Phenotypic switching of antibiotic resistance circumvents permanent costs in *Staphylococcus aureus*

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Bacterial antibiotic resistance is often associated with a fitness cost in the absence of the antibiotic [1, 2]. We have examined a resistance mechanism in *Staphylococcus aureus* that negates these costs. Exposure to gentamicin both in vitro and in vivo has been reported to result in the emergence of a gentamicin-resistant small colony variant (SCV) [3–8]. We show that the emergence of SCVs following exposure to gentamicin results from a rapid switch and that bacteria exposed to cycles of gentamicin followed by antibiotic-free medium repeatedly switched between a resistant SCV and a sensitive parental phenotype (revertants). The fitness of revertants relative to *S. aureus* with stable gentamicin resistance was greater in drug-free media, which suggests that *S. aureus* has evolved an inducible and reversible resistance mechanism that circumvents a permanent cost to fitness.

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Results and Discussion

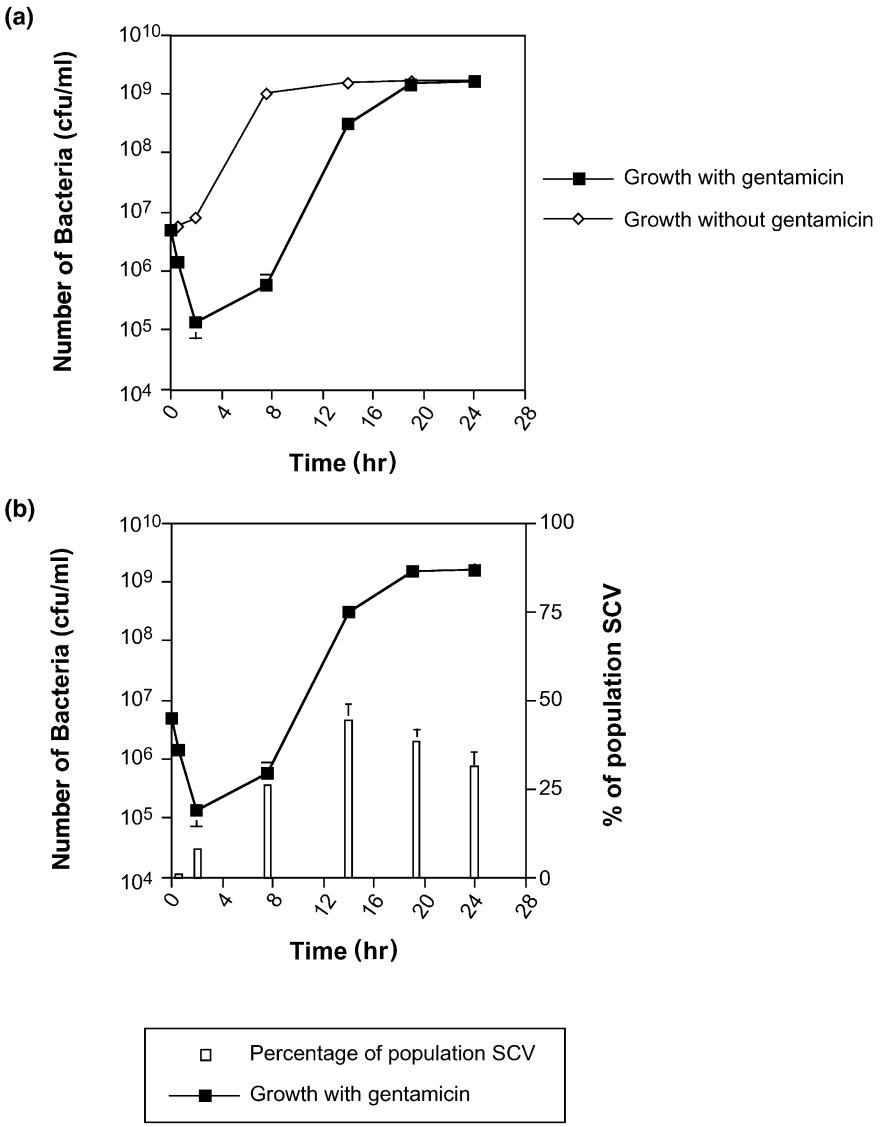
The acquisition of antibiotic resistance through genetic mutation is frequently associated with a fitness cost in the absence of the antibiotic [1, 2]. The evolution of fitness-compensatory mechanisms through second-site mutations has been defined in bacteria during serial passage in broth culture or experimental animals, but the relative fitness of the resulting strains often remains reduced [2–4]. A reversible antibiotic resistance mechanism that is not associated with a potentially lasting fitness cost is likely to be favored by selection.

Phenotypic variants of *Staphylococcus aureus*, known as small colony variants (SCVs), are believed to have a defect in electron transport and are resistant to aminoglycosides by virtue of poor drug uptake [5, 6]. These variants are characterized by a slow growth rate, absence of pigmentation, a reduced range of carbohydrate utilization, and failure to express several putative virulence factors [7]. SCVs have previously been considered to represent a small subpopulation of organisms that become auxotrophs following the acquisition of a mutation in genes involved in the electron transport chain. There are two classes of SCV, those that are stable following serial passage, or those that can revert back to having wild-type growth characteristics [8]. They have been reported to arise after uptake of bacteria by endothelial cells in vitro [9], following exposure to gentamicin in broth culture [10], and during human or experimental infection [11–14]. The significance of SCVs for human infection is that they are associated with cases of *S. aureus* infection that prove to be resistant to antibiotic cure, manifested by persistence or relapse.

The rate of emergence of SCVs upon exposure to gentamicin was determined for five independent isolates of *S. aureus*, all from cases of severe community-acquired disease. Bacteria were grown in a laboratory growth medium (BHI, brain heart infusion) containing gentamicin, and the number of SCVs as a percentage of the population was determined by serial plating and colony counts over a time course. Surprisingly, SCVs were present after only 30 min of exposure to gentamicin and made up more than 27% of the population by 7 hr (Figure 1). The relative proportion of SCVs reached a peak at 14 hr, subsequently declining with the emergence and outgrowth of gentamicin-resistant strains with wild-type characteristics. Stable resistance to gentamicin can result from point mutations of the genes encoding target proteins, and the emergence we see is consistent with normal mutation rates in combination with selection by the presence of gentamicin.

We examined whether the rate of emergence of SCVs could be explained by growth from the original inoculating population using two strategies. The frequency of SCVs in the population before exposure to gentamicin was found to be less than 10^{-5}. In order to reach the frequency observed at 30 min, the SCVs would need to have a mean generation time of approximately 3 min, and over 7 hr, the mean generation time would have to be 28 min. However, the actual mean generation time over 7 hr under the same conditions was found to be 3.5 hr. The second strategy was based on the fact that, if the emergence of SCVs depended on their presence in the inoculum, reducing the numbers of bacteria introduced into the gentamicin broth should reduce their frequency within the population. After reducing the bacterial inoculum 100-fold, the proportion of SCVs in the population after 24 hr of incubation increased from 32% ± 4.5% to

Figure 1



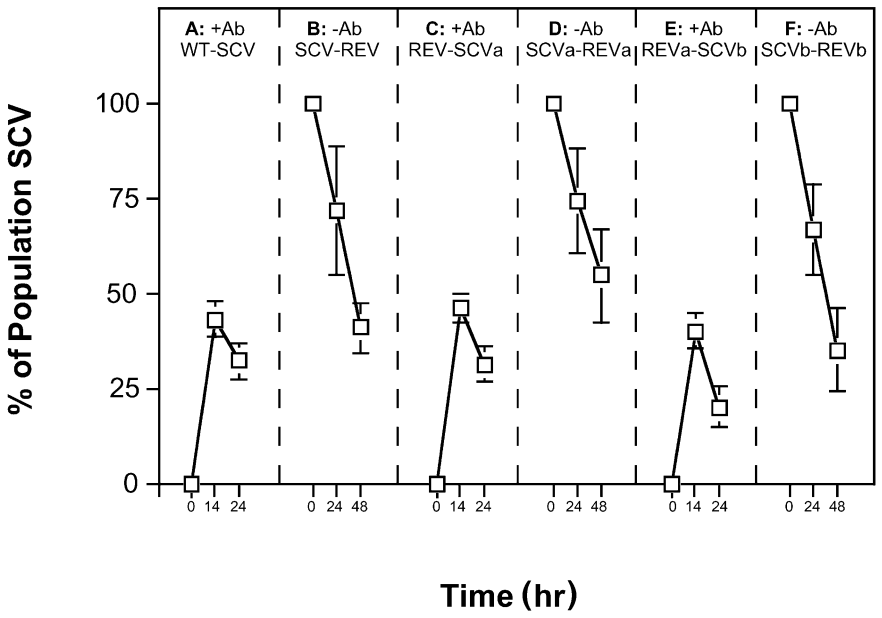
The effect on the growth of *S. aureus* upon exposure to gentamicin. **(a)** A growth curve of *S. aureus* in the presence or absence of a sublethal dose of gentamicin. The data is represented as the mean cfu/ml for five *S. aureus* strains ± standard error of the mean. **(b)** A graph showing the emergence of SCVs (as a percentage of the total population) after exposure to gentamicin over 24 hr. The data is represented as the mean value for five *S. aureus* strains ± standard error of the mean.

68.8% ± 14% ($p < 0.001$). This is consistent with switching to the SCV phenotype combined with a decreased chance of including stable gentamicin-resistant mutants in the inoculum. Together, these data suggest that the SCVs cannot have emerged as a result of the replication of existing variants in the inoculating population and must have emerged by switching from the wild-type to the SCV phenotype.

To test whether this was a two-way and repeatable switch, we picked SCVs from each of the five original isolates and exposed them to antibiotic-free media. In all cases, SCV clones were found to spontaneously revert to the wild-type phenotype. Rechallenge of revertant clones with gentamicin resulted in the emergence of SCVs at the same frequency as from the original wild-type population. This phenomenon was fully repeatable with induction of switching through cyclical exposure to gentamicin and gentamicin-free media five times (Figure 2). This unequivocally demonstrates a mechanism by which *S. aureus* can switch between gentamicin-sensitive and gentamicin-resistant forms. We extended our analysis to 30 independent isolates of *S. aureus* associated with either nasal carriage in healthy volunteers (n = 15) or invasive disease (n = 15, inclusive of the 5 initial isolates). The emergence of SCVs was demonstrated for all isolates, suggesting that this mechanism is common within natural populations of *S. aureus*.

Having established the existence of a switching mechanism, we next determined whether the switch was spontaneous or induced by gentamicin. No SCVs were observed at any time during 24 hr of growth in the absence of gentamicin, supporting the hypothesis of an inducible

Figure 2



Phenotypic switching of *S. aureus* in response to the presence or absence of gentamicin. (A) A graph showing the switch to the SCV phenotype of a population of wild-type (WT) bacteria following exposure to gentamicin (+Ab). (B) A graph showing the switch back to wild-type phenotype (revertants: REV) of populations of SCVs following removal of gentamicin (−Ab) from the environment. (C) A graph showing the switch to the SCV phenotype (SCVa) of a population of revertant (REV) bacteria following exposure to gentamicin (+Ab). (D) A graph showing the switch back to wild-type phenotype (REVa) of populations of SCVa following removal of gentamicin (−Ab) from the environment. (E) A graph showing the switch to the SCV phenotype (SCVb) of a population of REVa bacteria following exposure to gentamicin (+Ab). (F) A graph showing the switch back to wild-type phenotype (REVb) of populations of SCVb following removal of gentamicin (−Ab) from the environment. The data is represented as the mean percentage of SCV in the population for five *S. aureus* strains ± standard error of the mean.

switch. However, high-frequency spontaneous switching and antibiotic-imposed selection of resistant SCVs might explain these data if the rate of switching from wild-type to SCV was lower than the rate of switching from SCV to revertant. Our estimates suggest that the rate of switching from wild-type to SCV (95% confidence interval [CI]: 0.07%–0.3% cells/generation) is at least an order of magnitude greater than the rate of switching from an SCV to a revertant (95% CI: 0.0009%−0.04% cells/generation). We therefore consider it unlikely that our data can be explained by a high-rate switch that usually favors the sensitive variant in the absence of gentamicin, but which allows selection and capture of the resistant population in the presence of gentamicin. Whether or not the switch from SCV to revertant is spontaneous or induced by the absence of antibiotic is as yet unclear.

To examine whether stresses other than gentamicin induce switching to SCVs, the five *S. aureus* isolates were grown under either acidic, oxidative, anaerobic, starvation, or temperature stress. SCVs were not induced by these conditions. Consistent with this was the finding that the general stress-response sigma factor SigB did not play a role in the switching mechanism, as a strain deficient in SigB switched to the same degree as its parent ($SigB^+$) strain [15]. Bacteria were also grown in the minimum inhibitory concentration (MIC) [16] of either methicillin, vancomycin, erythromycin, ciprofloxacin, chloramphenicol, streptomycin, spectinomycin, amikacin, kanamycin, or tobramycin. The only conditions tested that led to switching to SCVs was growth in the presence of streptomycin or spectinomycin. It is possible that only certain members of the aminoglycoside family can induce switching to SCVs or that concentrations other than the MIC may be required to induce this switching event. We examined the effects of varying the concentration of gentamicin on the emergence of SCVs. The MIC of SCV was found to be 20-fold higher than wild-type/revertants (which had the same MICs). Growth in concentrations above the MIC of SCV resulted in death of the entire inoculum, while the emergence of SCVs was favorably selected by a concentration between the MIC of wild-type and SCV. Concentrations below the MIC of wild-type initially induced switching, but by 24 hr, the SCVs were outgrown by sensitive wild-type. It has been suggested previously that the SCV phenotype confers increased resistance to other antibiotics [17]. We found that SCVs were no more resistant to methicillin, vancomycin, erythromycin, ciprofloxacin, rifampicin, fusidic acid, and tetracycline.

We next considered whether the ability to switch between gentamicin-resistant and -sensitive forms provided a fitness advantage over mutants of *S. aureus* that are stably resistant to gentamicin. Mutants with stable gentamicin resistance were isolated from the five *S. aureus* isolates and were found to have a 40-fold increase in MIC relative to wild-type. SCVs had a growth rate disadvantage relative to the stable mutants when grown in the presence of gentamicin, as can be seen in Figure 1 by the outgrowth of SCVs by resistant mutants after 14 hr of exposure to gentamicin. However, gentamicin-sensitive revertants outcompeted stable gentamicin-resistant strains when grown in antibiotic-free media where the stable mutants

had a relative fitness of 0.44 (standard error of 0.05; paired t test, $p < 0.0001$) [18]. We next examined whether the ability to switch during cycling conditions in the presence or absence of gentamicin was sufficient to allow the revertant phenotype to dominate once the antibiotic was permanently removed. After two successive cycles in the presence and absence of gentamicin, 54% (±15%) of the population was found to be sensitive to gentamicin. By adopting the SCV phenotype on exposure to gentamicin, *S. aureus* acquires antibiotic resistance together with a reduced rate of growth. The removal of gentamicin from the bacterial environment induces bacteria to revert to the wild-type phenotype, and, in so doing, avoids the acquisition of a fitness disadvantage associated with resistance by mutation.

Gentamicin is not routinely used to treat serious *S. aureus* infections [19], and so the use of gentamicin as a therapeutic agent is unlikely to have been the selective force responsible for the evolution of the switching mechanism. Gentamicin is naturally produced by *Micromonaspora* species, and streptomycin and spectinomycin are produced by *Streptomyces* species, so we hypothesize that long-term interactions with such species has selected for this switching mechanism. This is consistent with the fact that *S. aureus* was known to have evolved penicillin resistance by the time the drug was discovered, presumably again in response to interactions between microbial species in the environment. Evolutionary development of this switch suggests that other bacterial pathogens may have evolved similar strategies, a possibility that merits investigation.

These data provide the first demonstration that bacteria can switch between antibiotic-resistant and -sensitive forms in response to antibiotic pressure. We are working to elucidate the mechanism, but it is currently unclear how it compares with other phenotypic switches in bacteria. In the absence of mechanistic detail, we propose two models. The first is that an epigenetic event occurs and alters the regulation of a subset of genes, resulting in the SCV phenotype. Although SCVs revert once gentamicin is removed, it is not an immediate or a population-wide event, and so the possibility that a regulatory change could continue to exert effects through several generations is intriguing. For this to be true, the effector protein would need to have an extremely long half-life to result in the observed maintenance of the phenotype once gentamicin is removed. It would also need to be highly active at very low concentrations, such that titration following multiple cell divisions ultimately leads to a state in which the threshold concentration for activity is maintained in one daughter cell (which maintains the SCV phenotype) but lost in another (resulting in reversion), thereby explaining why reversion is not population wide. We do not believe a protein with such activity has previously been characterized and suggest that this would be a novel mechanism for antibiotic resistance.

Our second proposed mechanism is that a recombinational switch is causing an alteration at the level of DNA sequence. The frequency of recombination causing the phase-variation of the type 1 fimbriae of *E. coli* can be directionally modulated in response to alterations in the level of DNA supercoiling, which in itself is caused by many environmental factors [20]. We propose that aminoglycosides, either directly or indirectly, may induce a recombination event resulting in the SCV phenotype. The long-term association between *S. aureus* and aminoglycoside-producing microbes and the fitness costs associated with permanent antibiotic resistance make the evolution by natural selection of such an environmentally induced recombination event feasible.

Materials and methods

SCV emergence assays

BHI (brain heart infusion) broth containing the published breakpoint MIC of gentamicin, 2 μg/ml (GIBCO BRL,) was inoculated with 5×10^6 cfu/ml (colony forming units/ml) of *S. aureus* strains that had grown overnight at 37°C in air on horse blood agar. The broth cultures were incubated with constant rotation in air at 37°C. Aliquots were removed at allotted time points, serially diluted, and plated onto antibiotic-free blood agar. SCVs were identified by having a colony size a tenth of the size of a normal colony and having no pigmentation or hemolytic activity. The percentage of the population that consisted of SCVs was counted after 24 hr of growth at 37°C. The data shown is the mean percentage for the five independent strains. Error bars indicate the standard error of the mean.

Frequency of SCVs in an unstressed population

To measure the prevalence of SCVs in a population of *S. aureus*, bacteria were grown overnight on blood agar at 37°C. The bacteria were diluted and plated onto fresh blood agar such that individual colonies of SCVs, if present, could be identified, i.e.: 200–500 cfu/plate. A total of 10^5 cfu were plated, and no SCVs were identified.

Growth rate of SCVs

The frequency of SCVs in the inoculating population is $< 10^{-5}$, so if we assign this a value of 10^{-6}, the number of SCVs introduced into the BHI with gentamicin was 5 cfu/ml. After 30 min, the SCV make up 0.1% of 1.4×10^6 cfu/ml. The number of doublings from 5 to 1.4×10^3 is approximately 8, resulting in a mean generation time after 30 min of exposure to gentamicin of 3.75 min. After 7 hr, the SCV make up 27% of 5×10^5 cfu/ml, which is 1.4×10^5 cfu/ml. The number of doublings from 5 to 1.4×10^5 is approximately 15. Therefore, the mean generation time for SCVs to have grown from the inoculating population over 7 hr would be 28 min. SCVs from each of the five *S. aureus* strains were grown overnight at 37°C on blood agar. BHI containing 2 μg/ml gentamicin was inoculated with 1×10^6 SCVs cfu/ml; after 7 hr of incubation, the cfu/ml for each strain was estimated by diluting and plating onto fresh blood agar and was found to be 4.2×10^6 ($\pm 3 \times 10^5$). This suggests that SCVs grown in BHI with gentamicin have a mean generation time of 3.5 hr.

Reversion assay

The experiment to measure the rate of switching from SCV to revertant phenotype was carried out as described above for the SCV emergence assay with the following exceptions: the BHI broth contained no antibiotics; the inoculating population consisted of SCVs; and the assay was continued for an additional 24 hr.

Estimation of switching rates

Accurately estimating the rate of switching (% cells/generation) from wild-type to SCV is problematic because the generation time of the wild-type may be extended indefinitely by the antibiotic. We provide a conservative

estimate by determining net switching (ignoring the high initial rate of mortality of wild-type cells) over a 2-hr period, during which very few wild-type or SCVs (generation time = 3.5 hr) would have undergone binary fission: the density of SCVs after 2 hr, divided by the initial wild-type inoculum.

We used a simple, discrete generation simulation to estimate the switching rate of SCVs to revertants. Densities of SCVs (S) and revertants (R) were calculated for every SCV generation time step, $S_{t+1} = 2(S_t - c)$; $R_{t+1} = 2R_t/x + S_t c$, where c is the switching rate and x is the relative fitness (ratio of malthusian parameters, see Competitions below) of SCVs to revertants. The 95% CI of the data (final densities and percentage of SCVs) were used to determine 95% CI of c, assuming that x varies between 0.33 and 0.45. The value of 0.45 is a maximum for the relative fitness of SCVs to revertants and will overestimate the switching rate; the mean relative fitness of stably resistant mutants, which themselves consistently outcompeted SCVs, was 0.44.

Alternative stress conditions

The following growth conditions were assayed for the ability to induce switching to SCVs. In each case, the assay was performed as described above for SCV emergence in 10 ml media. Acidic stress, pH of BHI broth reduced to 3 with HCl; oxidative stress, BHI broth containing 7.5 mM H_2O_2; anaerobic stress, BHI broth in an anaerobic growth chamber; starvation stress, growth in PBS; temperature stress, BHI broth at 55°C and 10°C. The following concentrations of antibiotics were used in accordance with the published breakpoint MIC for *S. aureus* [16]: streptomycin, 4 μg/ml; methicillin, 4 μg/ml, vancomycin, 8 μg/ml; erythromycin, 1 μg/ml; ciprofloxacin, 2 μg/ml; chloramphenicol, 4 μg/ml.

MIC estimation

The minimum inhibitory concentration of antibiotics was estimated using Etest strips (AB BIODISK) in accordance with the manufacturer's instructions.

Isolation of stable gentamicin-resistant S. aureus *strains*

Each of the five *S. aureus* strains described above were inoculated into BHI containing 2 μg/ml gentamicin and were incubated for 24 hr at 37°C in air. These cultures were diluted and plated onto TSA containing 5 μg/ml gentamicin. Gentamicin-resistant strains with wild-type growth characteristics were shown to be stably resistant following serial passage on antibiotic free media.

Competitions

The fitness of stably gentamicin-resistant strains of *S. aureus* relative to revertants was measured by competitions in BHI without antibiotics. BHI was inoculated with a 1:1 ratio of each strain and incubated with constant rotation at 37°C for 24 hr. The culture was serially diluted and plated onto media with and without gentamicin. Relative fitness was calculated from the ratio of the resistant:revertant malthusian parameters, $m = \ln (N_f/N_0)$, where N_f and N_0 are the final and starting densities, respectively [18].

Natural competitions under alternating conditions

BHI broth containing gentamicin (2 μg/ml) was inoculated with each of the five wild-type strains. After 2 days of incubation, an aliquot was taken from these cultures and was used to inoculate fresh BHI broth without antibiotics. After 2 days of incubation at 37°C, these cultures were used to inoculate fresh BHI broth containing gentamicin, incubated for 2 days at 37°C, and then used to inoculate fresh BHI broth without antibiotics for a second cycle of growth conditions. Following this second cycle, the bacteria were diluted and plated onto blood agar. A total of 100 colonies were selected at random and tested for gentamicin resistance.

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