RESEARCH ARTICLE

Antibiotic-Mediated Selection of Quorum-Sensing-Negative *Staphylococcus aureus*

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ABSTRACT ***Staphylococcus aureus*** **is a human commensal that at times turns into a serious bacterial pathogen causing life-threatening infections. For the delicate control of virulence, *S. aureus* employs the *agr* quorum-sensing system that, via the intracellular effector molecule RNAIII, regulates virulence gene expression. We demonstrate that the presence of the *agr* locus imposes a fitness cost on *S. aureus* that is mediated by the expression of RNAIII. Further, we show that exposure to sublethal levels of the antibiotics ciprofloxacin, mupirocin, and rifampin, each targeting separate cellular functions, markedly increases the *agr*-mediated fitness cost by inducing the expression of RNAIII. Thus, the extensive use of antibiotics in hospitals may explain why *agr*-negative variants are frequently isolated from hospital-acquired *S. aureus* infections but rarely found among community-acquired *S. aureus* strains. Importantly, *agr* deficiency correlates with increased duration of and mortality due to bacteremia during antibiotic treatment and with a higher frequency of glycopeptide resistance than in *agr*-carrying strains. Our results provide an explanation for the frequent isolation of *agr*-defective strains from hospital-acquired *S. aureus* infections and suggest that the adaptability of *S. aureus* to antibiotics involves the *agr* locus.**

IMPORTANCE ***Staphylococcus aureus*** **is the most frequently isolated pathogen in intensive care units and a common cause of nosocomial infections, resulting in a high degree of morbidity and mortality. Surprisingly, a large fraction (15 to 60%) of hospital-isolated *S. aureus* strains are *agr* defective and lack the main quorum-sensing-controlled virulence regulatory system. This is a problem, as *agr*-defective strains are associated with a mortality level in bacteremic infections and a probability of glycopeptide resistance greater than those of other strains. We show here that *agr*-negative strains have a fitness advantage over *agr*-positive strains in the presence of sublethal concentrations of some antibiotics and that the fitness defect of *agr*-positive cells is caused by antibiotic-mediated expression of the *agr* effector molecule RNAIII. These results offer an explanation of the frequent isolation of *agr*-defective *S. aureus* strains in hospitals and will influence how we treat *S. aureus* infections.**

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In growing bacterial populations, even small changes in fitness are rapidly manifested in subpopulations with different growth rates (1). A classic example is resistance to streptomycin. In the presence of the antibiotic, resistant cells have an enormous selective advantage, whereas in its absence, the resistance imposes a fitness cost that results in a large reduction in the growth rate compared to that of sensitive cells (2, 3). Similarly for bacterial pathogens, virulence factor expression may be disadvantageous outside a host but needed for infection, as in the case of the *Salmonella enterica* serovar Typhimurium type III secretion system (4). In *Pseudomonas aeruginosa*, it is less obvious what confers the fitness difference than for the LasR quorum-sensing (QS) system, where virulence factor expression is selected against both *in vitro* and *in vivo* (5). Thus, what confers maximized fitness under one set of conditions may be counterselected under different environmental conditions (6, 7, 4) and the exact components providing the selective pressure are often not known.

QS allows for a coordinated response to cell density and environmental changes and is commonly employed by bacteria to control virulence gene expression (8, 9). A particularly well-studied QS system is encoded by the *agr* (accessory gene regulator) locus in the human pathogen *Staphylococcus aureus* (10). The signal molecule of *agr* is a posttranslationally modified peptide termed the autoinducing peptide (AIP) that is formed and excreted by the combined activity of AgrB and ArgD. At high concentrations, the signal is perceived by a classical two-component signal transduction system composed of the membrane-bound histidine kinase AgrC and the response regulator AgrA, both of which are encoded by the *agr* locus. Upon the binding of AIPs, AgrC activates AgrA by His-dependent phosphorylation. AgrA, in turn, induces the expression of a stable RNA, RNAIII, as well as that of the RNAII transcript containing *agrA*, *agrB*, *agrC*, and *agrD*, resulting in a feedback loop (11, 12). RNAIII is the key intracellular effector molecule of *agr*, and as its concentration increases with cell density, it induces the expression of extracellular virulence factors while repressing the expression of cell wall-associated proteins. Independently of RNAIII, AgrA directly controls the expression of α and β phenol-soluble modulins and, via an unknown mechanism, participates in the downregulation of genes involved in carbohydrate and amino acid metabolism (13).

Together, AgrA and RNAIII interconnect metabolism and virulence gene expression in response to cell density (14, 13).

The central role of *agr* in *S. aureus* virulence has been verified in a large number of *in vivo* models, including septic arthritis (15), skin abscesses (16, 17), osteomyelitis (18), and endocarditis (19), in which *agr*-defective strains display less virulence than wild-type (WT) strains. The *agr* locus is functional in essentially all community-acquired *S. aureus* strains, and the locus is considered important for the high virulence of these strains (20), as well as for their transmission between hosts (21). Also, subinhibitory concentrations of antibiotics are known to modulate virulence gene expression in *S. aureus* in a process likely involving *agr* (22). In contrast, *agr*-negative isolates frequently arise in hospital infections (23). Here, it has been estimated that 15 to 60% of *S. aureus*-associated infections display *agr* dysfunctions, whereas carriage of *agr*-negative strains by healthy individuals is unusual outside hospital settings (prevalence, ~4%) and is associated with previous hospital exposure (21, 24–28). Even though virulence gene expression is compromised in *agr*-deficient isolates, they still give rise to concern. Clinical studies indicate that *agr*-defective variants have reduced susceptibility to thrombin-induced platelet microbicidal proteins and are linked with an increased duration of and mortality due to *S. aureus* bacteremia (24, 29–31). In terms of resistance to antimicrobials, *agr*-negative strains are known to display intermediate resistance or heteroresistance to glycopeptides such as vancomycin (glycopeptide intermediate-level resistant *S. aureus* [GISA] and hetero-GISA) (32) and a laboratory-generated *agr*-negative strain demonstrated a small but reproducible increase in vancomycin heteroresistance (32).

The observation that *agr*-deficient strains frequently arise in the hospital environment, where the antibiotic pressure is expected to be high, led us to investigate (i) if there is a fitness cost associated with *agr* and (ii) if this effect is enhanced during growth in the presence of antibiotics. Growth competition experiments demonstrated that *agr*-negative strains displayed greater fitness than the isogenic WT strain in the presence of the antibiotics ciprofloxacin, mupirocin, and rifampin but not vancomycin. The fitness cost of carrying an intact *agr* operon in the presence of antibiotics was correlated with the ability to induce RNAIII expression. The study described here possibly explains the frequent isolation of *agr*-defective strains in clinical settings and identifies antibiotics as a factor that modulates the competition between QS-positive and -negative cells.

RESULTS

Fitness cost of *agr* expression. To assess if there is an impact on fitness associated with *agr*, we compared the growth of WT *S. aureus* Newman to that of a Newman Δ*agrA* mutant strain that does not produce any detectable amounts of RNAIII, as determined by quantitative PCR (qPCR). Fitness was assessed by using three growth parameters, namely, the exponential growth rate, the CFU count at stationary phase, and the outcome of competition between the two strains when inoculated at a 1:1 ratio and grown for ~8 cell divisions. The competition assay showed that the Δ*agrA* mutant strain exhibited a fitness advantage over the WT strain, with a relative competitive fitness of 1.07 determined as previously described (33, 34) (Fig. 1, TSB [tryptic soy broth]). However, when cultured individually, the WT and Δ*agrA* mutant strains multiplied with identical growth rates in exponential phase (OD [optical density], 0.02 to 0.08) and reached the same final cell

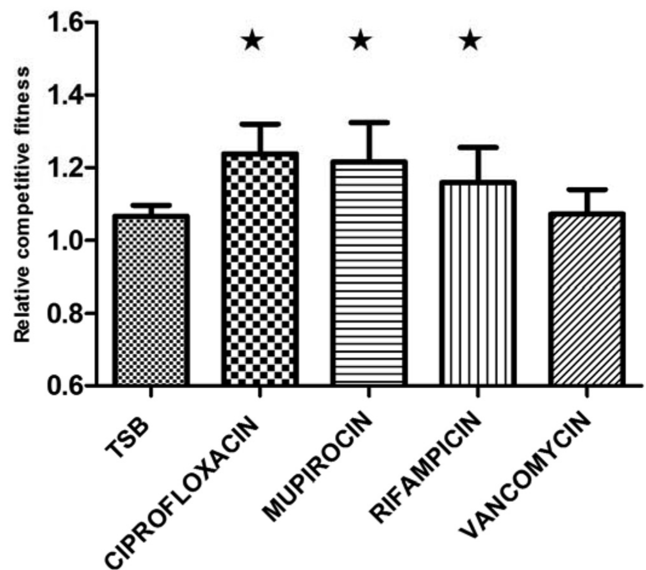

FIG 1 *agr* and antibiotics influence fitness. Relative competitive fitness of the Δ*agrA* mutant compared to that of the WT grown in the absence or presence of antibiotics after 48 h. The relative competitive fitness of the WT is 1. A star indicates a significant difference ($P < 0.05$) from the TSB control. Means with error bars indicating 95% confidence intervals are presented.

density, as measured by CFU counting at stationary phase (see Fig. S1 in the supplemental material). The difference in fitness between the two strains when grown in competition was observed in late exponential phase/early stationary phase and continued until stationary phase (Fig. 2A). Thus, the reduced fitness of the WT compared with that of the Δ*agrA* mutant may be related to the induction of *agr* at this growth stage (Fig. 2B).

***agr* imposes a fitness cost during antibiotic treatment.** To determine if the presence of antibiotics affects the cost of carrying an intact *agr* operon, we conducted competition assays (~8 cell divisions) of the WT and Δ*agrA* mutant strains in TSB growth medium supplemented with 1.0 μg/ml ciprofloxacin, 0.2 μg/ml mupirocin, 0.02 μg/ml rifampin, or 1.0 μg/ml vancomycin. These antibiotics were chosen to be clinically relevant and to target various cellular functions and were supplied at concentrations near their MICs in order to mimic a failed, sublethal antibiotic treatment (MICs are shown in Table S1 in the supplemental material).

In the growth medium supplemented with ciprofloxacin, mupirocin, or rifampin, the Δ*agrA* mutant strain exhibited a significantly higher relative competitive fitness than the WT strain, with values of 1.24 (*P* value of 0.0129) in ciprofloxacin, 1.21 (*P* value of 0.0018) in mupirocin, and 1.16 (*P* value of 0.05) in rifampin (Fig. 1). In the presence of vancomycin, however, no significant fitness increase was observed in the Δ*agrA* mutant strain, with a relative competitive fitness of 1.07 (*P* value of 0.65) (Fig. 1). Again, the higher relative competitive fitness of the Δ*agrA* mutant could not be attributed to differences in the exponential growth rates or final yields (endpoint CFU counts) of the individual strains (see Fig. S1 in the supplemental material). Thus, the observed fitness advantage of the Δ*agrA* mutant strain in competition with the WT is substantially enhanced by the presence of certain antibiotics.

RNAIII is responsible for the fitness cost imposed by *agr*. The *agr* QS regulon is composed of (i) the RNAIII-controlled viru-

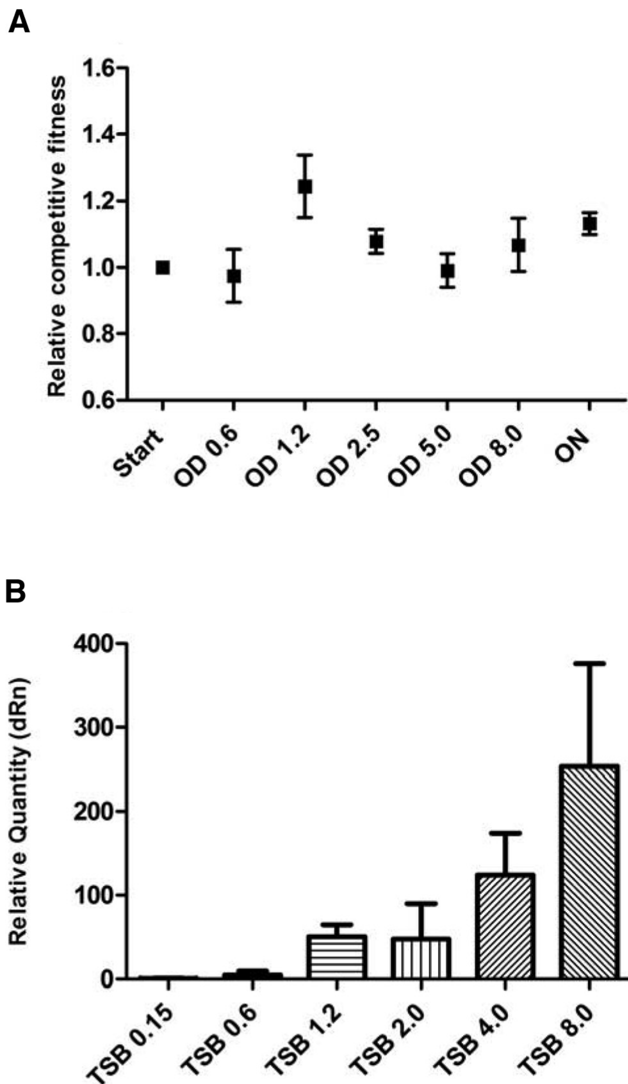

FIG 2 Effect of *agr* status on the competition ratio at various cell ODs. (A) Competitive fitness of the Δ*agrA* mutant relative to that of the WT grown in TSB as a function of OD with the last sampling point after 24 h of incubation. (B) RNAIII expression levels of WT cells in TSB normalized to the expression level of RNAIII at an OD of 0.15. Means with error bars indicating 95% confidence intervals are presented. ON, 24 hours; dRn, baseline subtracted fluorescent reading normalized to the reference dye.

lence factors and (ii) the AgrA-regulated genes (13). In order to determine which of these regulatory pathways is responsible for the *agr*-mediated decrease in fitness, we assayed the relative fitness in TSB without antibiotics of WT cells and a ΔRNAIII mutant constitutively overexpressing either the AgrA response regulator or RNAIII. The fitness assay was designed with pairwise competitions between the plasmid-lacking WT and strains constitutively overexpressing either *agrA* (pTX::agrA) or RNAIII (pTX::RNAIII) or carrying an empty expression vector as a control. The competition assay showed that overexpression of *agrA* did not alter fitness (0.96; *P* value of 0.98) relative to that of the control strain with the empty pTX vector. In contrast, a large fitness cost was observed when RNAIII was overexpressed, decreasing the relative competitive fitness to 0.40 (*P* value of $<$0.0001), in contrast to the 0.96 relative competitive fitness of the strain carrying the pTX plasmid (Fig. 3A).

In order to confirm that RNAIII expression is increased in cells carrying pTX::RNAIII but not in cells carrying pTX::agrA, we determined the RNAIII levels by reverse transcription (RT)-qPCR at three separate ODs, 0.15, 0.6, and 1.2. At two out of three OD sample points (0.15 and 0.6), we observed an increase in the RNAIII levels of the pTX::RNAIII-carrying strain over that of cells carrying the pTX::agrA or pTX plasmid (Fig. 3B). In order to corroborate the observed fitness cost due to increased RNAIII levels in the WT strain, we conducted competitions between the WT lacking the pTX plasmid and the ΔRNAIII mutant strain (no RNAIII expression) constitutively overexpressing *agrA* (pTX::agrA) or RNAIII (pTX::RNAIII) or carrying the vector (pTX) (Fig. 3C). Overexpression of RNAIII in the ΔRNAIII background caused a large decrease in fitness (0.89; *P* value of $<$0.018) relative to that of the ΔRNAIII mutant strain carrying the empty pTX vector (Fig. 3C). Compared to the WT strain carrying the pTX vector (Fig. 3A) the ΔRNAIII mutant (pTX::RNAIII) strain exhibited a significant increase in fitness (1.11; *P* value of $<$0.005) (Fig. 3C). Overexpression of *agrA* in the ΔRNAIII background did not significantly alter fitness (1.02, *P* value of 0.34) relative to that seen with the overexpression of *agrA* in the WT background (fitness, 0.96) (Fig. 3A). On the basis of these observations, we conclude that RNAIII expression is responsible for the fitness cost to *agr*-positive *S. aureus* cells.

RNAIII expression levels in the presence of antibiotics. Previous studies have shown that translational inhibitors at sublethal concentrations affect virulence gene expression in *S. aureus* (22, 35) and that mupirocin induces RNAIII expression in strain Newman (36). Therefore, we examined the hypothesis that the fitness decrease associated with some antibiotics may be mediated via an antibiotic-dependent increase in RNAIII expression. For this purpose, RNA was isolated from WT cells at various ODs in the absence or presence of antibiotics supplemented at the concentrations used in the competition assays. RNAIII transcript levels in antibiotic-treated cultures relative to those in nontreated TSB cultures were assessed by RT-qPCR. The results show that, relative to untreated cultures, RNAIII expression was increased by mupirocin at ODs (600 nm) of 0.15 and 0.6 and by rifampin and ciprofloxacin at ODs of 2.0 and 4.0, whereas vancomycin did not affect RNAIII levels (Fig. 4). In conclusion, the antibiotics that decrease the fitness of strains carrying *agr* relative to that of the Δ*agrA* mutant strain also increase RNAIII expression, particularly at high cell densities and in the presence of vancomycin, which did not enhance the *agr*-mediated fitness cost or stimulate RNAIII expression (Fig. 4).

DISCUSSION

S. aureus strains defective in the *agr* QS system are known to arise spontaneously under laboratory conditions (37–39), and among clinical isolates, *agr*-negative variants are surprisingly common (23, 24). The data presented here show that the expression of RNAIII, the effector molecule of the *agr* QS system, is responsible for the fitness cost of *agr* and, importantly, that the fitness cost is enhanced by the presence of antibiotics that induce the expression of RNAIII. The inverse correlation between fitness and RNAIII expression is corroborated by the findings that (i) ectopic expression of RNAIII is sufficient to reduce fitness (Fig. 3); (ii) the fitness cost is displayed in the transition to and during the stationary

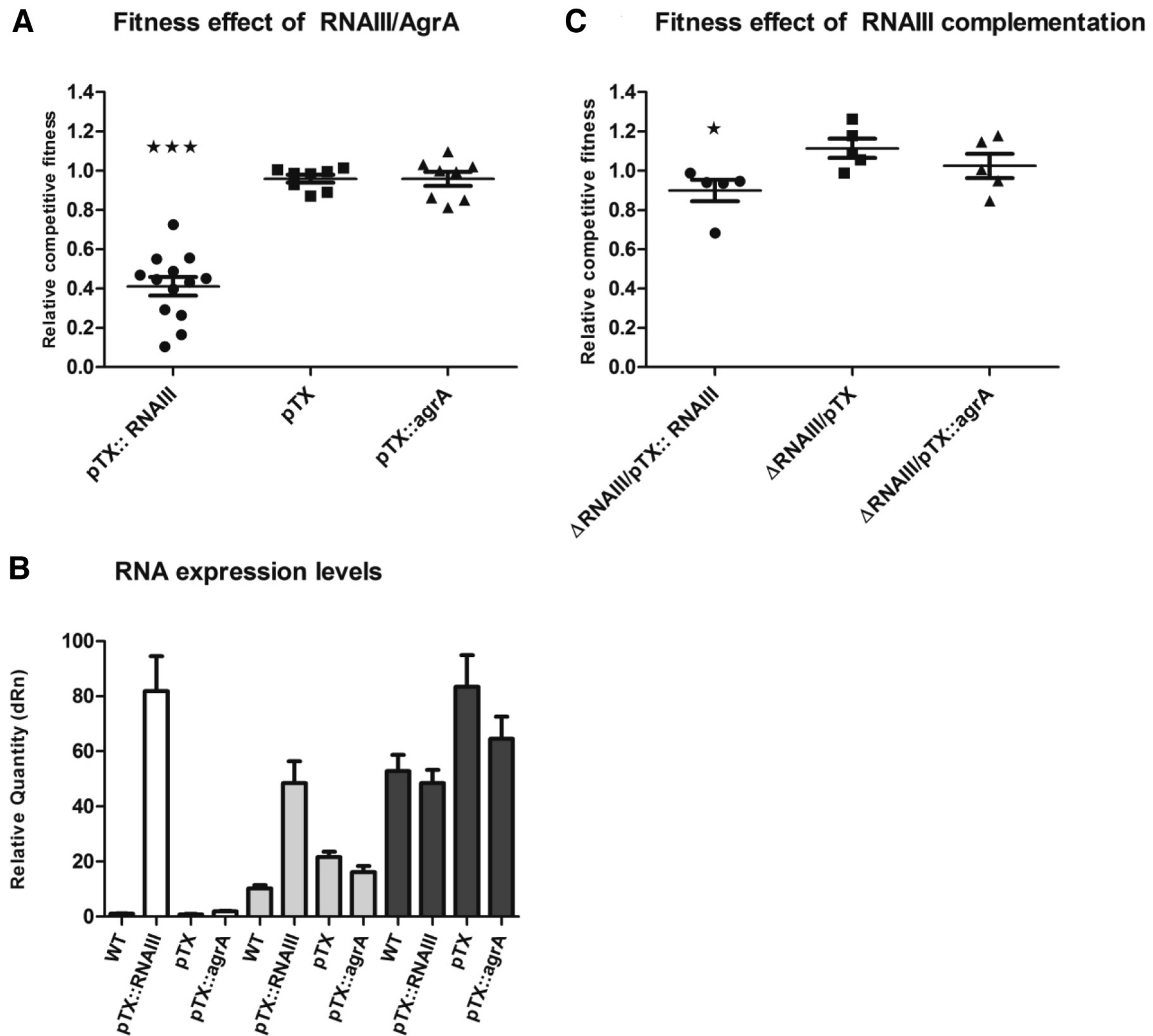

FIG 3 Overexpression of RNAIII reduces fitness. (A) Relative competitive fitness in TSB of the WT strain carrying the pTX vector expressing RNAIII or *agrA* or without an insert. A relative competitive fitness level of <1 is a result of decreased fitness of pTX-related gene expression. Three stars indicate a significant difference ($P < 0.001$) from the WT strain carrying the pTX vector without an insert. Means with error bars indicating 95% confidence intervals are shown. (B) RNAIII expression levels in TSB of WT cells carrying the pTX vector, pTX::RNAIII, or pTX::agrA and WT cells not carrying the pTX vector quantified at ODs of 0.15 (white), 0.6 (gray), and 1.2 (black). The relative expression level of RNAIII is set to the WT strain's RNAIII expression levels at an OD of 0.15. Means with standard deviations are shown. (C) Relative competitive fitness in TSB of ΔRNAIII mutant strains carrying the pTX vector expressing RNAIII or *agrA* or without an insert. Means with error bars indicating 95% confidence intervals are shown. A star indicates a significant difference ($P < 0.05$) from the ΔRNAIII mutant strain carrying the pTX vector without an insert. dRn, baseline subtracted fluorescent reading normalized to the reference dye.

growth phase, where RNAIII is maximally expressed (Fig. 2); and (iii) conditions that do not affect RNAIII expression, such as vancomycin exposure, do not influence fitness (Fig. 1 and 4).

Fitness costs associated with QS are not restricted to *S. aureus*. In the opportunistic human pathogen *P. aeruginosa*, the LasR-LasI QS system controls the expression of a number of genes needed for infection (40). Mutants lacking LasR arise spontaneously and show a selective advantage in competition with WT bacteria (41). Importantly, they are also isolated from a variety of infections, where they can develop from QS-positive cells within a matter of days (42–46). In a social context, *lasR* and *agr* mutants may be considered "cheaters" that fail to contribute to the production of "public goods," such as the catabolic enzymes needed during infection (41). However, mixed infections carrying both WT and QS-deficient cells occur, suggesting that there could be an interplay between cheaters and providers and that labor division is a deliberate strategy during the infection process (47, 48).

We show that in *S. aureus*, the expression of RNAIII is instrumental in the *agr*-associated fitness cost and that antibiotics that induce RNAIII expression also have a negative impact on fitness (Fig. 3). Beyond QS-controlled gene expression, little is known of the elements that impose the fitness burden. It has been speculated that the expression of a large number of gene products in response to a quorum signal creates a metabolic burden in WT cells and selects for QS-deficient variants (42). However, when the strains are cultivated individually, there are only minor differences in growth, although in some cases, QS mutants grow to a final cell density greater than that of the WT (41, 49, 50). In our studies, both the WT Newman and Δ*agrA* mutant strains multiplied with identical growth rates and reached the same final cell density;

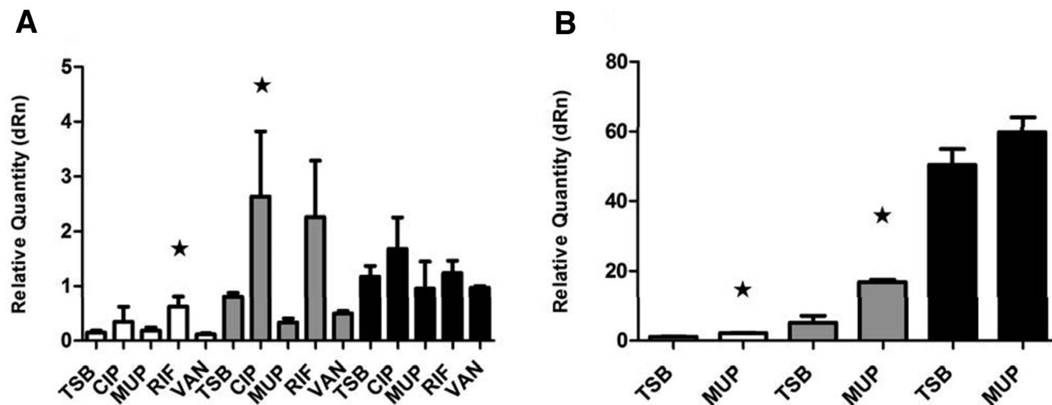

FIG 4 Induction of RNAIII expression by antibiotics. (A and B) RNAIII expression levels of WT cells in TSB in the absence or presence of the antibiotics ciprofloxacin (CIP), mupirocin (MUP), rifampin (RIF), and vancomycin (VAN). A star indicates a significant difference in the relative RNAIII expression level from that of the TSB control at the same OD. The ODs tested were 2.0 (white), 4.0 (gray), and 8.0 (black) in panel A and 0.15 (white), 0.6 (gray), and 1.2 (black) in panel B. The relative expression levels are normalized to the TSB control RNAIII expression levels of one sample at an OD of 8.0 in panel A and an OD of 0.15 in panel B. Means with standard deviations are shown. dRn, baseline subtracted fluorescent reading normalized to the reference dye.

therefore, if fitness costs are to be explained by growth differences, they must be subtle and apparent only in limited parts of the growth cycle. Another explanation for the fitness differences seen may be the quorum-controlled production of autolysins that occurs in both *S. aureus* and *P. aeruginosa* in the stationary growth phase (50, 51). At high pHs, *lasR* mutant cells reach up to 10-fold greater cell numbers than WT cells because of pH-mediated autolysis (50). If QS-dependent lysis is prominent in stationary phase, the QS-negative cells that fail to undergo lysis may have an advantage observed as increased fitness.

The results reported here have important clinical implications. It has long been recognized that *agr*-deficient mutants are often isolated in the hospital setting but rarely in the community (25). Given the central role of *agr* in virulence, these observations are puzzling. Our findings suggest that the fitness cost of carrying *agr* is enhanced by the presence of some antibiotics and that treatment with those antibiotics will select for *agr*-deficient mutants. This notion is supported by a study reporting that bacteremic patients who had received fluoroquinolone or beta-lactam antibiotic treatment prior to hospitalization displayed an approximately 2-fold greater probability of harboring *agr*-dysfunctional strains than those who had not received any treatment prior to admission (26). In another study, isogenic *S. aureus* isolates were periodically recovered from the bloodstream of a patient undergoing chemotherapy. Among the 31 loci affected in the last multidrug-resistant isolate was *agrC*, resulting in the inactivation of *agr* (52). In this case, the ability of *agr*-negative strains to develop a GISA or hetero-GISA phenotype (32) may have been instrumental in the final development of vancomycin resistance and in the fatal outcome of the infection. Our results show that some antibiotics select for *agr*-negative variants of *S. aureus*, and this phenomenon should be taken into consideration when designing antimicrobial chemotherapy.

MATERIALS AND METHODS

Strains, media, and MIC determination. The strains used in this study are derivatives of *S. aureus* Newman if not otherwise specified (see Table S2 in the supplemental material). All strains were grown at 37°C in TSB with or without ciprofloxacin (Bayer Schering Pharma), mupirocin (GlaxoSmithKline), rifampin (Sigma-Aldrich), and vancomycin (Sigma-Aldrich). MICs were determined by (i) using E-test strips (Biodisk) according to the manufacturer's instructions and (ii) broth dilution assay according to EUCAST instructions (E.Dis 5) including the reference strain *S. aureus* ATCC 25923. Construction of Δ*agrA* and ΔRNAIII mutants was performed by transduction with phage $\varphi 80\alpha$ (53).

Quantitation of RNAIII expression by qPCR. RNA was isolated by using the SV RNeasy Mini Kit (Qiagen). RNA was converted to cDNA by using the high-capacity cDNA RT kit (Applied Biosystems) with an RNase inhibitor. The cDNA was used as the template for real-time qPCRs with the primers listed in Table S3 in the supplemental material and the Maxima SYBR green/ROX qPCR Master Mix. PCR products were detected by using the MX3000P qPCR system (Stratagene Products/Agilent Technologies), and the results were analyzed with the MxPro software (version 4.10; Stratagene).

Fitness measurements. Three different assays were used to estimate fitness. (i) Growth rates were determined by growing bacteria in TSB medium with or without antibiotics at 37°C and measuring OD (600 nm) over time with a Bioscreen C reader (Labsystems) by using a 100-well honeycomb plate filled at 300 μl/well with an overnight culture diluted to 10^6 bacteria/ml in TSB growth medium. The relative fitness of the strains was calculated as the ratio of their doubling times (t_Ds) as follows: t_D(WT)/t_D(mutant). (ii) CFU counts at stationary phase were determined after 24 and 48 h for cells grown in 30 ml of TSB medium in a 300-ml narrow-neck Erlenmeyer flask with or without antibiotics shaken at 200 rpm with a starting inoculum of ~10^7 bacteria/ml. (iii) Competition experiments to assay relative competitive fitness after 24 and 48 h, *W*, of the tetracycline-resistant Δ*agrA* mutant or a plasmid (pTX, pTX::RNAIII, or pTX::*agrA*)-carrying strain compared to that of the tetracycline-susceptible WT strain was calculated by using the following formula (33): $W = \ln(RF/RI)/\ln(SF/SI)$, where *RI* and *SI* refer to the CFU counts of resistant and susceptible cells at the start of the competition assay, respectively, and *RF* and *SF* refer to the numbers of resistant and susceptible cells at the endpoint of the competition assay. The experimental conditions of the competition assay were as follows. Overnight cultures of mutant and WT cells were diluted to ~10^7 bacteria/ml, mixed at a 1:1 ratio, and allowed to compete for 7 to 8 generations, reaching stationary phase (under the same culturing conditions as those used to determine CFU counts). The ratio of the endpoint CFU counts of the mutant and WT cells (competition ratio) was determined by spreading suitable dilutions on TSB agar plates with and without tetracycline at 2 μg/ml. The number of biological replicates used in the competition assay was between 5 and 12 for each condition assayed.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00459-12/-/DCSupplemental.

Figure S1, PDF file, 0.3 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.3 MB.
Table S3, PDF file, 0.1 MB.

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