Research Article

Journal of Molecular Microbiology and Biotechnology

J Mol Microbiol Biotechnol 2012;22:381–391
DOI: 10.1159/000346073

Published online: January 25, 2013

Interplay between Population Dynamics and Drug Tolerance of *Staphylococcus aureus* Persister Cells

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Key Words

Persister cells · Population dynamics · Multi-drug tolerance · Antibiotics · Antimicrobials · *Staphylococcus aureus*

Abstract

Population dynamics parameters of *Staphylococcus aureus* strain SA113 were quantified based on growth and killing experiments with batch culture cells in rich medium. Eradication kinetics and the concomitant isolation of a subpopulation of drug-tolerant SA113 persisters upon treatment with super-minimal inhibitory concentrations of antibiotics such as ciprofloxacin, daptomycin, and tobramycin served as a basis for mathematical analyses. According to a two-state model for stochastic phenotype switching, levels of persister cells and their eradication rates were influenced by the antibiotics used for isolation, clearly indicating a heterogeneous pool of *S. aureus* persisters. Judging from time-dependent experiments, the persisters' degree of drug tolerance correlated with the duration of antibiotic challenge. Moreover, cross-tolerance experiments with cells consecutively treated with two different antibiotics revealed that multi-drug tolerance is not a necessary trait of *S. aureus* persisters isolated by antibiotic challenge. In some cases, the results depended on the order of the two antibiotic treatments, suggesting that antibiotic tolerance may be achieved by a combination of preexisting persisters and an adaptive response to drug exposure. Counts of live cells which had endured drug treatment increased only after lag phases of at least 3 h after the shift to non-selective conditions. Thus, this study provides quantitative insights into population dynamics of *S. aureus* persisters with regard to antibiotic challenge.

Introduction

Bacterial persister cells are dormant variants of regular cells that neither grow nor die in the presence of bactericidal compounds. Since persisters make up a small fraction within a culture dominated by isogenic sibling cells, the persister state is a paradigm of bacterial phenotypic heterogeneity [Dhar and McKinney, 2007; Lewis, 2010]. Wiuff et al. [2005] noted that a decrease in the overall bacterial mortality of a culture upon antibiotic challenge could be attributed to drug-tolerant persisters. Our present understanding of the heterogeneous response to antibiotics within a genetically uniform population is predicated on studies addressing persistence on two different levels of description. One type of studies asks for the regulatory network motifs that enable the coexistence

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of persistent and non-persistent cells. Positive feedback loop-mediated bistability and threshold amplification of intracellular regulatory noise were identified as critical for bacterial persistence [Balaban, 2011]. Genes associated with altered persister levels clearly point towards toxin-antitoxin systems as a prime instance [Cataudella et al., 2012; Keren et al., 2004a; Maisonneuve et al., 2011; Moyed and Bertrand, 1983; Shah et al., 2006; Vazquez-Laslop et al., 2006]. On a population scale, a mathematical analysis defined the important parameters that control the dynamics of the normal and persistent subpopulations to calculate switching rates between the two phenotypes [Gefen and Balaban, 2009]. Two types of persisters can be discriminated dependent on whether or not a triggering signal to enter the dormant state is required [Balaban, 2011; Balaban et al., 2004; Dhar and McKinney, 2007; Gefen et al., 2008]. Whereas the level of type I persisters increases at the onset of stationary growth phase, type II persisters appear to be formed continuously, irrespective of environmental stimuli [Balaban et al., 2004; Dhar and McKinney, 2007; Gefen and Balaban, 2009; Gefen et al., 2008; Kussell et al., 2005]. It seems that not one single mechanism is responsible for persister formation, but instead, the activation of different stress modules results in growth arrest, and various different genetic pathways may converge towards persistence [Allison et al., 2011; Dhar and McKinney, 2007]. The change between the dormant and the growing state is a hallmark of persister cells [Buerger et al., 2012; Gefen and Balaban, 2009; Jõers et al., 2010]. In variable environments, phenotype switching provides a bet-hedging strategy that has been proposed to be superior to sensing as an adaptive mechanism to ensure the population's survival [Kussell and Leibler, 2005; Kussell et al., 2005]. The exit of bacteria from dormancy may be triggered by extracellular compounds [Dworkin and Shah, 2010; Mukamolova et al., 1998] or in a stochastic manner [Buerger et al., 2012] and may be accompanied by specific lag-phases during the resuscitation process [Gefen et al., 2008; Jõers et al., 2010].

Although the persister state was described for staphylococci more than 65 years ago [Bigger, 1944], only few studies addressed persisters in this genus so far [Keren et al., 2004a; Möker et al., 2010; Shapiro et al., 2011; Singh et al., 2009]. Keren et al. [2004a] conjectured that a stationary culture might exclusively consist of persisters, whereas we found that the pool of stationary phase *Staphylococcus aureus* cells represents a mixture of persisters and drug-susceptible cells [Lechner et al., 2012]. Upon aminoglycoside treatment, selection of persister and small colony variant (SCV) cells, representing another and possibly related dormant form of bacteria, was observed [Wiuff et al., 2005]. *S. aureus* SCVs frequently have genetically manifested defects in their electron transport chain or auxotrophies for hemine, menadione, or thymine [reviewed by Proctor et al., 2006]. Most SCVs are inherently tolerant to aminoglycosides [von Eiff, 2008], and are capable of switching between the normal and the small colony phenotype, even during the course of an infection [Massey et al., 2001; Tuchscherr et al., 2011].

Based on our previous work on the identification of conditions for *S. aureus* persister isolation [Lechner et al., 2012], the present study aims at providing parameters of population dynamics for *S. aureus* cells grown in liquid culture and challenged by antibiotics. We analyzed persisters that were isolated by treatment with various antibiotics for cross-tolerance and resuscitation dynamics. Growth, death and switching rates of normal and persister cells were calculated, and subpopulation fractions of these cell types were defined. Our results demonstrate that the choice of antimicrobial compounds applied for isolating drug-tolerant *S. aureus* persisters is critical for the behavior of the population. The observed dependence on the choice of antibiotics suggests that there may be multiple types of persisters with complex patterns of cross-tolerance and that the response of the population to antibiotic challenge may be due to a combination of selection for preexisting persisters and an adaptive response.

Results and Discussion

To provide a basis for modeling of staphylococcal persister dynamics, we first revisited key results described in our previous study on *S. aureus* persisters [Lechner et al., 2012]. Therein, a number of *S. aureus* strains and mutants had been challenged with various antibiotics applied at different magnitudes of the minimal inhibitory concentration (MIC; 1-fold, 10-fold and 100-fold) at exponential or stationary growth phase, and bacterial killing had been monitored over time.

Mathematical Analysis of Killing Curves Indicates the Existence of Multiple S. aureus *Persister Types*

Antibiotic treatment experiments indicated a variety of dynamic behaviors of *S. aureus* cells of an isogenic inoculum in a common batch culture reflected by different killing kinetics. Eradication of exponential phase cells had frequently been biphasic, as expected in the presence of two different subpopulations, normal cells and persisters [Lechner et al., 2012]. We here analyzed mathemati-

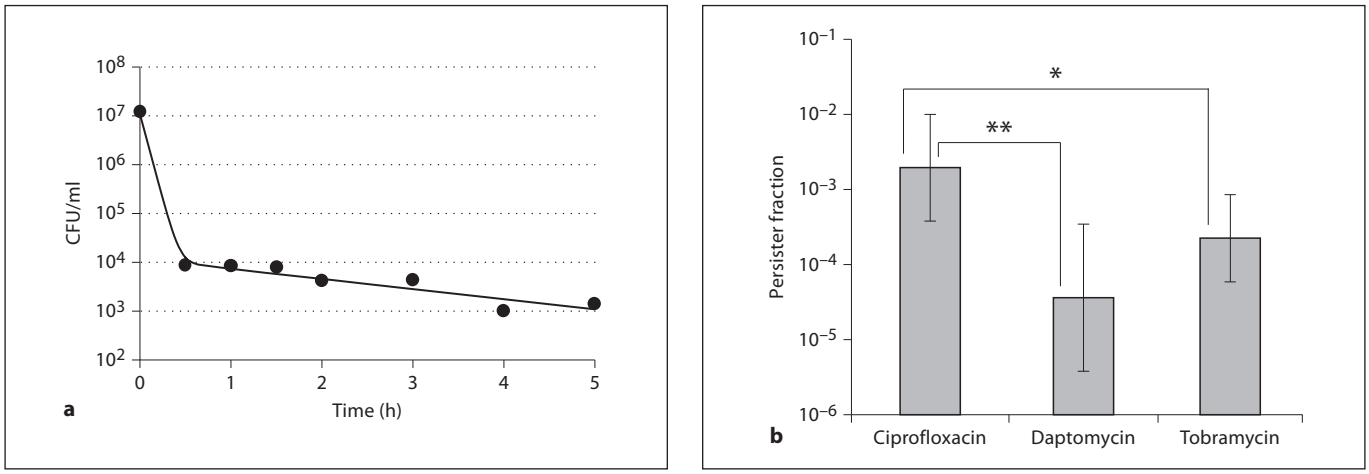

Fig. 1. Fitting of killing curves. **a** Example of a measured killing curve (data points, for strain SA113 taken from exponential growth phase and treated with 100-fold MIC of tobramycin) and a double-exponential fit to it (solid line). Fits like this one were performed for individual experiments and the resulting parameters averaged over repeat experiments to obtain the parameters given in table 1. **b** Initial persister fractions (i.e. fraction of persisters present in the population at the time of addition of the drug) obtained from the fits based on values obtained with ciprofloxacin (100-fold MIC), tobramycin (100-fold MIC) and daptomycin [10-fold MIC; mean plus standard deviation of $\log(p_0/n_0)$, see table 1]. Differences were found to be significant between ciprofloxacin and daptomycin (** p = 0.01) and ciprofloxacin and tobramycin (* p = 0.045).

Table 1. Parameters obtained from the fitting of killing curves

Antibiotic	Concentration	Number of experiments	Death rate of normal cells $\mu_n^{(AB)}$ (h^{-1})	Death rate of persister cells $\mu_p^{(AB)}$ (h^{-1})	Fraction of persisters $\log_{10}(p_0/n_0) = \log_{10} f_0$	Switching rate normal→ persister a (h^{-1})
Ciprofloxacin	10-fold MIC	3	−6.96 ± 0.20	−0.55 ± 0.12	−3.22 ± 0.21	7.4×10^{-4}
	100-fold MIC	6	−4.37 ± 1.62	−0.56 ± 0.20	−2.71 ± 0.71	2.4×10^{-3}
Rifampicin	10-fold MIC	3	−7.05 ± 2.47	−0.81 ± 0.36	−2.62 ± 1.13	2.9×10^{-3}
Tobramycin	10-fold MIC	3	−9.87 ± 1.66	−0.14 ± 0.08	−3.83 ± 0.26	1.9×10^{-4}
	100-fold MIC	6	−13.65 ± 4.82	−0.32 ± 0.22	−3.65 ± 0.58	2.8×10^{-4}
Daptomycin	10-fold MIC	6	−9.31 ± 0.81	−0.55 ± 0.55	−4.44 ± 0.98	4.6×10^{-5}
Daptomycin/Ca^{2+}	1-fold MIC	3	−3.13 ± 2.18	0.22 ± 0.68	−2.31 ± 0.31	6.0×10^{-3}

All values are for strain SA113, mean ± standard deviation from three or six experiments as indicated. Calculations are based upon results of Lechner et al. [2012].

cally the killing dynamics observed with the antimicrobials tobramycin, which inhibits translation, ciprofloxacin, which corrupts the function of topoisomerase, or daptomycin, which targets the cell envelope. A model for phenotype switching (defined by eq. 1 in 'Methods') was applied that describes isolation of preexisting persisters by these antibiotics. To that end, killing curves of different experiments using strain SA113 were fitted with a double-exponential expression (eq. 2) to obtain killing rates of the two subpopulations and the initial fraction of persister cells (table 1; fig. 1). The switching rate from the normal phenotype to the persister phenotype was estimated from the persister fraction. Not surprisingly, the death rates of the two subpopulations depended on the type and concentration of the antibiotic used, as also shown in other bacteria [Keren et al., 2004a, 2011; Maisonneuve et al., 2011]. In addition, considerable variation between the initial persister fractions (and thus the switching rates) was obtained from these fits, which argues against a single preexisting persister subpopulation isolated by the antibiotics, also in agreement with previous reports [Allison et al., 2011; Wiuff et al., 2005]. In

many cases however, the difference between persister levels observed in experiments with two different antibiotics was similar to the variation in persister fraction between repeats of experiments with the same antibiotic. Hence, we tested for the statistical significance of these differences using only experiments that were repeated six times (fig. 1b). The most striking difference was found between the persister fractions for SA113 cultures that had been treated with ciprofloxacin (100-fold MIC) and daptomycin (10-fold MIC) ($p = 0.01$; fig. 1b), but the difference between treatment with ciprofloxacin and tobramycin (100-fold MIC) was also found to be significant ($p = 0.045$). A dependence of the persister fraction on the drug used to isolate the persisters is not expected for a homogeneous subpopulation of preexisting persisters as assumed in the model of eq. 1. It may however be obtained if the persister subpopulation is heterogeneous with different types of persisters exhibiting tolerance to different antibiotics or if the observed tolerance to the drug involves an adaptive reaction to the drug exposure. The absolute values of the switching rates are in the range of $\sim 10^{-5}$ to 10^{-3} per hour. Previous estimates for the switching rate of *E. coli* upon treatment with ampicillin were in the same range [Balaban et al., 2004], with values for wild-type *E. coli* being slightly lower and values for a high-persistence mutant *(hipA7)* being slightly higher than in the present study.

Degree of Drug Tolerance of Isolated S. aureus *Persisters Is Dependent on the Kind and Duration of Antibiotic Treatment*

Deduced from our data and from studies in other bacteria [Allison et al., 2011; Gefen and Balaban, 2009; Lechner et al., 2012; Lewis, 2007], an *S. aureus* culture in the logarithmic phase was expected to mainly consist of susceptible cells and a tiny subpopulation of bacteria (that may collectively be classified as type II persisters) which exhibit different degrees of drug tolerance. The addition of an antibiotic should then isolate cells gradually more tolerant over time to yield a population dominated by highly robust persisters. To further corroborate this picture, we performed a test for phenotypic tolerance to antibiotics proposed earlier [Wiuff et al., 2005], in which tolerant cells isolated with an antibiotic are reexposed to fresh medium with the same antibiotic. To this end, SA113 cells were treated at exponential growth phase with 100-fold MIC of ciprofloxacin, 10-fold MIC of daptomycin, or 100-fold MIC of tobramycin for 30 min, 1.5 h, and 3 h (primary culture), and the washed pellets were transferred to fresh media supplemented with the identical antibiotics at the same concentration (secondary culture). Cells grown under nonselective conditions in the first culture, but exposed to the respective drugs in the second culture served as controls. The same experiment was also conducted with stationary-phase cells treated with 100-fold MIC of daptomycin/Ca^{2+}. Generally, cells taken from selective primary cultures were more tolerant to the respective antibiotics than naïve cells first grown under antibiotic-free conditions. Killing rates of secondary culture cells exposed to ciprofloxacin or daptomycin were reciprocally correlated with the length of drug exposure in the primary culture (fig. 2a, b, d), whereas this was not the case for bacteria sampled from tobramycin-containing medium. There, the slower colony-forming unit (CFU) decrease was independent of whether cells in the first culture had been treated with the drug for 30 min or longer (fig. 2c). Comparable findings were described by Wiuff et al. [2005] for *E. coli*. In line with data of our first study [Lechner et al., 2012], killing kinetics of secondary culture cells corroborate the assumption that a highly tobramycin-tolerant pool of persisters was rapidly isolated by 100-fold MIC of the drug. In contrast, sorting of robust persister cells appears to occur rather slowly (within 3 h) after addition of 100-fold MIC of ciprofloxacin or 10-fold MIC of daptomycin. Cells with a lower degree of drug tolerance thus seem to be reprieved from killing at earlier time points. A comparison with the model using the parameters obtained from table 1 indicates that the reexposure experiments led to population sizes that were very similar to what was obtained with continued exposure to that antibiotic (correlation coefficients $R \geq 0.9$). This observation provides further quantitative support for the phenotypic tolerance model used to describe the killing assay above. Within the model, the difference between the antibiotics in the kinetics of persister isolation were mostly due to the death rate of the susceptible cells, which was significantly higher for tobramycin at 100-fold MIC than for the other two conditions (table 1).

Mono- and Multi-Drug Tolerance Is Not Necessarily Correlated in S. aureus *Persisters*

The analysis of the eradication experiments suggested that there may not be one single persister phenotype, but several types of dormant cells in different physiological states and with different patterns of drug tolerance, consistent with the model of Allison et al. [2011]. To shed further light on possible differences in drug tolerance among a pool of persisters, cultures were again exposed to one antibiotic for 0.5–3 h, but now cells were subsequently challenged by a different drug for an additional

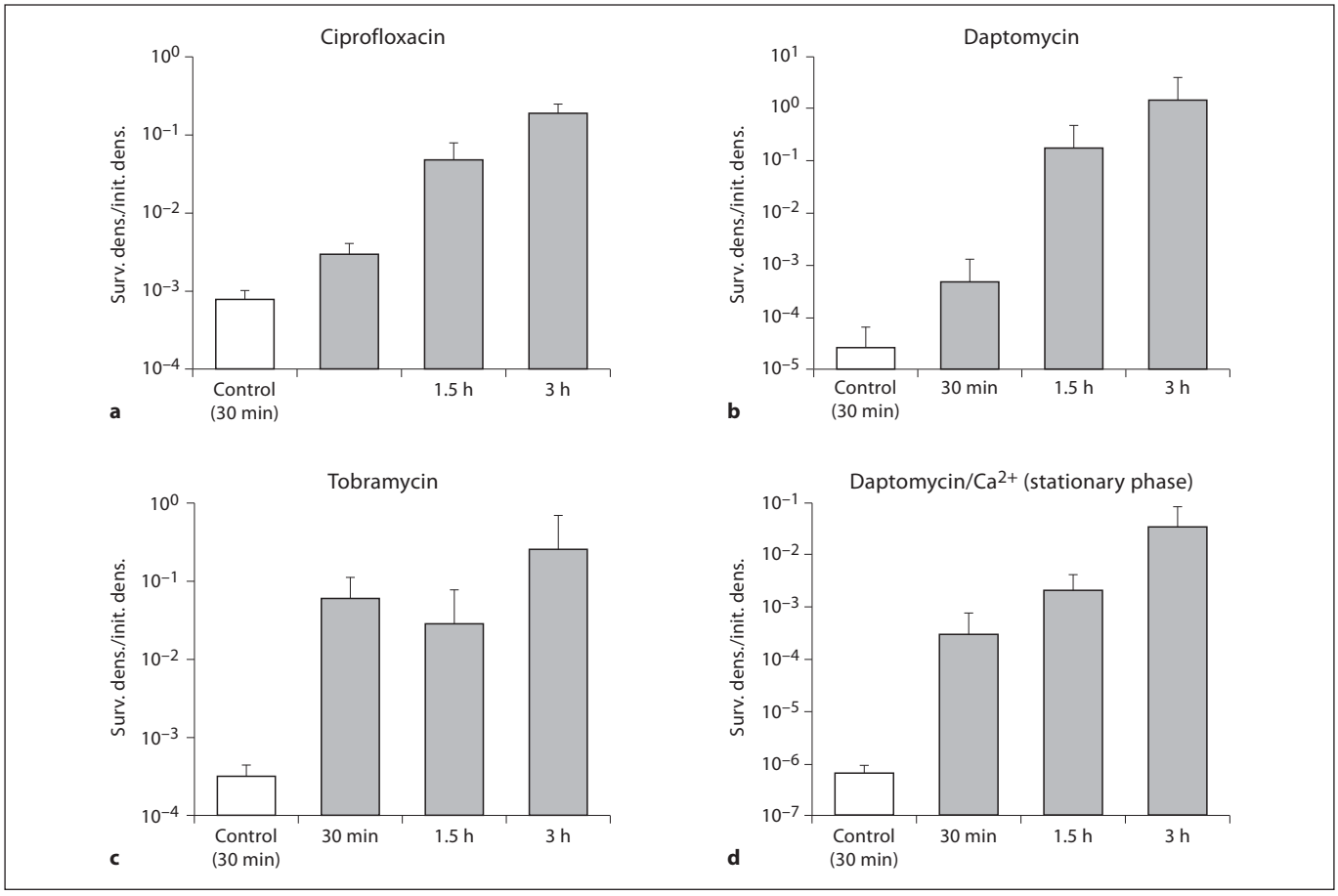

Fig. 2. Recurrent reexposure experiments. Exponential phase SA113 cells were treated with 100-fold MIC of ciprofloxacin (**a**), 10-fold MIC of daptomycin (**b**), or 100-fold MIC of tobramycin (**c**), or stationary phase cells were challenged with 100-fold MIC of daptomycin/Ca^{2+} (**d**). Cells from samples taken after 30 min, 1.5 h, and 3 h were then treated again with the same antibiotic at the same concentration. The values at the y-axis display the $\log_{10}$ ratios between CFU counts of surviving cells after 3 h of reexposure and CFU counts of cells prior to reexposure. The values are averages of three replicates, and the error bars indicate standard deviations.

3 h. Cross-tolerance was assumed when a similar or greater number of bacteria recovered after treatment with the second antibiotic compared to recurrent reexposure to the same drug. Results shown in figure 3 demonstrate that cross-tolerance was usually not observed, with the exception of cells treated first with tobramycin and second with ciprofloxacin (fig. 3a). For a quantitative analysis, the two-phenotype model of persisters was extended towards including multiple persister phenotypes, falling into three categories: (1) tolerant to the first antibiotic but not to the second; (2) tolerant to the second but not to the first, and (3) tolerant to both antibiotics. To account for the biphasic nature of the observed killing curves for single antibiotics (as compared to more complex dynamical behavior that would be possible in models with several types of persisters), all cells susceptible to an antibiotic were assumed to be killed with the same rate as the normal cells. This assumption fixed all parameters of the model except the relative frequencies of the different types of persisters in the population. Figure 4 shows killing curves predicted from the extended model for the combination of three antibiotics and several scenarios. In case of full cross-tolerance (solid lines), as expected for a single type of persisters, the killing rate is abruptly switched upon exchange of the antibiotic to that of persisters under the influence of the second antibiotic. In the complete absence of cross-tolerance, which is obtained if there are two mutually disjunct persister populations for the two antibiotics, rapid eradication with a rate similar to killing of susceptible cells is expected after the ex-

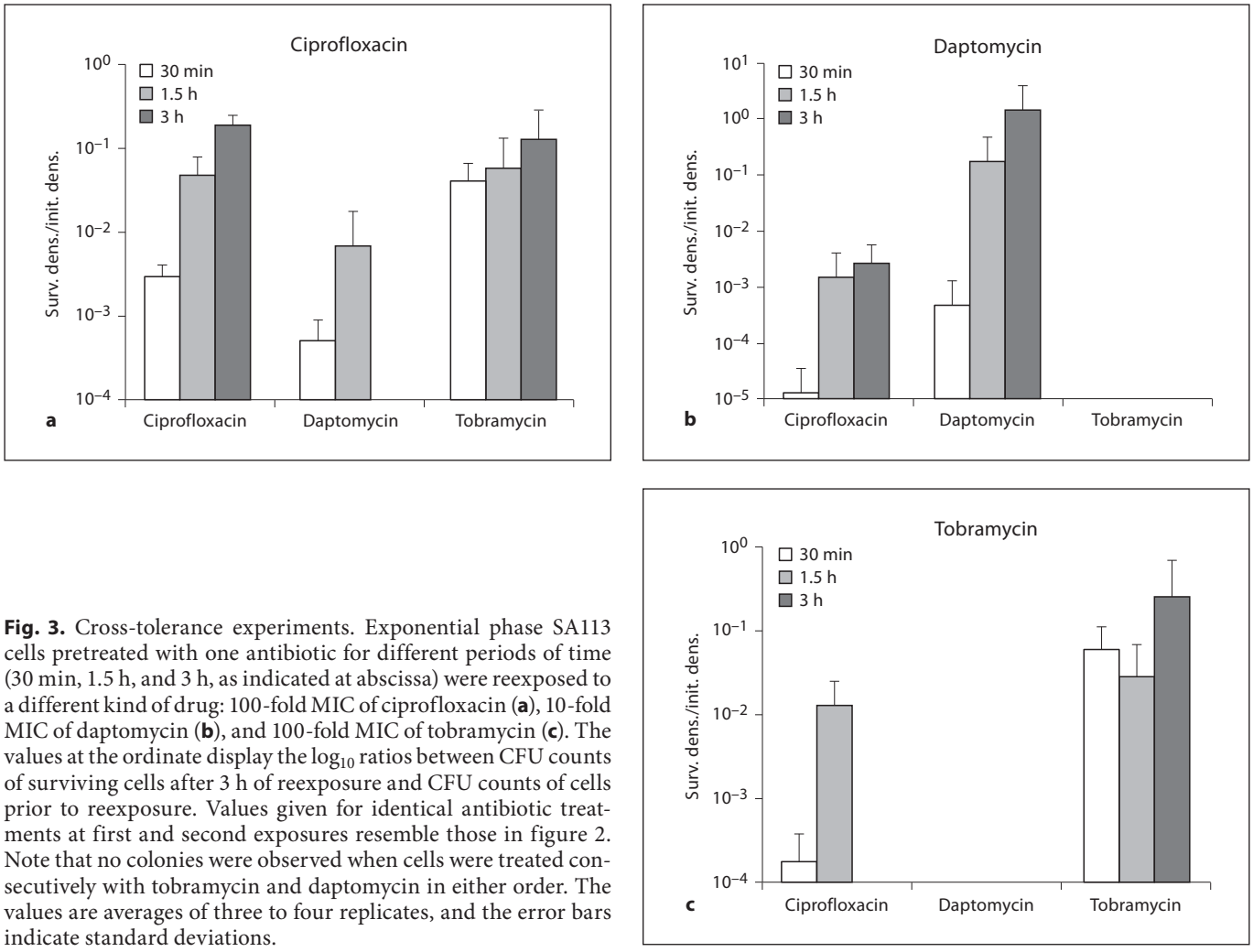

Fig. 3. Cross-tolerance experiments. Exponential phase SA113 cells pretreated with one antibiotic for different periods of time (30 min, 1.5 h, and 3 h, as indicated at abscissa) were reexposed to a different kind of drug: 100-fold MIC of ciprofloxacin (**a**), 10-fold MIC of daptomycin (**b**), and 100-fold MIC of tobramycin (**c**). The values at the ordinate display the $\log_{10}$ ratios between CFU counts of surviving cells after 3 h of reexposure and CFU counts of cells prior to reexposure. Values given for identical antibiotic treatments at first and second exposures resemble those in figure 2. Note that no colonies were observed when cells were treated consecutively with tobramycin and daptomycin in either order. The values are averages of three to four replicates, and the error bars indicate standard deviations.

change of antibiotics (dotted lines). Finally, partial cross-tolerance with a fraction of persisters tolerant to both antibiotics results in biphasic killing upon antibiotic exchange (dashed lines). The data for daptomycin and tobramycin (fig. 4e, f) clearly showed no cross-tolerance, as expected for two disjunct subpopulations constituted by persisters tolerant to either tobramycin or daptomycin. This in agreement with our previous observations, when stationary-phase bacteria had been killed completely upon simultaneous addition of both daptomycin and tobramycin at super-MICs [Lechner et al., 2012]. In the cross-tolerance experiments of either antibiotic with ciprofloxacin (fig. 4a–d), however, the order in which the antibiotics are applied was found to be crucial. This observation suggests that the behavior of the isolated persisters was influenced by the challenge with the first antibiotic and is inconsistent with models that assume pre-existing tolerant subpopulations, neglecting any response to the antibiotic challenge. It also indicates that care must be taken to separate cellular responses due to the antibiotic treatment from those characteristic for persisters, which underscores that methods for persister isolation should be as gentle as possible to obtain unstressed (ideally naïve) persisters for further characterization [Kaldalu et al., 2004; Keren et al., 2004b; Shah et al., 2006].

Delay in Resuscitation and Subsequent CFU Doubling Times Are Dependent on the Kind and Duration of Antibiotic Treatment

How long do *S. aureus* persisters, which have endured antibiotic treatment, require to reassume growth, and what are the CFU doubling times within the first hours

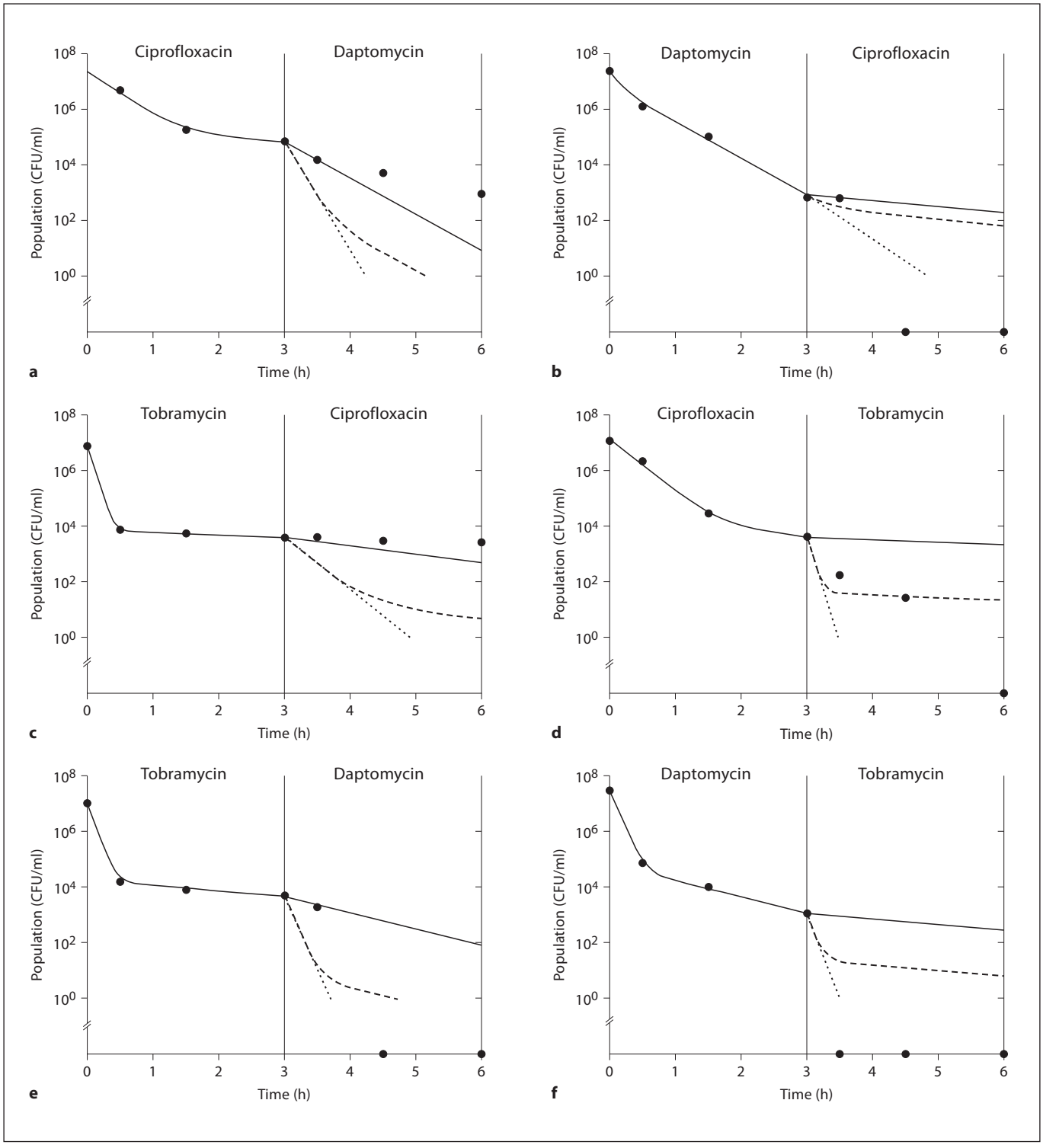

Fig. 4. Model for multiple persister types and cross-tolerance. **a–f** Time course of killing by exposure to one antibiotic for 3 h and to a second antibiotic for the following 3 h. The three lines in each panel indicate model predictions for a case with a single persister phenotype tolerant to both antibiotics (solid line), a case with two types of persisters that are tolerant to one antibiotic each, without any cross-tolerance (dotted line), and a case with partial cross-tolerance or three persister phenotypes (dashed line). In all models, the antibiotics are assumed to select for cells with a preexisting phenotype from a heterogeneous population. The data points indicate measured population sizes (from the experiments of fig. 3).

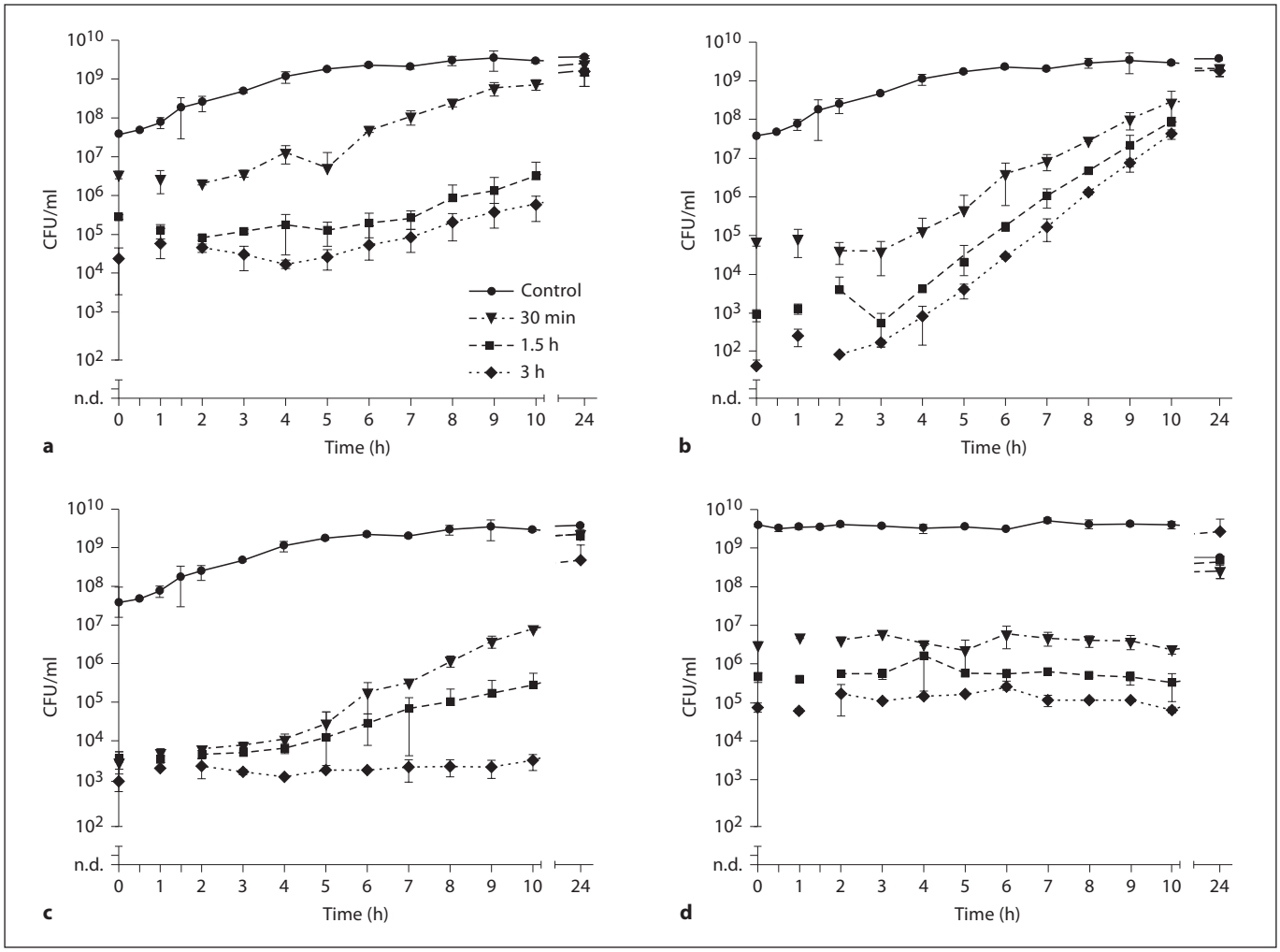

Fig. 5. Resuscitation experiments. Exponential phase SA113 cells were treated with 100-fold MIC of ciprofloxacin (**a**), 10-fold MIC of daptomycin (**b**), or 100-fold MIC of tobramycin (**c**), or stationary phase cells were challenged with 100-fold MIC of daptomycin/Ca^{2+} (**d**). Cells from samples taken after 30 min, 1.5 h, and 3 h were then cultured in fresh medium without antibiotic for 24 h to determine resuscitation times. The values are averages of two replicates, and the error bars indicate standard deviations. The limit of detection was 100 CFU/ml.

after resuscitation? To answer these questions, SA113 cells were challenged by single drugs for 0.5–3 h, and were then transferred to fresh non-selective media. Cells treated with 100-fold MIC of ciprofloxacin exhibited a consistent increase in live counts approximately 4 h after the shift to fresh media (fig. 5a). Resulting cultures exhibited relatively similar CFU doubling times (calculated on the basis of t = 10 h and t = 5 h values) ranging between 43 and 67 min, irrespective of the duration of the ciprofloxacin pretreatment. CFU values of cells challenged with 10-fold daptomycin also increased rather uniformly (23–33 min doubling times) starting approximately 3 h after the shift (fig. 5b). By contrast, live counts of cultures treated for 30 min or 1.5 h with 100-fold tobramycin rose after about 3 h, whereas those of cultures challenged with the drug for 3 h remained constant for at least 9 h (fig. 5c). This is reflected by doubling times of 37 min, 67 min or more than 6 h, respectively. Similar behavior as for the 3-hour exposure to tobramycin was also observed for stationary-phase cultures treated with 100-fold MIC of daptomycin/Ca^{2+}. As shown in figure 5d, CFU counts for this case leveled off within 10 h after the cells had been inoculated into fresh medium, but values increased by two to four orders of magnitude after 24 h. Growth resumption in our experiments thus occurred not before 3 h (fig. 5a–c), in relation to a period of about 1.5 h as deter-

Table 2. Parameters obtained from the fitting of resuscitation curves

Antibiotic	Concentration and exposure time[1]	Number of experiments	Growth rate of normal cells μ_n (h^{-1})	Growth rate of persisters μ_p (h^{-1})	Fraction of normal cells $\log_{10}(n_0/p_0) = \log_{10} f_1$	Switching rate persister → normal[2] b (h^{-1})
Ciprofloxacin	100-fold MIC, 3 h	3	0.88 ± 0.16	–0.15 ± 0.11	–2.02 ± 0.04	2.1×10^{-2}
Daptomycin	10-fold MIC, 3 h	3	1.84 ± 0.17	–0.57 ± 0.51	–2.7 ± 0.52	1.6×10^{-2}
Tobramycin	100-fold MIC, 1.5 h	3	0.74 ± 0.01	0.06 ± 0.11	–1.63 ± 0.16	4.0×10^{-1}

All data are for strain SA113, mean ± standard deviation from three repeats. Calculations are based on results graphically shown in figure 5a–c.

[1] Very similar results were obtained for shorter exposure times; 3-hour exposure to tobramycin resulted in different dynamics (fig. 5c).

[2] Estimated via $f_1 = b/[\mu_p^{(AB)} - \mu_n^{(AB)}]$ using the killing rates measured with the same culture.

mined for *E. coli* [Gefen et al., 2008], which, however, depends on the growth medium used [Jõers et al., 2010].

For a quantitative analysis, the results of these experiments were fitted with a double exponential outgrowth model (eq. 3), from which switching rates from the persistent to the regular growing phenotype were obtained (table 2). Consistent with the observations described above, the parameters obtained for different exposure times to either ciprofloxacin or daptomycin were very similar, while for tobramycin only the two shorter exposure times could be described well with the model. The latter observation suggests that prolonged exposure to tobramycin affects the cells beyond simple random phenotype switching. Instead, it possibly induces a phenotype of 'deep dormancy' (with an as of yet unknown molecular basis) that results in delayed wake-up kinetics. Parameters obtained for the longest exposure time for each antibiotic that is well described by the model are summarized in table 2. For all three antibiotics, the switching rates from the persistent to the regular growing phenotype are in the same range as previously found for *E. coli* persisters [Balaban et al., 2004]. For tobramycin, the switching rate is similar to the killing rate of persisters during antibiotic treatment, consistent with the idea that the observed killing of persisters is due to so-called 'scout' cells, persisters resuming growth stochastically [Buerger et al., 2012]. On the other hand, the switching rates observed using ciprofloxacin and daptomycin are smaller than the killing rates of persisters exposed to these drugs, indicating that these two drugs kill persisters independently of growth resumption. Intriguingly, CFU doubling times of daptomycin-treated cells fell below usual generation times of naïve SA113 bacteria (which we determined as approximately 37 min under comparable antibiotic-free conditions) after being shifted to non-selective media (fig. 5b). We attribute this effect to the presence of a fraction of cells that start to proliferate at very late time points on the agar plates (whereas most cells initiate multiplication already in liquid medium). If such cells are missed at early time CFU counts, the population is given an apparent growth boost. It should be noted that the observed results in these resuscitation experiments show striking parallels to the 'post-antibiotic effect' phenomenon. Well established in pharmacodynamics, it describes delayed growth of bacteria after antibiotic treatment [Drusano, 2004]. The post-antibiotic effect of *S. aureus* cultures treated with different antibiotics has extensively been studied before. For ciprofloxacin, a delay of 1.5–2.5 h was determined [Davidson et al., 1991]. Cells after tobramycin treatment resumed growth after 6.6–12 h [Isaksson et al., 1993]. For daptomycin-treated *S. aureus* cells, Hanberger et al. [1991] observed a PAE of 1–6.3 h. Obviously, differences among the studies with regard to different antibiotic concentrations, growth phase, media, strains backgrounds and other variables impede direct comparisons, but we note that time delays in our resuscitation experiments fit well into the window of previously described PAE values for the three drugs. Future studies may more precisely define the possible role of persisters in the PAE.

Conclusions

Exploiting rich nutrient media conditions in vitro, we here define switching rates of *S. aureus* cells between growth and the dormant persister state. Upon isolation of persisters by super-MICs of antibiotics, it was observed

that the choice of the drug influenced population dynamics of staphylococcal cultures with regard to cross-tolerance to another antibiotic and the behavior of growth resumption. The complex patterns of cross-tolerance indicate the existence of multiple types of persisters and suggest (via the observed dependence on the order of treatment) that adaptive responses to drugs as well as preformation of dormant subpopulations are involved in persister formation. The quantitative data gathered here provide a basis for more comprehensive studies in the future and for a more refined, molecular-level modeling of *S. aureus* population dynamics in relation to antibiotic challenge. A quantitative understanding of *S. aureus* susceptibility to antibiotics incorporating information about physiologic downshift phenomena such as SCV and persister cells as well as strain background and mutations will help develop tailored antibiotic therapies to treat staphylococcal infections.

Experimental Procedures

Bacterial Strains, Media, and Culture Conditions

Throughout this study, *S. aureus* SA113 was used [Iordanescu and Surdeanu, 1976]. Bacteria were grown at 37°C with aeration in baffle flasks containing tryptic soy broth (Sigma) at a 1:6 culture-to-flask ratio or on tryptic soy agar. Liquid cultures were shaken at 150 rpm. To prepare exponential phase cultures, cells grown overnight were transferred to 16 ml of fresh media to an initial OD_{578} of 0.07 and were shaken for about 1.5 h until an OD_{578} of approximately 0.5 was reached. Overnight cultures were used to work with stationary phase cells. Numbers of viable cells were determined in retrospect by CFU analysis. Therefore, cells from respective cultures were collected, washed and suspended in 1% saline and spotted as 10 μl aliquots of serial dilutions on tryptic soy agar as described [Lechner et al., 2012].

Antibiotics

Daptomycin analytic grade powder (designated 'Cubicin') was purchased from Novartis Pharma. Facultatively, Ca^{2+} cations (50 μg/ml final concentration), provided as $CaCl_2$, were added to daptomycin-treated cultures, to increase antibiotic activity, where indicated. Ciprofloxacin was obtained from Fluka and tobramycin was from Sigma. Solutions of antimicrobials were prepared freshly prior to each application and were sterilized using a filter of 0.2 μm pore size (Whatman).

Reexposure and Resuscitation Experiments

Cells were grown to exponential or stationary phase in baffle flasks under different antibiotic selective conditions (as indicated in the main text and in figure legends) to provide primary cultures. After 30 min, 1.5 h, and 3 h, 2.24 ml samples were withdrawn, cells were pelleted, washed in 1% saline and transferred to 14-ml tubes which contained 2.24 ml of fresh tryptic soy broth media. The medium of this secondary culture was supplemented with either the same antibiotic at the same concentration as used in the primary culture (in recurrent reexposure experiments), with a different drug to check for cross-tolerance, or antibiotics were omitted to monitor resuscitation. The CFU content was determined 30 min, 1.5 h, and 3 h after addition of the first and second antibiotic, or on an hourly basis for up to 10 h and after 24 h in case of the resuscitation tests. Experiments were conducted at least twice, using two to four biological replicates. Samples from cultures not exposed to antibiotics in the primary culture but subsequently treated in an identical manner in the secondary culture served as controls.

Theoretical Analysis

Killing and resuscitation data were analyzed quantitatively with a model for stochastic phenotype switching by Balaban et al. [2004]. It describes a population of cells as consisting of two subpopulations representing persisters *(p)* and normal cells *(n)* that exhibit different exponential growth or death rates (μ_n and μ_p). According to this model, cells switch between these two phenotypes with rates *a* and *b*, respectively. These rates are assumed to be independent of environmental triggers (type II persisters). The full dynamics of the model is given by

$$\begin{aligned}\frac{d}{dt}n &= \mu_n n - an + bp \\ \frac{d}{dt}p &= \mu_p p + an - bp\end{aligned} \tag{1}$$

A shift from a growth medium (with $\mu_n > \mu_p \geq 0$) to a medium containing an antibiotic (for which $\mu_n < \mu_p \leq 0$) is described by a change in the growth/death rate starting from a steady state of eq. 1. It is based on the growth rates from the first medium and results in double-exponential decay of the population:

$$N(t) = n_0\left[\exp(-\mu_1 t) + f_0\exp(-\mu_2 t)\right] \tag{2}$$

where the three parameters μ_1, μ_2 and f_0 can be identified with the absolute values of the death rates of normal cells and persisters and to the initial fraction of persisters (the fraction of persisters extrapolated to the time of addition of the drug), respectively [$\mu_1 = -\mu_n^{(AB)}$, $\mu_2 = \mu_p^{(AB)}$, $f_0 = p_0/n_0 \approx p_0/(n_0 + p_0)$]. The latter identification is an approximation for small switching rates between the phenotypes, a condition that is generally valid in our experiments. The three parameters were determined by fitting killing curves with eq. 2. The switching rate from the normal to the persistent phenotype was estimated via $f_0 \approx a/\mu$ (where μ is the growth rate of the population before the addition of the antibiotic). Each killing experiment was fitted separately, and the resulting parameter values were averaged over three or six repeats (table 1). Fit results for individual experiments were used for the statistical analysis of the difference between experiments with different antibiotics, applying the t test to the logarithm of the persister fraction $\log_{10} f_0$.

For the analysis of cross-tolerance experiments, the model given by eq. 1 was extended to incorporate three types of persisters that are tolerant to only the first, only the second, or both antibiotics, assuming that all non-tolerant cells exhibit the same behavior.

Resuscitation curves were also fitted by the same procedure using again a double exponential expression:

$$N(t) = p_0\left[f_1\exp(\mu_1' t) + \exp(\mu_2' t)\right] \tag{3}$$

The parameters μ_1', μ_2' and $f_1 = n_0/p_0 \approx n_0/(n_0 + p_0)$ correspond to the growth rates of normal and persister cells after the removal of the antibiotic and to the initial fraction of normal cells. The switching rate b from the persistent to the normal phenotype is estimated via $f_1 \approx b/(\mu_p^{A\,B} - \mu_n^{A\,B})$. Each resuscitation experiment was fitted separately, and the resulting parameter values were averaged over three repeats (table 2).

Acknowledgements

We thank Tanja Hildebrandt for technical assistance and Friedrich Götz for support. This work was supported by grants within the priority programmes 1316 'Host Adapted Metabolism of Bacterial Pathogens' and 1617 'Phenotypic Heterogeneity and Sociobiology of Bacterial Populations' and by the research training group Graduate College 685 'Infection Biology: Human- and Plant-Pathogenic Bacteria and Fungi' of the Deutsche Forschungsgemeinschaft.

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