Selection for *Staphylococcus aureus* small-colony variants due to growth in the presence of *Pseudomonas aeruginosa*

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Opportunistic infections are often polymicrobial. Two of the most important bacterial opportunistic pathogens of humans, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, frequently are coisolated from infections of catheters, endotracheal tubes, skin, eyes, and the respiratory tract, including the airways of people with cystic fibrosis (CF). Here, we show that suppression of *S. aureus* respiration by a *P. aeruginosa* exoproduct, 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO), protects *S. aureus* during coculture from killing by commonly used aminoglycoside antibiotics such as tobramycin. Furthermore, prolonged growth of *S. aureus* with either *P. aeruginosa* or with physiological concentrations of pure HQNO selects for typical *S. aureus* small-colony variants (SCVs), well known for stable aminoglycoside resistance and persistence in chronic infections, including those found in CF. We detected HQNO in the sputum of CF patients infected with *P. aeruginosa*, but not in uninfected patients, suggesting that this HQNO-mediated interspecies interaction occurs in CF airways. Thus, in all coinfections with *P. aeruginosa*, *S. aureus* may be underappreciated as a pathogen because of the formation of antibiotic-resistant and difficult to detect small-colony variants. Interspecies microbial interactions, analogous to those mediated by HQNO, commonly may alter not only the course of disease and the response to therapy, but also the population structure of bacterial communities that promote the health of host animals, plants, and ecosystems.

4-hydroxy-2-heptylquinoline-*N*-oxide | antibiotic resistance | interspecies | polymicrobial | tobramycin

Bacterial communities consisting of multiple species exist in the soil and water and on animal and plant surfaces. These polymicrobial populations can be important determinants of host organism health. Commensal communities of animal flora contribute to normal digestion, intestinal development, and disease resistance, whereas imbalances in these communities can lead to pathogenic states such as colitis and periodontal disease. Bacteria infecting human tissues also commonly comprise mixed communities, particularly when mucosal barriers have been compromised (1). Relatively little is known regarding microbial interactions within such communities compared with what is known of individual bacterial species living in isolation (2–6).

Infection with mixed communities of bacterial pathogens is common in hospitalized and immunosuppressed patients (1). One of the best studied pathogenic bacterial communities occupies the airways of people with the disease cystic fibrosis (CF). The Gram-positive bacterium *Staphylococcus aureus* is the pathogen most commonly cultured from young children with CF (7), and in the preantibiotic era, many CF patients succumbed to *S. aureus* infection (8). CF patients infected only with *S. aureus* have airway inflammation (9), and treatment of these patients with antistaphylococcal antibiotics often leads to clinical improvement (9). Of the multiple opportunistic bacteria that may infect these patients, however, the Gram-negative bacterium *Pseudomonas aeruginosa* is considered to be the most significant pathogen (10). As patients acquire *P. aeruginosa*, *S. aureus* is cultured less frequently (7, 11), although both organisms are commonly coisolated from CF respiratory cultures [20–25% of cultures in one CF clinic population; supporting information (SI) Table 1]. Although this shift could result from antibiotic treatments, an alternative explanation is that *P. aeruginosa* competes with *S. aureus* within CF airways.

P. aeruginosa produces an antistaphylococcal substance, 4-hydroxy-2-heptylquinoline-*N*-oxide (HQNO), that suppresses the growth of many Gram-positive bacteria (11, 12). HQNO also allows some Gram-positive bacteria to grow, albeit slowly, in the presence of the first-generation aminoglycoside antibiotics streptomycin and dihydrostreptomycin (13). After these initial discoveries, the apparent paradox of how HQNO both could inhibit growth and protect against aminoglycosides remained unresolved, and the potential implications for bacterial mixed species infections never were pursued (12). Given that more recent research showed that electron transport is required for aminoglycoside uptake (14), we reasoned that HQNO protects *S. aureus* from killing by aminoglycosides by inhibiting electron transport. The newer aminoglycoside tobramycin commonly is used in treating *P. aeruginosa* infections such as those in CF (7, 10), and *in vitro* testing indicates that *S. aureus* and *P. aeruginosa* isolates from CF patients are usually tobramycin-susceptible (15, 16). We therefore hypothesized that *S. aureus* density within CF airways reflects a balance between the suppressive effects of antibiotics and HQNO, and HQNO-mediated protection from aminoglycosides (which would escape detection by routine susceptibility testing). To provide support for this hypothesis, we examined the effects of HQNO on aminoglycoside susceptibility of *S. aureus* under conditions that were clinically and physiologically relevant, and that had been previously unexamined. We used HQNO at concentrations normally produced by *P. aeruginosa*, together with aminoglycosides that are in clinical use (including tobramycin), and with both brief and extended bacterial incubations, so as to mimic conditions characteristic of acute and chronic infections, respectively.

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Abbreviations: CF, cystic fibrosis; HQNO, 4-hydroxy-2-heptylquinoline-*N*-oxide; MH, Mueller–Hinton; MIC, minimal inhibitory concentration; SCV, small-colony variant.

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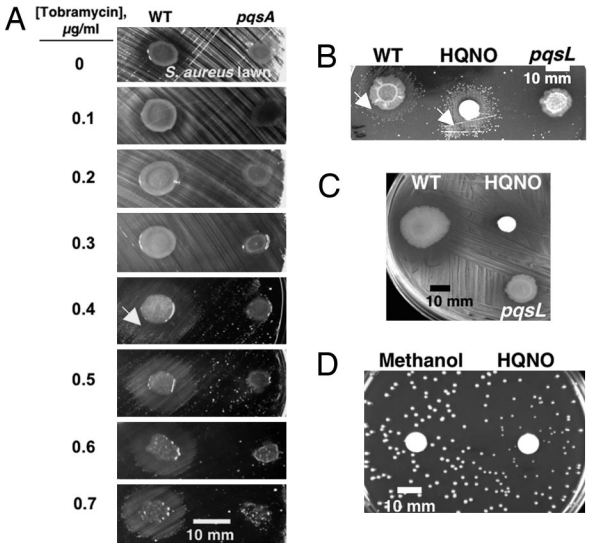

Fig. 1. HQNO produced by *P. aeruginosa* simultaneously suppresses the growth of *S. aureus* and protects it from tobramycin killing. (*A*) Colonies of *P. aeruginosa* grown on a lawn of *S. aureus* on LB agar plates with tobramycin added as indicated. WT, wild-type *P. aeruginosa* PAO1. The *pqsA* mutant is defective for HQNO production. The MIC of tobramycin for these *P. aeruginosa* strains is 1 μg/ml. White arrow, zone of *S. aureus* growth. *S. aureus* grows slowly in the zone surrounding the wild-type (WT) *P. aeruginosa* colony, most evident when the *S. aureus* lawn is suppressed by tobramycin concentrations >0.3 μg/ml, but similar *S. aureus* densities are present in the zones under each condition (data not shown). (*B*) A paper disk (containing 15 μg of pure HQNO) and colonies of *P. aeruginosa* on a lawn of *S. aureus* cells on agar media containing 0.6 μg/ml tobramycin. WT, wild-type *P. aeruginosa* PA14. The *pqsL* mutant is defective for HQNO production. White arrow, zone of *S. aureus* growth. (*C*) Experiment performed as in *B*, except without tobramycin. (*D*) *S. aureus* colonies (of cells from a diluted culture) growing on LB agar near disks containing HQNO or methanol solvent alone. For all *P. aeruginosa* experiments shown, equivalent results were obtained by using *P. aeruginosa* strains PAO1 and PA14 and their derived *pqsA* and *pqsL* mutants.

Results

HQNO at Physiological Concentrations Has Short-Term Effects on *S. aureus*. The short-term effects on *S. aureus* of HQNO produced by *P. aeruginosa* were examined by coculturing the two organisms overnight in the presence of various antibiotics. Significantly, *S. aureus* grew in the presence of tobramycin levels above the minimal inhibitory concentration (MIC; 0.4 μg/ml) in a zone surrounding *P. aeruginosa* colonies (Fig. 1*A*). *P. aeruginosa* conferred protection against additional aminoglycosides (gentamicin, amikacin, and kanamycin) but not against any other antibiotic classes tested: β-lactams (carbenicillin and ceftazidime), macrolides (azithromycin), and chloramphenicol (data not shown). Colonies of *P. aeruginosa* mutants unable to produce HQNO (*pqsA* and *pqsL* mutants; refs. 17 and 18) did not protect *S. aureus* from tobramycin (Fig. 1 *A* and *B*), whereas pure HQNO did (Fig. 1*B*). These results indicate that HQNO production by *P. aeruginosa* colonies protects *S. aureus* from the antibiotic effect of tobramycin.

In the absence of tobramycin, wild-type *P. aeruginosa* colonies, but not *pqsA* (Fig. 1*A*) or *pqsL* (Fig. 1*C*) mutant colonies, visibly suppressed *S. aureus* growth, as demonstrated in refs. 11, 13, 17, and 19. We found that both wild-type *P. aeruginosa* culture supernatants (data not shown) and pure HQNO (Fig. 1*C*) suppressed *S. aureus* growth to a degree similar to *P. aeruginosa* colonies. Furthermore, *S. aureus* formed smaller colonies when near an HQNO source than when distant from such a source (Fig. 1*D*). Cells from the smaller colonies, when transferred to media without HQNO, formed colonies of wild-type size, indicating that growth limitation by HQNO was reversible. When either HQNO or *P. aeruginosa* was added to *S. aureus* liquid cultures, neither resulted in a decrease in the number of viable *S. aureus* cells (data not shown). Reversible, incomplete growth suppression by HQNO is consistent with the fact that *S. aureus* could grow adjacent to *P. aeruginosa* colonies in the presence (Fig. 1*A*) or absence (data not shown) of aminoglycosides and adjacent to a source of pure HQNO (Fig. 1*D*).

Long-Term Exposure of *S. aureus* to HQNO Selects for Small-Colony Variants (SCVs). The two phenotypes associated with *S. aureus* grown in the presence of HQNO, aminoglycoside resistance and small colony formation, are also characteristic of genetically stable *S. aureus* SCVs (20). *S. aureus* SCVs are observed in diverse infections, including the airways of as many as 50% of CF patients (21). Such SCVs are selected by aminoglycoside exposure (20, 22), frequently arise because of mutations that impair electron transport (20, 21), and are resistant to aminoglycosides because of diminished electron transport-mediated drug uptake (23). Given the similarities between SCVs and the transient, HQNO-induced *S. aureus* phenotypes (Fig. 1), we explored whether prolonged growth in the presence of HQNO ultimately selects genetically stable SCVs.

Growth of *S. aureus* for 5 days in the presence of 10 μg/ml HQNO (a concentration typically found in stationary-phase *P. aeruginosa* cultures; ref. 17) yielded a high proportion of SCVs (Fig. 2*A*). Similarly, after 5 days in coculture with wild-type *P. aeruginosa*, SCVs represented the majority of the *S. aureus* population (Fig. 2*A*). In contrast, cocultures with HQNO-deficient *P. aeruginosa* strains (*pqsA* or *pqsL* mutants, data not shown) and *S. aureus* monocultures (Fig. 2*A*) yielded no SCVs. A significant number of *S. aureus* SCVs were obtained after a shorter incubation period (overnight growth) when 50 μg/ml tobramycin was added to the growth medium (Fig. 2*B*), as reported for the aminoglycoside gentamicin (24). Although overnight growth in the presence of either 6–12 μg/ml tobramycin or 10 μg/ml HQNO yielded rare SCVs (Fig. 3), the combination of these conditions led to a population composed nearly entirely of SCVs (Figs. 2*B* and 3). Therefore, the effect of the two compounds acting together is greater than the sum of their individual effects, consistent with synergy between aminoglycosides and HQNO during selection for *S. aureus* SCVs. Such synergy is likely to be clinically relevant, because the two compounds would be predicted to be found together during treatment of many clinical coinfections with *S. aureus* and *P. aeruginosa*.

***S. aureus* SCVs Derived from Selection with HQNO Have the Characteristics of Clinical SCVs.** To determine whether the *S. aureus* SCVs selected *in vitro* had the reduced respiration characteristic of clinical SCVs, we used the redox-sensitive dye Alamar blue (ref. 25; Fig. 2*C*). HQNO (10 μg/ml) inhibited dye reduction by wild-type cells by 80%, consistent with the known ability of HQNO to inhibit *S. aureus* cytochrome activity (12). Using this assay, the respiratory activity of an SCV selected by coculture with *P. aeruginosa* was 40% of that of wild-type cells, and it was not further inhibited by HQNO (Fig. 2*C*). Clinical SCVs commonly have respiratory defects that can be complemented by hemin or menadione (vitamin K3), which are required for biosynthesis of electron transport chain components, although the mutations responsible for these defects have not been described for clinical isolates (20, 21). Menadione restored wild-type colony size to *S. aureus* SCVs selected *in vitro* when grown with either chemically defined medium (data not shown) or with rich medium (Fig. 2*D*), suggesting that they are menadione auxotrophs (20). Menadione-auxotrophic *S. aureus* SCVs are known to accumulate aminoglycosides more slowly than wild-type cells, and they have been isolated from clinical infec-

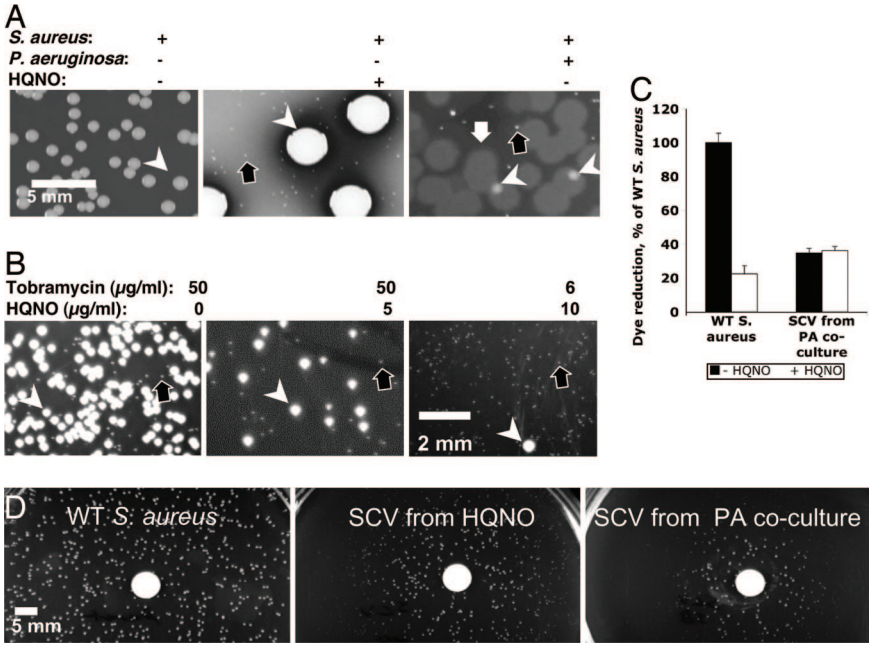

Fig. 2. HQNO inhibits *S. aureus* electron transport, ultimately selecting for SCVs. (*A*) *S. aureus* grown for 5 days in static cultures alone, with *P. aeruginosa* PAO1, or with HQNO as indicated, before plating on selective media to distinguish species and morphotypes. Results shown are on sheep's blood agar. Cells from the culture with HQNO were incubated longer to better display SCVs. Results are representative of three separate experiments; equivalent results were obtained with *P. aeruginosa* strains PAO1 and PA14 and with *S. aureus* clinical isolates from five separate CF patients. White arrowheads, normal *S. aureus*. Black arrows, SCV *S. aureus*. White arrow, *P. aeruginosa*. (*B*) Colonies of cells from *S. aureus* cultures grown overnight in the presence of the indicated tobramycin and/or HQNO concentrations. Results are representative of two separate experiments. (*C*) Alamar blue, a redox-sensitive dye, was used to quantify reduction potential (as a measure of electron transport) of the *S. aureus* strains indicated in the presence and absence of HQNO. Results shown are the average of triplicates ± SD and are representative of three separate experiments. (*D*) Cells of *S. aureus* SCVs isolated after HQNO exposure form colonies of wild-type size when growing in close proximity to a disk containing 1.5 μg of menadione and with colony size diminishing with distance from the disk. No such colony changes were observed with disks containing thymidine or hemin. Results are representative of three separate experiments.

tions, including those in CF airways (20, 23, 26). Similarly, the *S. aureus* SCVs selected *in vitro* had increased resistance to tobramycin (Fig. 4 *A* and *B*), and susceptibility to tobramycin was restored to wild-type levels in the presence of menadione (Fig. 4*C*).

Given that laboratory growth of clinical *S. aureus* SCVs can yield revertants with wild-type colony morphology (20), we examined the genetic stability of SCVs selected *in vitro*. Wild-type revertants appeared at a rate of ≈0.5% per hour of culture in the absence of HQNO or *P. aeruginosa*, whereas no such revertants were observed after culture in the presence of tobramycin (data not shown). In environments where *P. aeruginosa* and *S. aureus* coexist, there is likely to be a dynamic interaction among the number of *P. aeruginosa* cells, the proportion of *S. aureus* SCVs in the bacterial population, and the response of each pathogen to tobramycin. Adding further complexity, subinhibitory levels of aminoglycosides induce *P. aeruginosa* biofilm formation, a form of bacterial growth characterized by high cell density (27). Because HQNO biosynthesis is up-regulated by the cell density-dependent quorum sensing signaling system (17), HQNO production would be predicted to be increased in biofilms, increasing the selection pressure for *S. aureus* SCVs. The recent identification in the CF lung of SCVs of *P. aeruginosa*, which have an increased capacity for biofilm formation (28) and also have been selected by aminoglycoside exposure (29), would be predicted to further augment these effects.

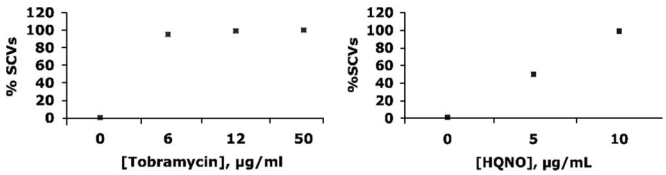

Fig. 3. Synergy between HQNO and tobramycin for *S. aureus* SCV formation. Quantitative results of the experiment are described in Fig. 2*B*. The relative proportions of SCVs were determined among 100–300 total colony-forming units in overnight cultures of *S. aureus* grown in HQNO and tobramycin. Growth in the presence of either 10 μg/ml HQNO (*Left*) or 12 μg/ml tobramycin alone (*Right*) yielded ≤5% SCVs, whereas in combination, they yielded nearly 100% SCVs, consistent with synergy. Results are representative of two separate experiments, each with at least two technical replicates.

HQNO Is Present in Sputum from CF Patients Infected with *P. aeruginosa*. The biosynthetic pathway that produces HQNO generates several additional 4-hydroxy-2-alkylquinolines (17), two of which have been found in CF respiratory secretions (11, 30). We therefore sought to detect HQNO in sputum from CF patients. The reducing agents used in previous studies to liquify these highly viscous samples (11) maximize solvent extraction but destroy HQNO, and we therefore performed these extractions without liquification. Despite these limitations, we detected HQNO and only in samples from patients infected with *P. aeruginosa* (Table 1). These results suggest that *P. aeruginosa* in the CF airway produces HQNO and affects *S. aureus* as it does *in vitro*. Indeed, HQNO produced by *P. aeruginosa* could select for *S. aureus* SCVs not only in the CF airway, but wherever these two opportunistic pathogens occur together (Fig. 5).

Discussion

S. aureus SCVs have been observed in infections for decades, but our current understanding of their clinical importance is limited (20, 26). *S. aureus* SCVs are of particular clinical concern

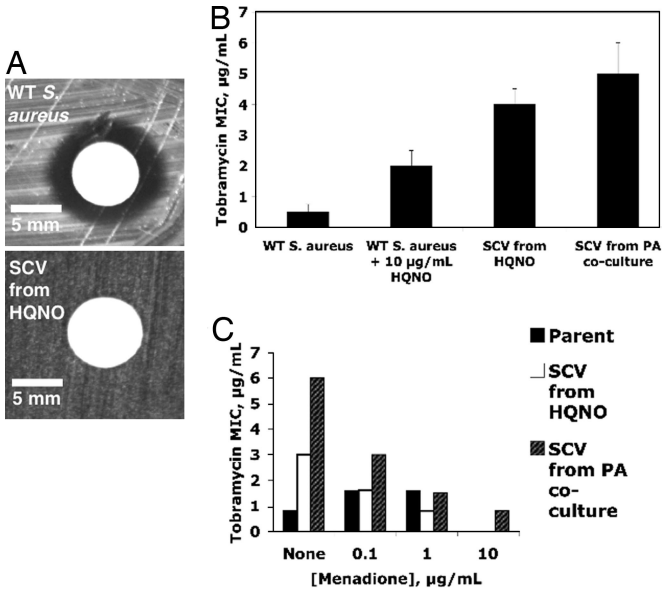

Fig. 4. HQNO selects for *S. aureus* SCVs that are resistant to tobramycin. (*A*) Tobramycin susceptibility of wild-type *S. aureus* and of an SCV isolated after HQNO exposure. A disk containing 1.5 μg of tobramycin was placed on a *S. aureus* lawn on LB agar. (*B*) The MIC of tobramycin for wild-type *S. aureus* growing in the absence and in the presence of HQNO, and for SCVs derived as indicated. Results shown are averages of technical duplicates ± SD and are representative of three separate experiments. (*C*) The MIC of tobramycin, with and without menadione, for wild-type *S. aureus* and SCVs derived as indicated. Menadione restored sensitivity to tobramycin to *S. aureus* SCVs (but menadione itself was toxic to wild-type *S. aureus* at 10 μg/ml); results shown are representative of two separate experiments.

because they are resistant to aminoglycosides and, perhaps, to other antibiotics (20). *S. aureus* SCVs are persistent, infecting CF patients for many years (20), and are capable of intracellular growth (20), which may facilitate their persistence in chronic infections (Fig. 5). SCVs also may be missed by clinical laboratories because of their slow growth and atypical phenotype relative to other bacteria in mixed cultures (20, 21). We propose that the observation that *S. aureus* is cultured less frequently from CF patients after *P. aeruginosa* is acquired (7, 11) in part is attributable to HQNO-mediated selection for persistent, yet poorly detectable, SCVs. Furthermore, *S. aureus* may be underappreciated as a pathogen not only in CF, but in all coinfections with *P. aeruginosa*, including hospital-acquired pneumonia and infections associated with burns, catheters, and endotracheal tubes. Hospitalized patients are frequently colonized or infected with multiple pathogens, including *S. aureus* and *P. aeruginosa* (6, 31). Our results argue for routine investigation into potential undiagnosed roles of *S. aureus* SCVs in hospital-acquired infections when *P. aeruginosa* is present, and the detection and antibiotic treatment of *S. aureus* SCVs may improve patient outcomes.

Table 1. *P. aeruginosa* and *S. aureus* cfu vs. HQNO content extractable with ethyl acetate from unliquified, frozen sputum samples from six CF patients

Sputum specimen no.	*S. aureus*, cfu/ml*	*P. aeruginosa*, cfu/ml	Extractable HQNO, μg/g of dry weight sputum
1	$>10^5$	0	0
3	0	0	0
4	1,400	10^5	1.24
6	$>10^5$	0	0
7	80	4,000	0.39
11	70	0	0

*The presence or absence of *S. aureus* SCVs was not noted.

In considering the physiologic role for *P. aeruginosa* production of HQNO, a molecule that inhibits Gram-positive bacterial respiration, it is important to note that *P. aeruginosa* dwells predominantly in environmental niches, such as soil and water, rather than mammalian tissues. Only in the setting of compromised mucosal barriers or prolonged antibiotic therapy does *P. aeruginosa* opportunistically infect humans. *P. aeruginosa* commonly coinhabits soil with antibiotic-producing Gram-positive bacteria such as *Streptomyces tenebrarius*, the source of tobramycin (32). Therefore, HQNO production could have evolved, at least in part, to metabolically restrain environmental bacteria that produce antipseudomonal compounds. In support of this idea, we found that wild-type *P. aeruginosa* suppressed the growth of at least two antibiotic-producing, soil-dwelling Gram-positive bacteria: *S. tenebrarius* and *Paenibacillus polymyxa* (data not shown). Such microbial competition has been proposed to contribute to the evolution of *P. aeruginosa* acute virulence factors (4).

Given the complexity of microbial communities in the environment, *P. aeruginosa* exoproducts are likely to have effects that extend beyond straightforward restraint or elimination of competitors. The majority of antibiotics that revolutionized medicine in the mid-20th century are simply microbial products deliberately administered at supraphysiologic concentrations, although these products can have distinctly different effects at naturally occurring concentrations (27, 33). Thus, under natural conditions, these compounds could maintain equilibrium between microbial community members rather than eradicating them. For example, HQNO produced by *P. aeruginosa* at physiologic levels impairs *S. aureus* growth, perhaps allowing *P. aeruginosa* to benefit from useful *S. aureus* metabolites (19). Community interactions also may aid the bacterial colonization of animal mucosal surfaces by facilitating resistance to host defenses, including the activities of phagocytes (20, 22). Such interactions may have been important in the development of the commensal bacterial communities that colonize multicellular organisms.

We have shown that *P. aeruginosa* simultaneously suppresses the growth and enhances the aminoglycoside resistance of *S. aureus* by producing HQNO, a compound found in the sputum of infected CF patients. Prolonged exposure to HQNO selects for aminoglycoside-resistant genetic variant (SCV) *S. aureus*, which are difficult to detect. Our results highlight the potentially important roles for bacterial interspecies interactions in virulence and response to therapy. In any large and complex bacterial community, including polymicrobial infections and commensal bacterial populations, diverse interspecies interactions ranging from antagonism to cooperation are likely to exist and to significantly impact the health of host plants and animals.

Materials and Methods

Bacteria, Chemicals, and Growth Conditions. *P. aeruginosa* wild-type and mutant strains derived from PAO1 (Iglewski strain), including *pqsA* mutants, were gifts from L. Gallagher and C. Manoil (University of Washington) (34), except for the *pqsL* mutant, which was described by D.A.D. in ref. 35. Equivalent strains in the PA14 background were reported in refs. 17 and 18. *S. aureus* Newman strain was a gift from C. Rubens (University of Washington). Five CF clinical isolates of *S. aureus* with a normal colony size were provided by the University of Washington CF Core Microbiology Laboratory, directed by J. Burns. HQNO, menadione, and tobramycin were from Sigma (St. Louis, MO); Alamar blue was from Trek

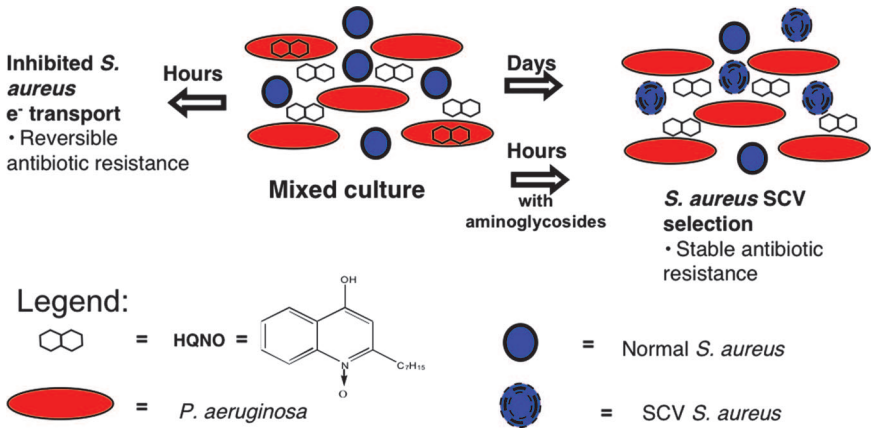

Fig. 5. Model for interspecies interactions between *P. aeruginosa* and *S. aureus*. After short-term exposure (hours), *S. aureus* grown in the presence of *P. aeruginosa* producing HQNO becomes transiently resistant to aminoglycosides and, possibly, other antibiotics (20, 21). After long-term exposure (days), *S. aureus* becomes stably resistant because of selection for *S. aureus* SCVs. Such SCVs can persist intracellularly in nonprofessional phagocytes and are difficult to detect (20). The presence of tobramycin also can select for *S. aureus* SCVs either independently (20) or synergistically with HQNO (Fig. 3). The production of HQNO and, thus, selection pressure for *S. aureus* SCVs, could be augmented by increased biofilm formation, either due to reversible induction by aminoglycosides (27) or associated with hyperadherent *P. aeruginosa* SCVs (28).

Diagnostics (Cleveland, OH); and hemin and thymidine were from Fluka Biochemica (Buchs, Switzerland). Mueller–Hinton (MH) broth, tryptic soy broth, and Luria–Bertani (LB) broth were from GIBCO (Grand Island, NY). Mannitol salt and sheep's blood agar plates were from Fisher (Pittsburgh, PA). M9 minimal medium plates were made as described in ref. 36, except using UltraPure Agarose (Invitrogen, Carlsbad, CA) at 1.5% and with the addition of all 20 aa plus hydroxy-L-proline at 20 μg/ml, cystine at 10 μg/ml, and biotin at 0.2 μg/ml. All incubations were performed at 37°C unless otherwise indicated. Sputum from CF patients was obtained from a collection (stored frozen) that was maintained by the Cystic Fibrosis Research Development Program at Children's Hospital and Regional Medical Center (CHRMC), Seattle, WA. Access to and the use of these samples, and the use of clinical *S. aureus* isolates, were approved by the CHRMC Human Subjects Institutional Review Board.

Bacterial Susceptibility Assays. Antibiotic susceptibility was determined by standard broth microdilution MIC assays in 96-well microtiter plates in MH broth by using 10^5 cells in 100 μl per well.

Assays with *S. aureus* on Agar Surfaces. *S. aureus* cultures grown overnight with LB broth were diluted 1:10 and applied to the surface of MH agar by using a cotton swab, followed by the addition of *P. aeruginosa* cells or test compounds, as described below, and incubation overnight at 37°C. To test for inhibition by *P. aeruginosa* colonies, 5 μl of *P. aeruginosa* cultures, grown overnight with LB broth and diluted 1:10, was added as spots to the *S. aureus* lawn. To test for inhibition by *P. aeruginosa* culture supernatants, *P. aeruginosa* overnight cultures grown with LB broth were centrifuged to pellet cells, the supernatants were filtered through a 0.22-μm filter, and 20 μl of the supernatant was placed in a well in the MH agar made by removing a plug of agar. For disk assays, the indicated chemicals (in methanol or water, as appropriate) were added to sterilized 9-mm-diameter Whatman filter paper disks, which were allowed to dry before being placed on the agar surface. Chemical complementation experiments were performed on M9 minimal medium/agarose (and in parallel on MH agar for improved photographic images), using disks containing thymidine, menadione, or hemin to examine for auxotrophy as described in ref. 37.

Alamar Blue Reduction Assay. *S. aureus* cells were resuspended from agar surfaces into PBS (to minimize SCV reversion to wild-type phenotype) to an OD_{600} of 1.0. The resulting suspension was diluted 1:10 in MH broth for a final volume of 1 ml. HQNO (3.3 μl of 3 mg/ml methanol stock) or methanol alone was added to each sample, which was mixed before and after addition of 50 μl of Alamar blue. The samples were incubated for 1 h at 22°C and centrifuged to pellet cells, and supernatant was removed for analysis. HQNO in methanol (or methanol alone as control) was added so that each sample contained a final concentration of 10 μg/ml HQNO (to control for direct effects of HQNO on dye reduction). Percent reduction of Alamar blue was calculated as described in ref. 25.

***S. aureus* SCV Selection.** *S. aureus* Newman strain and *P. aeruginosa* wild-type PAO1 or *pqsL* mutant strains were grown overnight with MH broth and diluted 1:1,000 with MH broth with and without HQNO. These cultures, alone or combined as indicated, were incubated statically for 120 h at 37°C, then vortexed and serially diluted with PBS before being added onto four types of agar media (sheep's blood, mannitol salts, LB, and LB with ampicillin) to discriminate between and enumerate the two different species and normal versus SCV *S. aureus*. Alternatively, *S. aureus* Newman strain was grown statically in tryptic soy broth at 37°C with and without 10 μg/ml HQNO and subcultured daily into equivalent conditions for 10 days. Each day, an aliquot of each culture was spread on tryptic soy agar to screen for SCVs. SCVs for this analysis were defined as colonies at least 10-fold smaller in size than normal colonies (20, 38). For all *S. aureus* strains isolated for further study, the presence of catalase and coagulase activities was confirmed by using commercially available tests (BBL, Cockeysville, MD) per manufacturer's instructions.

Analysis of CF Sputum. The sputum samples tested were selected as the first six from a collection of 20 to have both an adequate volume for the analysis and culture positivity for only *S. aureus* and/or *P. aeruginosa*, or neither. For these samples, ≈500 μl of thawed sputum was diluted 1:1 with distilled water, vortexed in the presence of 10 2-mm glass beads, then extracted three times with 900 μl of ethyl acetate. The solvent was removed from pooled extract, and the residue was redissolved in 20 μl of 98% acetonitrile:2% acetic acid before LC/MS analysis and HQNO

quantitation by using an internal standard as described in refs. 18 and 39.

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