable values for up to 24 h, indicating the presence of ciprofloxacin-tolerant persisters (fig. 1a). SCV strains were more tolerant to the drug than strains HG001, HG002, and HG003 (in the following referred to as 'HG strains' due to mostly uniform behavior in this study) and SA113, which were killed by ciprofloxacin at comparable rates. In the presence of 100-fold MIC of rifampin, live counts of SA113 decreased drastically,

Table 1. Strains used in this study

<i>S. aureus</i> strains	Relevant characteristic(s)	Reference
HG001	NCTC8325 derivative, <i>rsbU</i> repaired	Herbert et al., 2010
HG002	NCTC8325 derivative, <i>tcaR</i> repaired	Herbert et al., 2010
HG003	NCTC8325 derivative, <i>rsbU</i> and <i>tcaR</i> repaired	Herbert et al., 2010
SA113 (ATCC 35556)	NCTC8325 derivative, <i>agr</i> ⁻ , 11-bp deletion in <i>rsbU</i>	Iordanescu and Surdeanu, 1976
SA113 <i>hemB</i>	SA113 <i>hemB:ermB</i> , SCV phenotype	Gaupp et al., 2010
SA113 <i>menD</i>	SA113 <i>menD:ermC</i> , SCV phenotype	Gaupp et al., 2010

Table 2. MIC values of antimicrobial agents (μg/ml)

Strain	Penicillin G	Ciprofloxacin	Rifampin	Tobramycin	Daptomycin	Daptomycin + Ca ²⁺	BAC
HG001, HG002, and HG003	0.02	0.31	0.01	1.25	50	4.0	1.6
SA113	0.02	0.3	0.02	0.6	25	4.0	1.6
SA113 <i>hemB</i>	0.01	0.2	0.02	31	25	6.5	1.4
SA113 <i>menD</i>	0.01	0.2	0.02	31	25	6.5	1.4

BAC = Benzalkonium chloride.

whereas killing of HG strains and particularly SCV strains was considerably slower during the first 3 h (fig. 1b). When cultures were challenged by 10-fold MIC of tobramycin, live counts of all strains decreased by three or four orders of magnitude during the 1st hour and leveled off within the next 7 h in non-SCV strains (fig. 1c). Colonies grown from HG cells surviving this treatment gave rise to two different morphotypes. About 78% of them had the size of the parent strains, whereas ~22% had a stable, non-reverting SCV appearance. Treatment with tobramycin at 100-fold of the MIC resulted in rapid killing of the entire *hemB* and *menD* population, whereas CFU values of SA113 decreased by about four orders of magnitude after 1 h, followed by a rather slow decrease during the next 5 h (fig. 1d). Inexplicable fluctuations in case of HG strains precluded unequivocal conclusions under these conditions. Treatment of *hemB* and *menD* with 10-fold MIC of daptomycin resulted in a slightly bi-phasic killing pattern (fig. 1e), whereas the majority of SA113 cells were killed within the 1st hour and the remainder was eradicated completely after 4 h. Again, respective values were less meaningful with HG strains. The activity of daptomycin is strongly increased in the presence of calcium ions [Steenbergen et al., 2005], and indeed applying 100- or 10-fold the MIC of daptomycin

with 50 μg/ml Ca²⁺ eradicated all cultures within up to 4 or 5 h, respectively. The complete set of killing curves with exponential-phase *S. aureus* cultures, including treatments with additional antimicrobials listed in table 2, is provided in online supplementary figure 1A–G (for all online supplementary material, see www.karger.com/doi/10.1159/000342449).

Isolation of Persister Cells in Stationary Phase *S. aureus* Cultures

In order to reappraise whether all stationary growth phase *S. aureus* cells actually represent persisters, overnight cultures were treated with the same set of antimicrobials (except penicillin G) applied at identical concentrations as before. In contrast to treatment with higher concentrations of the quaternary ammonium compound benzalkonium chloride, which resulted in the complete eradication of all strains tested within 3 h or less (online suppl. fig. 1G), a high degree of drug tolerance irrespective of strains or antibiotic concentration was observed in most cases. Notably, tobramycin or daptomycin challenge resulted in selective eradication of cells. CFU values of most strains declined slowly upon treatment with 100-fold MIC of tobramycin whereas live counts of *menD* cells decreased by 94.6% within the 1st hour or by 99.9% with-

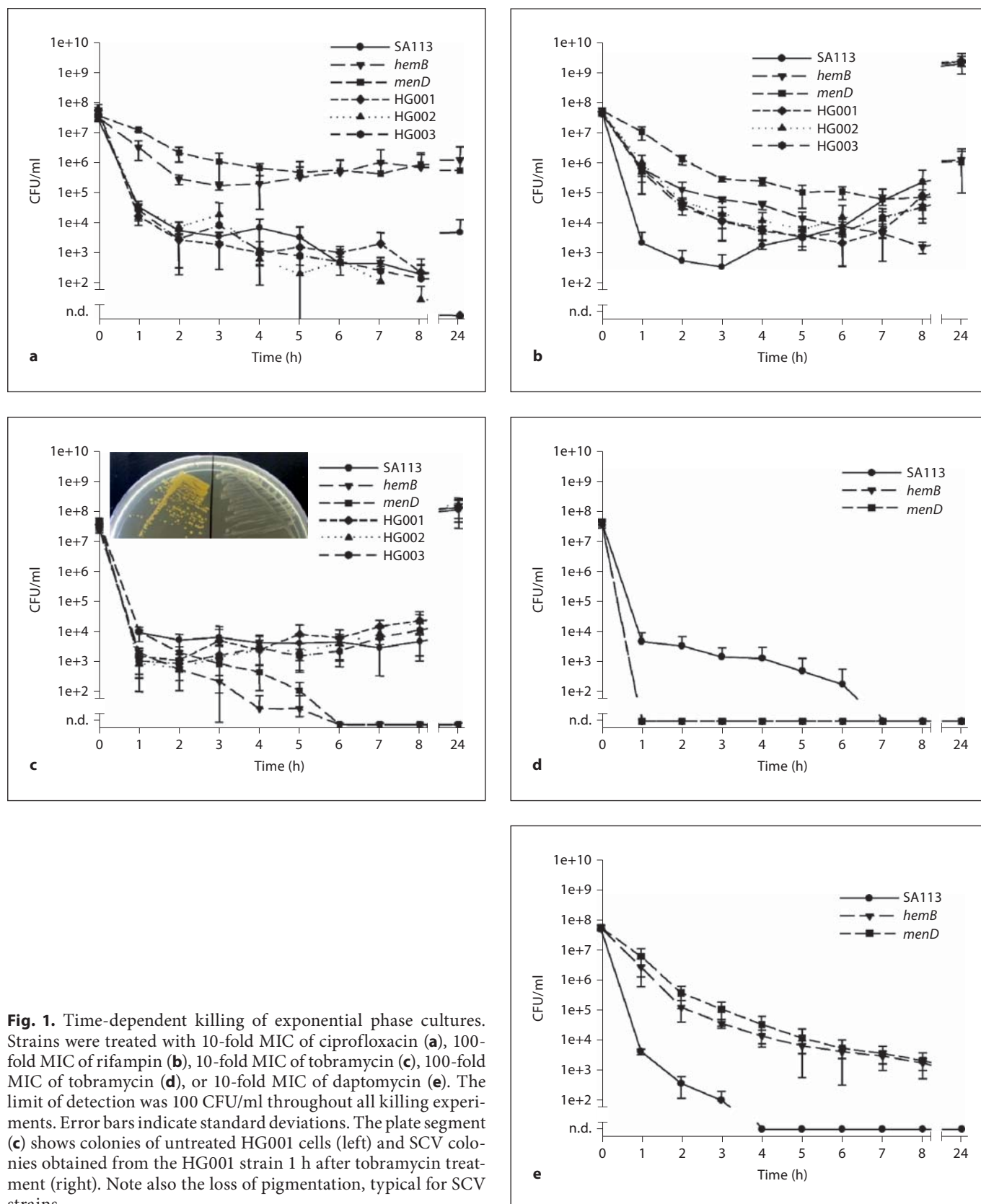


Fig. 1. Time-dependent killing of exponential phase cultures. Strains were treated with 10-fold MIC of ciprofloxacin (**a**), 100-fold MIC of rifampin (**b**), 10-fold MIC of tobramycin (**c**), 100-fold MIC of tobramycin (**d**), or 10-fold MIC of daptomycin (**e**). The limit of detection was 100 CFU/ml throughout all killing experiments. Error bars indicate standard deviations. The plate segment (**c**) shows colonies of untreated HG001 cells (left) and SCV colonies obtained from the HG001 strain 1 h after tobramycin treatment (right). Note also the loss of pigmentation, typical for SCV strains.

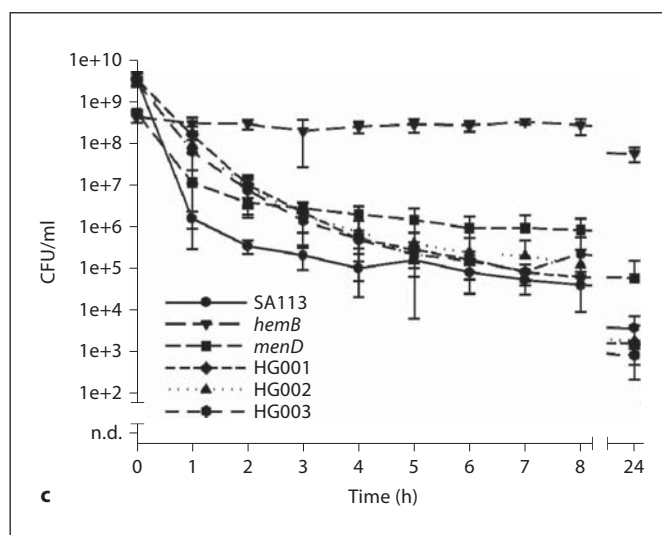
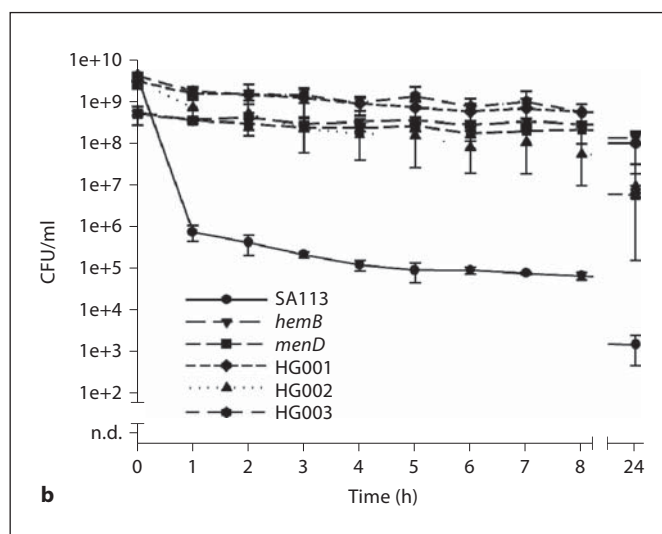
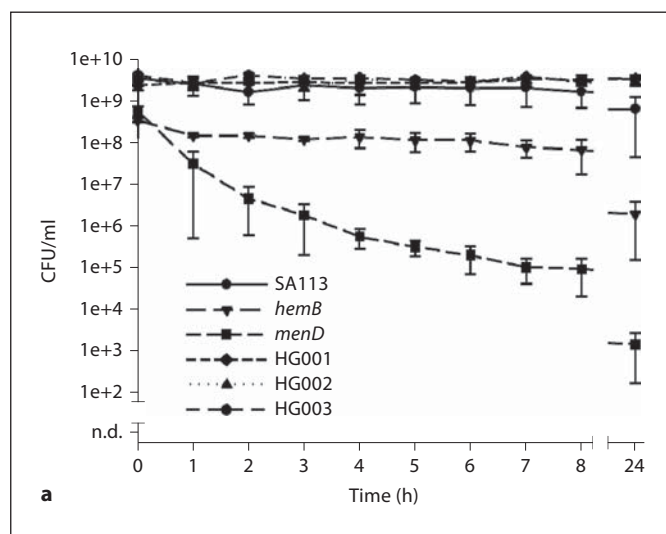


Fig. 2. Time-dependent killing of stationary phase cultures. Strains were treated with 100-fold MIC of tobramycin (a), 100-fold MIC of daptomycin (b), or 100-fold MIC of daptomycin/Ca²⁺ (c).

in 4 h, respectively, followed by an 84% reduction between time points $t = 4$ h and $t = 8$ h (fig. 2a). Challenging SA113 with 100-fold MIC of daptomycin yielded a killing curve that obeyed a very distinct biphasic pattern (fig. 2b). The bulk of cells (99.98%) were eradicated within 1 h, followed by a subsequent CFU decrease by only about 13%/h for the next 7 h or by about 4.3%/h for the next 23 h on average, respectively. Of note, killing curves observed with 100-fold MIC of daptomycin/Ca²⁺ were biphasic not only in case of SA113 but clearly also for the *menD* strain which had been unaffected by daptomycin alone (fig. 2c). CFU counts of HG strains declined rather continuously whereas the *hemB* population remained relatively stable. Addition of 1 μ g/ml hemin to the *hemB* culture resulted

in killing kinetics almost indistinguishable to those of SA113 (online suppl. fig. 2). The complete set of killing curves observed with stationary phase *S. aureus* cultures can be found in online supplementary figure 3.

Stationary Phase SA113 Cells Are Rapidly Eradicated by Treatment with Combined Super-MICs of Daptomycin and Tobramycin

An overnight culture of SA113 was challenged simultaneously with 100-fold MIC each of daptomycin and tobramycin, to combine both conditions which had resulted in pronounced isolation of persisters before. Killing kinetics observed were clearly different compared to those of cultures treated with either of the drugs alone

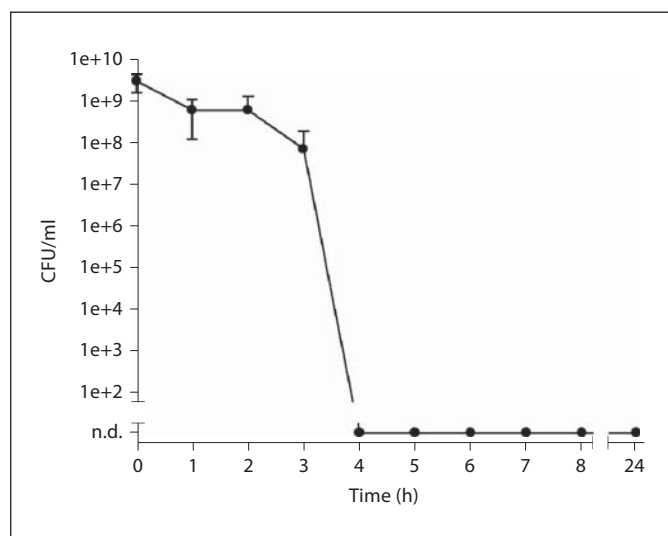


Fig. 3. Time-dependent killing of stationary phase SA113 by combined daptomycin and tobramycin treatment. Both antibiotics were applied at 100-fold MIC.

(fig. 3). After a 97% decline in CFU values during the first 3 h, the remaining population was entirely eradicated between time points $t = 3$ h and $t = 4$ h, which indicates a synergistic effect in antibacterial activity exerted by these two antibiotics.

Long-Term Antibiotic Challenge Leads to Complete Eradication of SA113 Cells

In order to assess the stability of persister levels over longer periods of time, CFU values of cultures treated with 100-fold MICs of antibiotics for 21 days were monitored. All cultures underwent considerable decreases in live counts within the first 7 days or earlier, whereas untreated cultures maintained high CFU levels during the entire course of the experiment. Formation of colonies on nonselective plates ceased, when cultures had been challenged with daptomycin or tobramycin for 3 days, with rifampin for 7 days or with ciprofloxacin for 21 days (fig. 4). The recurring CFU increase of cultures in the presence of penicillin G is rationalized by a concomitant constant decrease in antimicrobial activity within up to 10 days, whereas all other compounds tested were active up to the time of complete persister eradication or beyond (online suppl. fig. 4).

The Persister State Is Not Inherited in SA113 Cells

Exponential cultures of SA113 were repeatedly sorted out for persisters by treatment with 100-fold MIC of tobra-

mycin or 10-fold MIC of daptomycin to reveal whether antibiotic tolerance of *S. aureus* persisters is inherited over many generations. As depicted in figure 5a and b, respective killing curves after up to four cycles still resembled those of the original populations. The fact that persister levels were not elevated in later cycles indicates that the persister state of *S. aureus* is not vertically transmitted.

Discussion

Although staphylococcal persisters had been described more than 60 years ago [Bigger, 1944], a profound analysis of this drug-tolerant and putatively dormant state for this genus is lacking. We hence addressed a number of different questions regarding *S. aureus* persisters: (1) Do all stationary phase cells of *S. aureus* reside in the persister state? (2) Do exponential phase cultures also contain persister cells and, if so, to what extent? (3) Is there a correlation between persister levels and strain and genotype backgrounds? (4) Does the kind and concentration of an antimicrobial agent applied to comparable cultures influence persister levels? To establish a method for isolating drug-tolerant *S. aureus* persisters, antibiotics were applied which act on most of the common bacterial target structures, such as the cell envelope (penicillin, daptomycin), ribosome (tobramycin), RNA polymerase (rifampin), or topoisomerase (ciprofloxacin). Sorting for persisters was observed independently of the chemical nature or the target of the drug in accordance with previous studies on *S. epidermidis* [Shapiro et al., 2011] and other bacteria [Keren et al., 2011; Spoering and Lewis, 2001]. Generally, *S. aureus* cells in stationary growth phase were less vulnerable by antibiotics than those in exponential phase, arguably due to elevated persister levels [Wiuff et al., 2005]. Under most of the conditions we tested, the survival rates of stationary phase cells were either unaffected by antibiotic treatment (particularly upon challenge with 1- or 10-fold MICs of drugs) or CFU counts decreased rather constantly during 24 h (see online suppl. fig. 3). By contrast, the strong recurring increase in CFU counts of exponential phase SA113 treated with 100-fold MIC rifampin (fig. 1b) indicated genetically determined changes in susceptibility. These can be achieved by single amino acid exchanges in RNA polymerase β -subunit (RpoB) of *S. aureus* [Aubry-Damon et al., 1998]. Indeed, rifampin-challenged cells forming colonies on nonselective plates also grew well when restreaked on rifampin-supplemented plates (data not shown). During the antibiotic challenge procedure, a

Fig. 4. Long-term killing experiment. Stationary phase SA113 cultures were treated with 100-fold MIC each of daptomycin, tobramycin, ciprofloxacin, rifampin, or penicillin for 21 days. An identical SA113 culture without any antibiotic challenge served as a control.

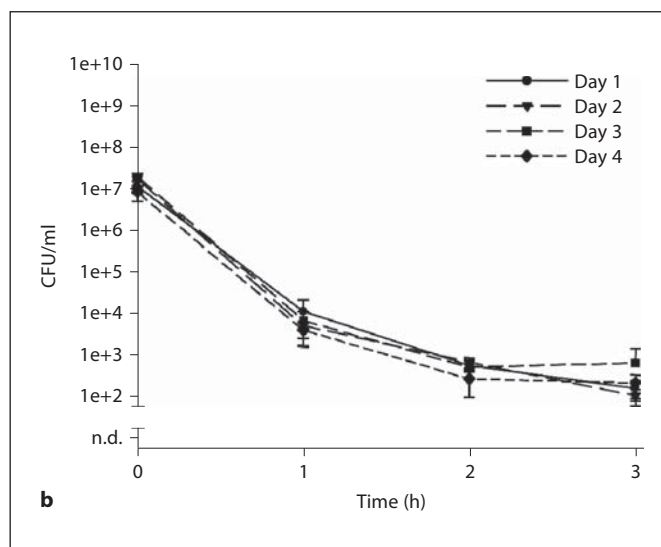
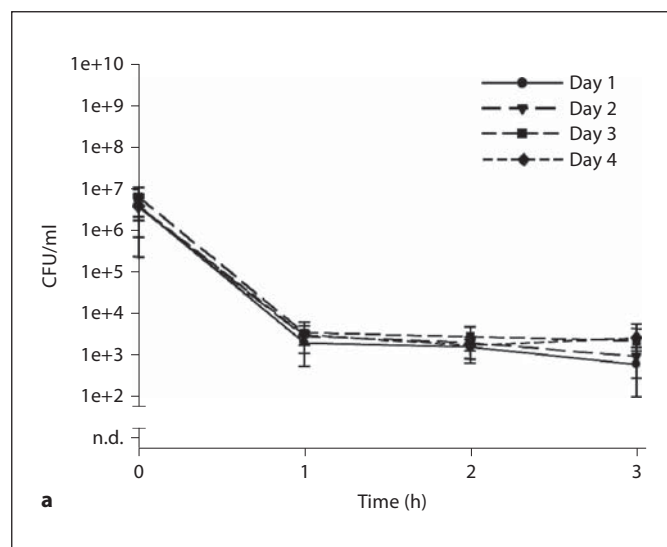
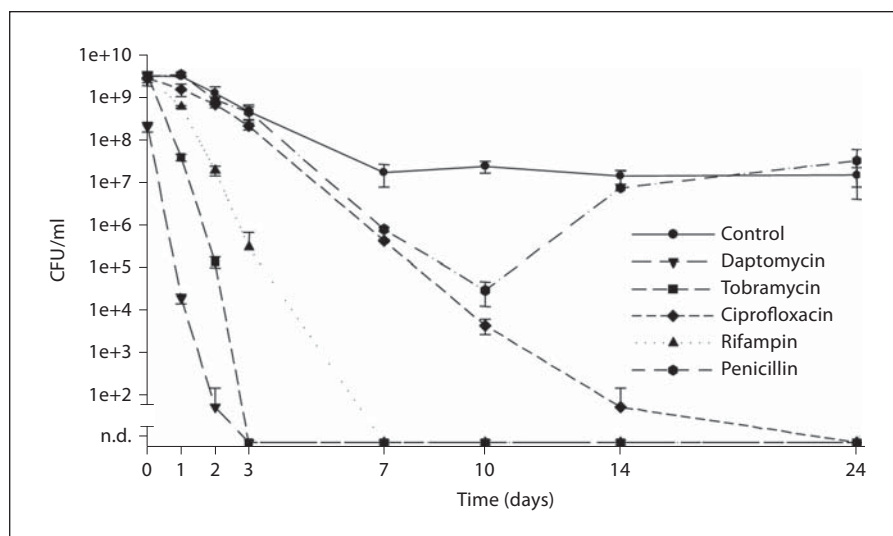


Fig. 5. Test for heritability of persistence. Exponential phase SA113 cells were challenged with 100-fold MIC of tobramycin (a) or 10-fold MIC of daptomycin/ Ca^{2+} (b) for 3 h in four consecutive cycles.

higher selective pressure for drug resistance was presumably exerted on populations mainly composed of rapidly killed cells. Accordingly, recovery of CFU counts was not observed with the more tolerant *hemB* and *menD* strains within 8 h. Eradication of exponential phase cells was most rapidly achieved with 10- and 100-fold MICs of the cyclic lipopeptide daptomycin, and this effect was augmented by the addition of Ca^{2+} cations [Steenbergen et al., 2005]. Indeed, daptomycin/ Ca^{2+} is effective against a number of *S. aureus* strains including *hemB* [Baltch et al., 2008; Begic et al., 2009; Mascio et al., 2007], and killing

kinetics of *S. aureus* ATCC 29213 cultures challenged with super-MICs of daptomycin/ Ca^{2+} had previously also displayed biphasic trajectories [Mascio et al., 2007], albeit not as pronounced as in the present study. Treatment of HG cells with 10-fold MIC of tobramycin in exponential growth phase isolated persisters that gave rise to both normally growing and small-colony variants in agreement with previous studies using the related aminoglycoside compound amikacin against *S. aureus* [Singh et al., 2009]. Tiny staphylococcal colonies had also been discovered by Bradley et al. [1978] when clinical isolates were

tested for tolerance towards oxacillin, cephalothin, or gentamicin. These had resumed the same size, pigmentation, and hemolytic characteristics as the parent strain after 24 h, which is in contrast to the phenotypically stable SCVs we obtained (fig. 1c). Whether the latter are the result of nongenetic heterogeneity [Tuchscher et al., 2011] or of mutations [von Eiff et al., 1997; Wiuff et al., 2005] may be clarified by analyzing possible hemin, thymidine, thiamine, or menadione auxotrophies due to defects in *hemB*, *menD*, or other factors.

Persisters of *S. aureus*, like other bacteria, appear to be physiologically heterogeneous in terms of robustness or fitness [Allison et al., 2011a]. Exposure of identical *S. aureus* cultures, assumed to contain a reproducible mixture of cells in different physiologic states, to different antibiotics resulted in a variety of killing curves. Some of these exhibited pronounced biphasic trajectories, indicating that the respective drug rapidly sorts for highly robust persisters (e.g. SA113 treated with 100-fold daptomycin in fig. 2b), whereas curves with rather smooth transitions from rapid to slower killing speak in favor of a compound which can be endured for longer periods also by less tolerant persisters. This is exemplified by *menD* cultures challenged in the stationary growth phase by tobramycin (fig. 2a), which kills nongrowing cells by interrupting translation. Resulting misfolded toxic peptides [Correia et al., 2006] are assumed to influence the persister state [Vazquez-Laslop et al., 2006].

As expected, not even very robust *S. aureus* persisters seem to be indefinitely inert against antimicrobials. During long-term experiments persisters of SA113 were eradicated completely when exposed to active drugs at high concentrations within 3–21 days (fig. 4; online suppl. fig. 4). Simultaneous treatment of stationary phase SA113 cultures with high concentrations of daptomycin and tobramycin eradicated persister cells completely within few hours, revealing a synergistic bactericidal effect exerted by this pair of drugs. It is conceivable that passive tobramycin entrance into cells was facilitated by daptomycin-mediated membrane damage, similar to the activated uptake of aminoglycosides into persister cells upon generation of a proton motive [Allison et al., 2011b]. In line with our results, Baltch et al. [2008] observed more efficient killing of *S. aureus* strains SH1000 and Newman upon treatment with a combination of daptomycin/Ca²⁺ and gentamicin than with either of the drugs alone. These results support the strategy to combat bacterial infections by applying suitable combinations of drugs [Cottarel and Wierzbowski, 2007]. It should be noted, though, that our study is generally restricted to provide model conditions

for isolating persisters, because conditions of 100-fold the MIC of antibiotics can hardly be achieved during clinical treatment.

The use of different well-established laboratory *S. aureus* strains and mutants demonstrates the genetic and genomic influence on bacterial persister levels. Generally, among the strains tested, SA113 was most susceptible to the applied antimicrobials, which may in part be due to its short generation time, particularly compared to SCV strains. Except for chromosomal resistance cassettes inactivating *hemB* or *menD*, SCVs of this study are isogenic to SA113 [Gaupp et al., 2010] and indeed chemical complementation of a *hemB* mutant resulted in an eradication pattern almost identical to SA113 (as tested with 100-fold MIC of daptomycin/Ca²⁺, see online suppl. fig. 2). By contrast, genomic comparison between HG strains and SA113, all derived from isolate RN1 [Novick, 1967], is less straightforward. SA113 was obtained by unspecific chemical mutagenesis and its genome sequence is unavailable, whereas HG strains resulted from targeted repairs of the transcriptional regulators RsbU and TcaR [Herbert et al., 2010]. The generally homogenous killing curves among HG strains indicate that neither of these two factors affects *S. aureus* persister levels. This is particularly remarkable in case of RsbU, which positively regulates the alternative transcription factor σ^B [Giachino et al., 2001], influences the expression of the *mazEF* toxin-antitoxin system [Donegan and Cheung, 2009; Senn et al., 2005], and downregulates the *agr* locus [Bischoff et al., 2001] encoding quorum sensing factors [Novick and Geisinger, 2008]. Overexpression of the MazF toxin has been described to induce bacteriostasis in *S. aureus* [Fu et al., 2009], which may have implications for persister formation. An interesting new finding points towards indole-induced persister formation in *E. coli* [Vega et al., 2012] and studies by Shapiro et al. [2011] showed that quorum sensing does not alter persister levels in *S. epidermidis*. Further studies will clarify whether small molecules influence persister levels in *S. aureus*.

Conclusions

We here demonstrate that growth phase, strain background, and genotype influence *S. aureus* persister levels. As in other bacteria, the concentration of these drug-tolerant cells clearly increased from exponential to stationary phase cultures and the persister state does not seem to be inherited [Keren et al., 2004a]. A systematic analysis of persister formation with clinical *S. aureus* isolates and

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