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Coculture of *Staphylococcus aureus* with *Pseudomonas aeruginosa* Drives *S. aureus* towards Fermentative Metabolism and Reduced Viability in a Cystic Fibrosis Model

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ABSTRACT

The airways of patients with cystic fibrosis are colonized with diverse bacterial communities that change dynamically during pediatric years and early adulthood. *Staphylococcus aureus* is the most prevalent pathogen during early childhood, but during late teens and early adulthood, a shift in microbial composition occurs leading to *Pseudomonas aeruginosa* community predominance in ~50% of adults. We developed a robust dual-bacterial *in vitro* coculture system of *P. aeruginosa* and *S. aureus* on monolayers of human bronchial epithelial cells homozygous for the Δ F508 cystic fibrosis transmembrane conductance regulator (CFTR) mutation to better model the mechanisms of this interaction. We show that *P. aeruginosa* drives the *S. aureus* expression profile from that of aerobic respiration to fermentation. This shift is dependent on the production of both 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) and siderophores by *P. aeruginosa*. Furthermore, *S. aureus*-produced lactate is a carbon source that *P. aeruginosa* preferentially consumes over medium-supplied glucose. We find that initially *S. aureus* and *P. aeruginosa* coexist; however, over extended coculture *P. aeruginosa* reduces *S. aureus* viability, also in an HQNO- and *P. aeruginosa* siderophore-dependent manner. Interestingly, *S. aureus* small-colony-variant (SCV) genetic mutant strains, which have defects in their electron transport chain, experience reduced killing by *P. aeruginosa* compared to their wild-type parent strains; thus, SCVs may provide a mechanism for persistence of *S. aureus* in the presence of *P. aeruginosa*. We propose that the mechanism of *P. aeruginosa*-mediated killing of *S. aureus* is multifactorial, requiring HQNO and *P. aeruginosa* siderophores as well as additional genetic, environmental, and nutritional factors.

IMPORTANCE

In individuals with cystic fibrosis, *Staphylococcus aureus* is the primary respiratory pathogen during childhood. During adulthood, *Pseudomonas aeruginosa* predominates and correlates with worse patient outcome. The mechanism(s) by which *P. aeruginosa* outcompetes or kills *S. aureus* is not well understood. We describe an *in vitro* dual-bacterial species coculture system on cystic fibrosis-derived airway cells, which models interactions relevant to patients with cystic fibrosis. Further, we show that molecules produced by *P. aeruginosa* additively induce a transition of *S. aureus* metabolism from aerobic respiration to fermentation and eventually lead to loss of *S. aureus* viability. Elucidating the molecular mechanisms of *P. aeruginosa* community predominance can provide new therapeutic targets and approaches to impede this microbial community transition and subsequent patient worsening.

Complex polymicrobial communities colonize the airways of cystic fibrosis (CF) patients within the first month of life (1). Culture-independent studies have revealed the simultaneous presence of numerous bacterial taxa, fungi, and viruses in respiratory samples from CF patients at all stages of life (2–8). This abundance of microbes colonizing the respiratory tract, and particularly the lower airways, is facilitated by thick airway mucus and deficient mucociliary clearance that result from mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene (9–11). These polymicrobial airway communities are diverse and dynamic, especially in young patients (12). During the first decade of life, bacterial diversity generally increases in patient samples. As patients age, diversity decreases until adulthood, when lower airway communities often become stable and resilient to significant perturbation by treatment (7, 12–15). In recent years, our understanding of the complexities of these polymicrobial communities has improved by characterizing patient samples using deep-sequencing technologies; however, how these communities impact interbacterial interactions, the immune re-

sponse, antibiotic efficacy, and overall patient health is not well understood.

Culture-independent studies have emphasized the relevance of several emerging pathogens, like *Streptococcus milleri* group species and *Mycobacterium abscessus*, as well as nonpathogenic com-

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munity members (16–19). Despite the identification of these emerging pathogens, *Staphylococcus aureus* and *Pseudomonas aeruginosa* remain two of the most relevant bacterial pathogens for CF patients. *S. aureus* is the most prevalent pathogen detected in pediatric patients clinically (20). The presence of *S. aureus* is correlated with worse lung function and lower forced expiratory volume (FEV), poorer nutrition, and increased inflammation in children (21, 22). Further, strains of *S. aureus* may persist in patients through adaptation of numerous virulence factors, including capsule, hemolysis, biofilm formation, and antibiotic resistance (particularly methicillin resistance) (23) and through the formation of small-colony variants (SCVs) (24). *P. aeruginosa* is detected in patients of all ages but is typically most prevalent and abundant in adult patients (20). Similarly to *S. aureus*, *P. aeruginosa* is associated with decreased FEV (25) and increased inflammation in children (26). Further, early colonization with *P. aeruginosa* is correlated with increased exacerbation and morbidity (27, 28). *P. aeruginosa* has a repertoire of virulence factors (including proteases, rhamnolipids, phospholipase C, hemolysin, and others) that may aid early-stage colonization. During chronic *P. aeruginosa* infection, expression of such virulence factors subsequently decreases over time to promote persistence (29). During long-term infections, hypermutators and adaptations like mucoidy and antibiotic resistance, as well as changes in gene expression, aid *P. aeruginosa*'s ability to chronically colonize patient airways (30–34).

While *S. aureus* is well recognized as the predominant CF pathogen throughout childhood, *P. aeruginosa* is arguably the second most relevant pathogen in CF children. In some patients, *P. aeruginosa* may be the first pathogen isolated in infants and is detected in about 25 to 40% of young children by culture-based methods (12, 20, 35). Early in life, *P. aeruginosa* acquisition is associated with increased isolation of methicillin-resistant *S. aureus* (28). Interestingly, during late adolescence and adulthood the frequency of *S. aureus* in CF patients steeply declines and this microbe is displaced by *P. aeruginosa* (20). *P. aeruginosa* is detected in 80 to 90% of adults and becomes the predominant organism in the sputum of at least half of patients (3, 12, 20).

This striking negative clinical correlation between *S. aureus* and *P. aeruginosa* during teenage years and young adulthood has driven several *in vitro* and *in vivo* studies geared toward characterizing the interbacterial interactions of these two organisms (36–44). These previous studies have found that *P. aeruginosa* secretes various antistaphylococcal products and proteases, such as LasA, that can cause both biofilm dispersion and cell lysis of *S. aureus* (42, 45). Further, *P. aeruginosa* produces several inhibitors of *S. aureus* respiration, including hydrogen cyanide, quinoline *N*-oxides, and the phenazine pyocyanin (41, 46–48). *P. aeruginosa* has also been described to benefit from coculture with *S. aureus* by using this Gram-positive organism as an iron source (43) and a cue to produce extracellular virulence factors against both prokaryotic neighbors and the host (49). In response to the hostile environment, *S. aureus* may adapt to *P. aeruginosa* exoproducts, particularly in the presence of 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO), through increased biofilm formation (41) or increased frequency of SCVs (50, 51).

Despite significant efforts to characterize *P. aeruginosa* and *S. aureus* in CF patients, the molecular mechanism of their interaction and the role of the host (52) in driving the dynamics of the transition between *P. aeruginosa* and *S. aureus* during teenage

years are not well understood. Studies of these two organisms, to date, have characterized isolated interactions using various *in vitro* or *in vivo* models. Here, we use a single *in vitro* model that can be adapted to study interactions on epithelial cells or plastic, during coculture in both the planktonic and biofilm phases. Using this system, we describe a mechanism by which *P. aeruginosa* drives *S. aureus* toward a lactic acid fermentative lifestyle through the action of HQNO and *P. aeruginosa*-produced siderophores early in coculture, eventually leading to killing of *S. aureus* and predominance of *P. aeruginosa* in the community.

MATERIALS AND METHODS

Strains and culture conditions. Overnight cultures were inoculated with a single colony of *P. aeruginosa* or *S. aureus* into 5 ml lysogeny broth (LB) or tryptic soy broth (TSB), respectively, and grown for ~12 h at 37°C with aeration on a roller drum. Methicillin-resistant *S. aureus* strains were grown in TSB plus 16 µg/ml oxacillin under these same conditions. SCV strains were grown statically for 20 h. A complete list of strains used throughout this study is provided in Table S1 in the supplemental material.

Coculture assays. (i) Coculture assay on CFBE monolayers. Coculture assays were performed as previously described (53), with indicated adaptations to accommodate multiple bacterial species, and are depicted in Fig. 1A. Liquid cultures of *P. aeruginosa* and *S. aureus* were individually centrifuged (10,000 × g, 3 min) and washed in minimal essential medium plus 2 mM L-glutamine (MEM + L-Gln). Washed bacterial cells were resuspended in MEM + L-Gln to an optical density at 600 nm (OD₆₀₀) of 0.1. For coculture samples, 250 µl of 0.1-OD₆₀₀ *P. aeruginosa* and 250 µl of 0.1-OD₆₀₀ *S. aureus* were added to triplicate wells of monolayers of CF bronchial epithelial (CFBE) airway cells in a 24-well plate. CFBE monolayers were grown as previously described (54). For monoculture controls, 250 µl of 0.1-OD₆₀₀ *P. aeruginosa* or 250 µl of 0.1-OD₆₀₀ *S. aureus* and 250 µl of MEM + L-Gln were added to triplicate wells. Target inputs were 0.5 × 10⁷ to 1 × 10⁷ CFU per well for each species in a 24-well plate. CFBE coculture plates were incubated at 37°C with 5% CO₂ for 1 h, at which point any unattached bacterial cells were removed and 500 µl of MEM + L-Gln + 0.4% L-arginine was added to each well and incubated for an additional 5 h. After this additional 5-h incubation, the planktonic cells were removed and 500 µl of fresh MEM + L-Gln + 0.4% L-arginine was once again replaced in each well. The established coculture biofilm was incubated for an additional 16 h, followed by viable cell counts. For planktonic CFU enumeration, culture supernatant was 10-fold serially diluted in phosphate-buffered saline (PBS) and plated on *Pseudomonas* isolation agar or mannitol salt agar, for *P. aeruginosa* and *S. aureus* selective growth, respectively. *S. aureus* SCV strains were selected on tryptic soy agar plates grown in an anaerobic chamber for 3 days. After removal of culture supernatants, the biofilm fraction was removed by treatment with 250 µl of 0.1% Triton X-100 in PBS, with gentle shaking for 15 min. Biofilm bacteria were then scraped and vortexed in the plate for 2 min (covered with aluminum tape). Biofilm fractions were then serially diluted and plated as described for the planktonic fraction.

(ii) Coculture assay on plastic substrate. In the plastic coculture assay, in which the bacterial biofilms are grown on a plastic substrate rather than on CFBE cells, bacterial culture preparation and inoculation were performed as described above. Cultures were inoculated directly into tissue culture-treated 24-well plastic plates. Unattached cells were removed after 1 h, and 500 µl MEM + L-Gln was added to each well. The cultures were then incubated for an additional 16 h, followed by CFU counts performed as described for the coculture assay on CFBE cells. For iron supplementation assays, bacterial cells were inoculated in MEM + L-Gln on plastic substrate. After 1 h, unattached cells were removed and MEM + L-Gln + 8 µM FeCl₃ was added to the well. Cocultures then proceeded as described above.

RNA isolation. Coculture assay mixtures for RNA analysis were prepared and inoculated as described in the CFBE and plastic assays and

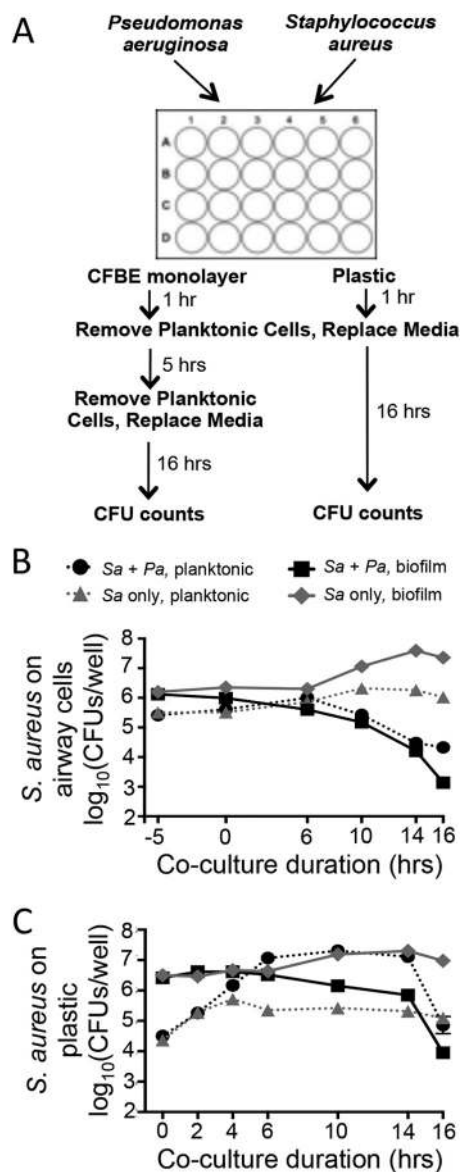


FIG 1 *In vitro* *P. aeruginosa*-*S. aureus* coculture assays on CFBE monolayers or plastic model early-stage coexistence and late-stage *S. aureus* killing by *P. aeruginosa*. (A) Assay design displaying *P. aeruginosa*-*S. aureus* coculture assays on CFBE monolayers and a plastic substrate. (B and C) Time course analyses were performed for *S. aureus* 8325-4 in planktonic and biofilm fractions. *S. aureus* viability was measured as log₁₀(CFU/well) in a 24-well plate either in coculture with *P. aeruginosa* PA14 or as a monoculture. The key for panels B and C is shown at the top of panel B. Assays were performed in the CFBE coculture (B) or plastic coculture (C). Error bars indicate standard errors of the means from a representative triplicate time course assay. *Sa*, *S. aureus*; *Pa*, *P. aeruginosa*.

incubated for 6 total hours. After 6 h, planktonic cells and supernatant were removed and surface-attached biofilms were treated with RNA*later*. Treated samples were scraped from the substrate using a cell scraper and collected. Samples were subsequently pelleted (16,000 × g, 5 min), and bacterial cells were lysed for 30 min at 37°C in 0.25 μg/μl lysostaphin and 2.5 μg/μl lysozyme in Tris-EDTA (TE) buffer. Total RNA was isolated using TRIzol and the Direct-Zol RNA miniprep kit (Zymo Research), followed by Turbo DNA-free DNase (Life Technologies) treatment, per the manufacturer's recommendation.

RNA-seq. One microgram of total RNA was treated for rRNA and tRNA removal before sequencing. Single-read transcriptome sequencing (RNA-seq) was performed on the HiSeq platform at the Helmholtz Centre for Infection Research (Braunschweig, Germany). Raw RNA-seq reads were processed using the CLC Genomics Workbench. Processing was performed using default parameter settings set by the manufacturer. For each sample, sequences were trimmed and mapped to the *P. aeruginosa* UCBPP-PA14 (*P. aeruginosa* PA14) and *S. aureus* 8325 reference genomes using the RNA-seq analysis tool. Mapped sequences were further analyzed using R-project and the Bioconductor package edgeR (55). Read count data sets were filtered to remove low-expression reads prior to RNA expression analysis, with a cutoff of at least 15 counts, to reduce bias from library size differences and to address the fact that there is little power to detect differential expression of very low detection reads. Only a single replicate was performed for each sample; therefore, a conservative cutoff of 10-fold increase or decrease in gene expression was applied to identify genes of interest.

Reverse transcription and qRT-PCR. cDNA was synthesized from total RNA for each coculture or monoculture using the Invitrogen Superscript III first-strand synthesis system according to the manufacturer's protocol. RNAs from plastic-grown coculture biofilm samples were synthesized using random hexamers and ~100 ng total RNA. cDNA for CFBE-grown cocultures was synthesized using gene-specific primers (GSP) (see Table S2 in the supplemental material) in a single reaction to decrease nonspecific detection from host RNA. Briefly, total RNA, 10 mM deoxynucleoside triphosphates (dNTPs), and primers (50 ng/μl random hexamers or 2 μM [each] GSP) were incubated for 5 min at 65°C in a 10-μl total volume. Following this incubation, 10 μl of cDNA synthesis mix was added for a final 20-μl reaction mixture of 1× reaction buffer, 5 mM MgCl₂, 0.01 M dithiothreitol (DTT), 2 U/μl RNaseOUT, and 10 U/μl Superscript III reverse transcriptase. Reaction mixtures were incubated at 25°C for 10 min, 50°C for 50 min, 85°C for 5 min, and 4°C for 5 min. Afterward, 1 μl RNase H was added to each reaction mixture and incubated at 37°C for 20 min. Quantitative real-time PCR (qRT-PCR) was performed with three technical replicates for each sample. *S. aureus*-specific primers were designed for each gene of interest and are specified in Table S2 in the supplemental material. For qRT-PCR, 1 μl of cDNA was diluted in a 10-μl reaction mixture with 0.25 μM forward and reverse primers and 1× iQ Sybr green Supermix (Bio-Rad). The qRT-PCR was as follows: 95°C for 3 min and then 95°C for 30 s, annealing temperature (*T*_{anneal}) for 30 s, and 72°C for 20 s, for 40 cycles. cDNA to each gene of interest was quantified based on cycle threshold (*C*_T) compared to a standard curve of purified *P. aeruginosa* PA14 *rplU* DNA and normalized from sample to sample based on *S. aureus rpoB* quantification (56). *S. aureus gyrB* was used as a second normalization control for initial experiments and showed results consistent with those of *rpoB*; therefore, a single gene, *rpoB*, was used for later assays and is reported here.

Quantification of metabolite accumulation and utilization. Sequential monocultures of *P. aeruginosa* PA14 and *S. aureus* 8325-4 were treated with culture supernatant containing exoproducts and metabolites from previous cultures, as depicted in Fig. 3A. Six-well tissue culture-treated plates were inoculated with 2 ml 0.05-OD₆₀₀ *P. aeruginosa* PA14 or *S. aureus* 8325-4 in MEM + L-Gln + 0.5 g/liter yeast extract. In many samples, lactate, acetate, and ethanol were produced at levels near the limit of detection; therefore, yeast extract (0.5 g/liter) was added to amplify production of these metabolites above background. Cultures were incubated at 37°C and 5% CO₂. Similarly to the plastic-grown coculture assay, supernatant and unattached cells were removed after 1 h and replaced with 2 ml fresh MEM + L-Gln + 0.5 g/liter yeast extract or 1 ml fresh medium plus 1 ml spent culture supernatant from the appropriate 16-h monoculture. A 16-h monoculture of *P. aeruginosa* PA14 was followed by a 16-h monoculture of *S. aureus* 8325-4 and, finally, a 12-h monoculture of *P. aeruginosa* PA14. Culture supernatants were collected at indicated time points throughout the 16-h or 12-h monocultures to examine the kinetics of metabolite accumulation and consumption. Lactate, ethanol, acetate,

formate, and glucose accumulation and/or utilization from culture supernatants was analyzed by high-performance liquid chromatography (HPLC) as previously described (57). Briefly, 400 μ l culture supernatant was filtered using 0.22- μ m nylon centrifugal filters and acidified with 10 μ l of 10% sulfuric acid sample. Samples were analyzed on a Bio-Rad HPX-87H column.

Statistical analysis. Differences in survival, bacterial load, or growth of monoculture versus cocultures of *S. aureus* or *P. aeruginosa* viabilities were compared for \log_{10} normalized values using Student's *t* tests with the Holm-Šidák method for correcting for multiple comparisons or analysis of variance (ANOVA) with Dunnett's or Šidák's multiple-comparison posttest, as specified. Normalized RNA expression, quantified by qRT-PCR, was compared using the Friedman rank test and Dunn's posttest.

RESULTS

***P. aeruginosa* coexists with and then kills *S. aureus* in late-stage coculture on airway cells and plastic.** Clinical culture data and non-culture-based microbial analyses from both pediatric and adult populations show dynamic community structure earlier in life, particularly pertaining to the evolving predominance of *P. aeruginosa* versus *S. aureus* (1, 7, 20, 58). To better understand the nature of these community dynamics and interbacterial interactions, we adapted the previously described *in vitro* airway cell-*P. aeruginosa* coculture assay, using human bronchial epithelial cells (CFBE41o⁻) homozygous for Δ F508 CFTR (referred to as CFBE here) (53), to include a dual-bacterial system (Fig. 1A). As depicted in Fig. 1A, during the *in vitro* CFBE coculture assay, *P. aeruginosa* PA14 and *S. aureus* 8325-4 are coinoculated onto a monolayer of CFBE cells. Unattached bacterial cells are removed after the first hour. A dual-species biofilm is allowed to form for 5 additional hours on the airway epithelium. After the initial 6 h, the medium is again replaced and the established biofilm coculture is incubated for an additional 16 h.

Over the course of 22 h, we observed that the population of *P. aeruginosa* PA14 is not impacted by the presence or absence of *S. aureus* 8325-4, in either the planktonic or the biofilm phase (see Fig. S1A in the supplemental material). In contrast, we observed a dynamic interaction between *P. aeruginosa* PA14 and *S. aureus* 8325-4 in regard to the *S. aureus* 8325-4 population. The kinetics of the CFBE-inoculated coculture assay displayed an extended period of coexistence, followed by late-stage killing of *S. aureus* 8325-4 by *P. aeruginosa* PA14 (Fig. 1B). It is important to note that we interpret the reduced population of *S. aureus* as attributable to the killing of this microbe as we are measuring viable counts of each microbe. A rapid decline in the *S. aureus* 8325-4 population occurs between 10 h and 16 h of cocubation with *P. aeruginosa* PA14. This *S. aureus* 8325-4-killing phenotype by *P. aeruginosa* PA14 occurs on airway cells but does not require the presence of CFBE cells, as a simplified coculture assay performed on plastic displays similar late-stage interaction kinetics (Fig. 1A and C). On both the CFBE and plastic substrates, *S. aureus* 8325-4 killing is observed in both the biofilm and planktonic phases (Fig. 1B and C). We will focus on *S. aureus* biofilm results here, but it should be noted that the reported phenotypes are consistent between the planktonic populations and the biofilm-grown bacteria.

The coculture assay performed on plastic also displays a strikingly enhanced planktonic *S. aureus* 8325-4 population in the presence of *P. aeruginosa* PA14 between 4 h and 6 h (Fig. 1C). The increased planktonic *S. aureus* 8325-4 population may be due, at least in part, to a biofilm dispersion phenotype in the presence of *P. aeruginosa* PA14. This conclusion is indicated by the decreased

biofilm population of *S. aureus* 8325-4 and a concomitant increase in the planktonic *S. aureus* 8325-4 population at 6 h (see Fig. S2 in the supplemental material). Less dramatic biofilm dispersion similarly occurs on CFBE monolayers prior to the second medium change in the airway cell coculture system. This *S. aureus* biofilm dispersion phenomenon was previously reported by Park et al. to be mediated by *P. aeruginosa* proteases (42). While the described *S. aureus* biofilm dispersion phenotype is consistent with the observations in our coculture model on plastic, other mechanisms leading to an increased planktonic population are possible. Additionally, similarly to the CFBE coculture model, when bacteria are cocubated on plastic, the *P. aeruginosa* PA14 population is not differentially impacted by the presence or absence of *S. aureus* 8325-4 (see Fig. S1B in the supplemental material).

Killing of *S. aureus* by *P. aeruginosa* is not specific to the *S. aureus* 8325-4-*P. aeruginosa* PA14 strain combination. There is, however, variation in the degree or kinetics of *S. aureus* killing by *P. aeruginosa* strains (see Fig. S3A in the supplemental material). Two other tested laboratory strains of *P. aeruginosa* (PAO1 and FRD1) killed *S. aureus* 8325-4, but less dramatically than *P. aeruginosa* PA14. *S. aureus* 8325-4 killing by seven clinical isolates of *P. aeruginosa* ranged from no killing (no difference in viable cell count from *S. aureus* 8325-4 monoculture) to killing that resulted in *S. aureus* 8325-4 viable cell counts below the limit of detection in our coculture assay ($<2 \log_{10}$ [CFU/well]). Notably, *P. aeruginosa* strains with lower biofilm populations at the end of the assay, likely due to decreased initial attachment and/or reduced growth/survival despite similar starting inocula, were unable to kill *S. aureus* 8325-4 (SMC1585, SMC5450, and SMC5451 [see Fig. S3B in the supplemental material]), indicating a dose-dependent relationship between *P. aeruginosa* population and *S. aureus* killing. However, end-of-assay *P. aeruginosa* population was not the only factor contributing to this variation, as demonstrated in the coculture with SMC1587, which developed a *P. aeruginosa* biofilm population comparable to that of *P. aeruginosa* PA14 at the end of assay but showed no killing of *S. aureus* 8325-4 (see Fig. S3 in the supplemental material). Importantly, both mucoid and nonmucoid strains of *P. aeruginosa* can reduce *S. aureus* 8325-4 populations (see Fig. S3A in the supplemental material). This finding was confirmed through equivalent *S. aureus* 8325-4 killing by the parent strain FRD1 (mucoid) and its transposon mutant derivative FRD1 *algT::Tn501*, which is nonmucoid due to the mutation in the alginate biosynthetic genes.

Furthermore, killing of *S. aureus* by *P. aeruginosa* PA14 in our coculture model is not specific to *S. aureus* 8325-4. All *S. aureus* laboratory strains tested (8325-4, Newman, MW2, and USA300) and clinical isolates of *S. aureus* (including both methicillin-sensitive and methicillin-resistant strains) were killed by *P. aeruginosa* PA14 (see Fig. S4 in the supplemental material). Susceptibility to killing did vary, but all strains experienced at least a 20-fold decrease in population when in coculture with *P. aeruginosa* PA14 compared to monoculture.

Taken together, our data show that multiple strains of *P. aeruginosa*, including mucoid and nonmucoid strains, are capable of killing a variety of *S. aureus* laboratory and clinical isolates, either in a biofilm or planktonically, indicating a general mechanism of interaction between these organisms. Furthermore, while the kinetics of the interaction shows some variation, the killing of *S. aureus* by *P. aeruginosa* does not require the presence of CFBE

cells. We took advantage of the latter conclusion in many of the subsequent experiments presented below.

Coculture with *P. aeruginosa* upregulates *S. aureus* fermentation pathway gene expression. To better understand the molecular nature of *P. aeruginosa*-*S. aureus* interactions, an unbiased approach to examine changes in gene expression was employed. RNA-seq analysis was performed on single samples of 6-h biofilms from the plastic coculture assay, as follows: (i) *P. aeruginosa* PA14 monoculture, (ii) *S. aureus* 8325-4 monoculture, and (iii) *P. aeruginosa* PA14 plus *S. aureus* 8325-4 coculture. *P. aeruginosa* PA14 and *S. aureus* 8325-4 gene read assignments for monocultures and cocultures are provided in Tables S3 and S4 in the supplemental material, respectively. We utilized the RNA-seq results as a hypothesis-generating approach; therefore, a conservative cutoff of 10-fold change or greater was used to identify differentially expressed genes of potential interest for further investigation.

P. aeruginosa PA14 gene expression was minimally impacted by the presence of *S. aureus* 8325-4 compared to growth of *P. aeruginosa* PA14 alone (see Fig. S5 in the supplemental material). Only one gene, PA_10470, displayed a greater-than-10-fold change in coculture compared to monoculture. PA_10470, which encodes a major facilitator superfamily (MFS) transporter, was downregulated 15-fold in the presence of *S. aureus* 8325-4.

In contrast, coculture of *P. aeruginosa* PA14 with *S. aureus* 8325-4 greatly impacted *S. aureus* 8325-4 gene expression compared to an *S. aureus* monoculture (Fig. 2A). A summary of genes with >10-fold change in expression (increased or decreased) in the presence of *P. aeruginosa* PA14 compared to *S. aureus* 8325-4 alone is provided in Table S5 in the supplemental material. The four most differentially upregulated *S. aureus* 8325-4 genes in coculture with *P. aeruginosa* PA14 compared to *S. aureus* 8325-4 monoculture were pyruvate formate-lyase 1-activating enzyme (*pflA*, SAOUHSC_00188), formate acetyltransferase (*pflB*, SAOUHSC_00187), L-lactate dehydrogenase (*ldh*, SAOUHSC_00206), and alcohol dehydrogenase (*adh*, SAOUHSC_00113), all of which are associated with fermentation pathways in *S. aureus* (Fig. 2A).

Upregulation of *S. aureus* 8325-4 fermentation pathway gene expression in coculture with *P. aeruginosa* PA14 on plastic was confirmed by qRT-PCR. Representative genes for formate (*pflB*, SAOUHSC_00187), lactate (*ldh*, SAOUHSC_00206), and ethanol (*adh*, SAOUHSC_00113) fermentation pathways were selected based on the RNA-seq results and quantified relative to the expression of a reference gene, *rpoB* (SAOUHSC_00524). *S. aureus* expression of *pflB* and *ldh* was upregulated >1,000-fold, and *adh* was upregulated >200-fold in coculture with *P. aeruginosa* PA14 compared to *S. aureus* 8325-4 monoculture in biofilm samples (Fig. 2B).

***S. aureus* shifts from aerobic respiration to lactic acid fermentation in the presence of *P. aeruginosa* secreted products.** The RNA-seq and qRT-PCR analyses strongly indicated that *S. aureus* upregulates fermentation pathways in the presence of *P. aeruginosa*. However, expression data alone do not definitively confirm a metabolic transition away from aerobic respiration. HPLC was performed to quantify the accumulation of metabolic products in culture supernatants and, thus, identify changes in *S. aureus* 8325-4 metabolism. Due to the complexity of the coculture system, simply analyzing monoculture versus coculture supernatants does not distinguish production and/or consumption of metabolic products by the two separate species. Therefore, a series

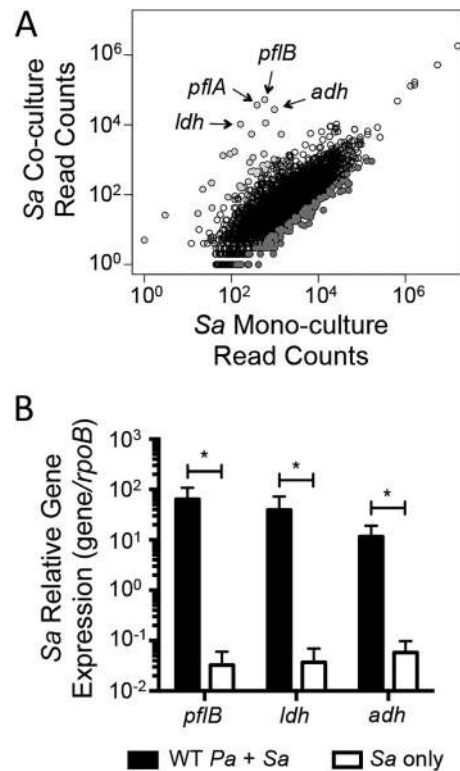


FIG 2 *S. aureus* fermentation pathway gene expression is upregulated in the presence of *P. aeruginosa*. *S. aureus* 8325-4 gene expression was compared in coculture with *P. aeruginosa* PA14 versus *S. aureus* monoculture. Total RNA was isolated from 6-h biofilm fractions on plastic. (A) Filtered RNA-seq reads corresponding to all expressed genes by *S. aureus* in coculture and monoculture. Highly differentially expressed genes in coculture with *P. aeruginosa* compared to *S. aureus* monoculture are indicated (light gray, >10-fold-increased expression in coculture; dark gray, >10-fold-decreased expression in coculture). Key fermentation pathway genes are indicated. (B) Relative expression of marker genes from formate (*pflB*, SAOUHSC_00187), lactate (*ldh*, SAOUHSC_00206), and ethanol (*adh*, SAOUHSC_00113) fermentation pathways. *S. aureus* gene expression in coculture with *P. aeruginosa* is compared with that in *S. aureus* monoculture. Statistical significance was determined for 9 replicate samples using multiple Student *t* tests and the Holm-Sidak method for correcting for multiple comparisons (*, $P < 0.05$). *Sa*, *S. aureus*; *Pa*, *P. aeruginosa*.

of monoculture assays were performed using culture supernatants of *P. aeruginosa* PA14 or *S. aureus* 8325-4 in the mixing experiments outlined in Fig. 3A. By separating the two organisms and sequentially analyzing culture supernatants, we were able to identify production, accumulation, and consumption of metabolites by the individual bacterial species.

When *S. aureus* 8325-4 was grown as a monoculture without the addition of *P. aeruginosa* PA14 supernatant over 16 h, *S. aureus* 8325-4 produced over 6 mM acetate, indicative of aerobic respiration (59, 60) (Fig. 3B). However, *S. aureus* 8325-4 treated with *P. aeruginosa* PA14 culture supernatants showed greatly reduced production of acetate, accumulating to only 1.5 mM rather than 6 mM over 16 h. Instead, *S. aureus* 8325-4 cultures treated with *P. aeruginosa* PA14 supernatants accumulated lactate in a linear fashion over the 16-h culture (to about 7 mM), whereas *S. aureus* 8325-4 cultures not treated with *P. aeruginosa* PA14 supernatants accumulated less than 1 mM lactate over 16 h (Fig. 3C). Accumulation of lactate in the *S. aureus* 8325-4 culture treated with *P.*

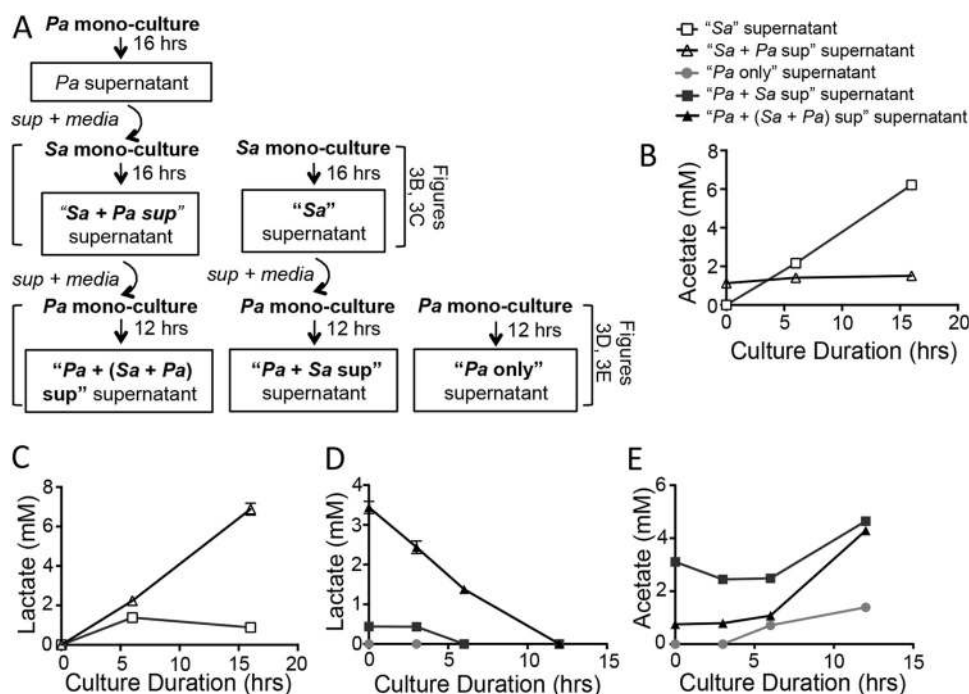


FIG 3 *P. aeruginosa* exoproducts induce lactate production by *S. aureus*, which is subsequently consumed by *P. aeruginosa*. (A) Schematic of the sequential *P. aeruginosa* PA14 and *S. aureus* 8325-4 monoculture and spent supernatant treatment protocol used to analyze metabolite accumulation and utilization. Metabolites were quantified by HPLC from clarified supernatants collected throughout the assay at the indicated time points (B to E). The key above panel B applies to the graphs in panels B to E. (B and C) Accumulation of acetate (B) or lactate (C) by *S. aureus* monoculture in the presence (white triangles) or absence (white squares) of *P. aeruginosa* culture supernatants. (D and E) Accumulation/utilization of lactate (D) or acetate (E) by *P. aeruginosa* monocultures in the absence of *S. aureus* culture supernatants (circles), the presence of *S. aureus* supernatants when *S. aureus* was grown in fresh MEM (black squares), or the presence of *S. aureus* supernatants when *S. aureus* was grown in MEM plus spent *P. aeruginosa* supernatants (black triangles). Sa, *S. aureus*; Pa, *P. aeruginosa*.

aeruginosa PA14 supernatants demonstrates a metabolic transition to fermentation in the presence of *P. aeruginosa* PA14 exoproducts.

Surprisingly, although formate and alcohol fermentation gene expression pathways were highly upregulated, formate and ethanol did not accumulate in *S. aureus* 8325-4 cultures treated with *P. aeruginosa* PA14 supernatants. After 16 h, formate and ethanol concentrations were detected at 0.5 mM and 0.6 mM, respectively (near the limit of detection for our instrument). These results are consistent with those seen in the *S. aureus* Col *hemB* mutant, previously reported by Kohler et al. (61).

In a coculture system, metabolites produced by one organism can serve as signals or nutrients for the other organism. Therefore, we were interested in whether *P. aeruginosa* utilizes the fermentation products secreted by *S. aureus*. When *P. aeruginosa* PA14 was grown in medium containing the supernatants from *S. aureus* 8325-4 cocultured with *P. aeruginosa* supernatants, the *S. aureus*-produced lactate was consumed by *P. aeruginosa* to below detectable levels (Fig. 3D). In fact, *P. aeruginosa* PA14 consumed the *S. aureus* 8325-4-produced lactate preferentially over the glucose in the medium (see Fig. S6 in the supplemental material), which was consistent with previous reports (62). *P. aeruginosa* can also consume acetate (63); however, it was not the preferred carbon source under the culture conditions used here; rather, additional acetate accumulated over time (Fig. 3E). The additional accumulation of acetate by *P. aeruginosa* monocultures was unexpected, given the lack of pyruvate in MEM. However, as shown previously, *P. aeruginosa* can produce pyruvic acid as an organic acid by-product

when grown in glucose-based minimal medium, in the absence of pyruvate (64). Perhaps, this glucose-derived pyruvate serves as the substrate for acetate production by *P. aeruginosa*.

Together, these results suggest interesting metabolic dynamics wherein as a monoculture, *S. aureus* primarily respire aerobically and produces acetate; however, in the presence of *P. aeruginosa* exoproducts, *S. aureus* is shifted to a fermentative metabolism and primarily produces lactate, which *P. aeruginosa* then preferentially consumes.

HQNO and *P. aeruginosa* siderophores are required for *S. aureus* killing by *P. aeruginosa*. While RNA expression and HPLC analyses yielded great insight into the *S. aureus* response to the presence of *P. aeruginosa* under the conditions of our coculture model, it did not inform the mechanism(s) by which *P. aeruginosa* causes altered viability of *S. aureus*. To investigate the mechanism behind *P. aeruginosa*-mediated killing of *S. aureus* in this coculture assay, a targeted candidate gene approach was used. Previously, *P. aeruginosa*-produced HQNO was reported to inhibit normal growth of *S. aureus* (44, 48, 51). Additionally, coculture of *P. aeruginosa* with *S. aureus* shifts the gene expression of *P. aeruginosa* toward that of a less iron-starved profile in a rat peritoneal coculture model (43). Therefore, we targeted the *Pseudomonas* quinolone signal (PQS) (Fig. 4A) pathway and *Pseudomonas* iron acquisition via siderophore production in our initial experiments.

P. aeruginosa PA14 strains with deletions in PQS pathway genes and/or genes required for siderophore biosynthesis were used in the airway epithelial cell and biofilm coculture assay. Sim-

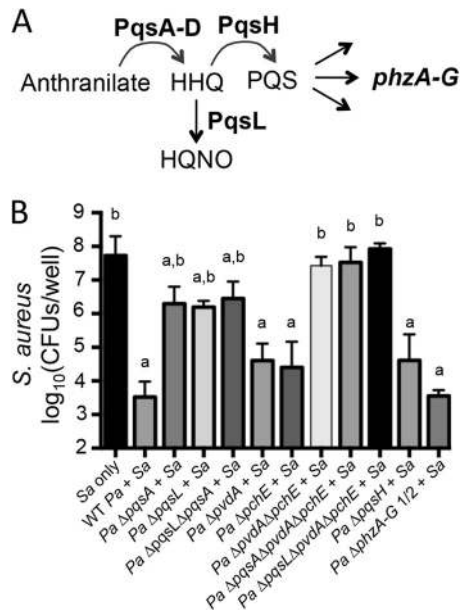


FIG 4 HQNO and siderophores produced by *P. aeruginosa* are required for late-stage killing of *S. aureus* *in vitro*. (A) Depiction of the PQS pathway in *P. aeruginosa* focusing on the genes/gene products analyzed in the coculture assays. (B) Coculture assays on a monolayer of epithelial cells were performed with *S. aureus* 8325-4 and *P. aeruginosa* PA14 WT or specified deletion mutant. *S. aureus* biofilm viability was measured at 16 h. Averages and errors (standard errors of the means) from three separate experiments, each run in triplicate, are shown. Differential survival of *S. aureus* in each coculture was compared to *S. aureus* monoculture using ANOVA and Dunnett's multiple-comparison posttest (a, $P < 0.05$). Differential survival of *S. aureus* monoculture or *S. aureus* in coculture with a *P. aeruginosa* mutant is compared to *S. aureus* coculture with WT *P. aeruginosa* also using ANOVA and Dunnett's multiple-comparison posttest (b, $P < 0.05$). Sa, *S. aureus*; Pa, *P. aeruginosa*.

ilarly to wild-type (WT) *P. aeruginosa* PA14, survival and growth of mutant strains of *P. aeruginosa* PA14 with the targeted deletions were not altered by the presence of *S. aureus* 8325-4 (see Fig. S7 in the supplemental material). In contrast, *S. aureus* 8325-4 survival was differentially impacted by coculture with various mutant strains of *P. aeruginosa* PA14 (Fig. 4B). As seen previously, after 16 h in the CFBE coculture biofilm, WT *P. aeruginosa* PA14 kills *S. aureus* 8325-4, resulting in about a 10,000-fold reduction in *S. aureus* 8325-4 CFU/well compared to *S. aureus* 8325-4 monoculture (Fig. 4B). Coculture with single mutants incapable of synthesizing pyoverdine ($\Delta pvdA$) or pyochelin ($\Delta pchE$), the two major siderophores produced by *P. aeruginosa* PA14, trends toward decreased killing of *S. aureus* 8325-4 compared to coculture with WT *P. aeruginosa* PA14. However, coculture with a mutant *P. aeruginosa* strain that lacks synthesis of both siderophores, the $\Delta pvdA \Delta pchE$ strain, markedly reduced *P. aeruginosa*-mediated killing of *S. aureus* 8325-4, allowing survival and growth of *S. aureus* to levels similar to those when this organism was grown in a monoculture. Interestingly, in a plastic-grown coculture biofilm, both siderophores are required for efficient *P. aeruginosa*-mediated killing of *S. aureus* 8325-4, as coculture with either single mutant, the $\Delta pvdA$ or $\Delta pchE$ strain, or the double mutant, the $\Delta pvdA \Delta pchE$ strain, results in greatly reduced killing of *S. aureus* compared to WT *P. aeruginosa* PA14 coculture (see Fig. S8 in the supplemental material). Thus, to kill *S. aureus* 8325-4 most efficiently in both the CFBE- and plastic-grown coculture models, *P.*

aeruginosa PA14 requires both major siderophores, pyoverdine and pyochelin.

Coculture on CFBE monolayers with the *P. aeruginosa* PA14 $\Delta pqsA$ mutant, which reduces all downstream products of the PQS biosynthetic pathway, similarly results in reduced *S. aureus*-killing (Fig. 4). Consistent with other previously reported coculture models of *P. aeruginosa* and *S. aureus* (51), HQNO plays an important role in *S. aureus* killing in our CFBE-coculture model; a *P. aeruginosa* PA14 $\Delta pqsL$ mutant, which does not produce HQNO (Fig. 4A), does not efficiently kill *S. aureus* 8325-4 (Fig. 4B). While *S. aureus* killing is significantly reduced in coculture with *P. aeruginosa* PA14 $\Delta pqsA$ or $\Delta pqsL$ mutants compared to WT *P. aeruginosa* PA14, *S. aureus* 8325-4 survival is not recovered to monoculture levels. Further, coculture with *P. aeruginosa* PA14 $\Delta pqsA \Delta pqsL$ does not further reduce killing compared to either the *P. aeruginosa* PA14 $\Delta pqsA$ or the *P. aeruginosa* PA14 $\Delta pqsL$ single mutant. Similarly to the pattern observed in coculture with the siderophore mutants, the requirements for *P. aeruginosa*-mediated killing of *S. aureus* on plastic are more stringent than in the CFBE coculture model; that is, coculture with *P. aeruginosa* PA14 $\Delta pqsA$, $\Delta pqsL$, or $\Delta pqsA \Delta pqsL$ mutants supports survival of *S. aureus* 8325-4, comparable to that of *S. aureus* 8325-4 monoculture (see Fig. S8 in the supplemental material). Interestingly, PQS plays a variable role in *S. aureus* 8325-4 killing in these coculture models. The *P. aeruginosa* PA14 $\Delta pqsH$ mutant, which does not produce PQS but instead accumulates its precursor 2-heptyl-4-quinolone (HHQ), trends toward a reduced ability to kill *S. aureus* 8325-4 in the CFBE coculture model, indicating a possible moderate impact of PQS or downstream signals in *S. aureus* killing (Fig. 4B). However, in our plastic-grown coculture model *P. aeruginosa* PA14 $\Delta pqsH$ trends toward more effective killing of *S. aureus* 8325-4 than that by WT *P. aeruginosa* PA14 (see Fig. S8 in the supplemental material). Together, our data are consistent with the model that it is the accumulation of HQNO that is primarily responsible for the role of the PQS pathway in *P. aeruginosa*-mediated killing of *S. aureus*.

Some redox-active phenazines produced by *P. aeruginosa*, like pyocyanin, inhibit respiration and have been reported to be anti-staphylococcal (47, 65). In both our CFBE- and plastic-grown coculture models, *P. aeruginosa* PA14 $\Delta phzA-G1/2$, a mutant lacking both phenazine biosynthetic clusters and thus unable to produce phenazines, including pyocyanin, still killed *S. aureus* 8325-4 to levels similar to that observed for the WT *P. aeruginosa* PA14 (Fig. 4B; see also Fig. S8 in the supplemental material). This result further indicates that HQNO is the primary effector in the PQS pathway required for *S. aureus* 8325-4-killing by *P. aeruginosa* PA14 in this coculture model. Finally, consistent with the requirement for both siderophores and HQNO for *P. aeruginosa*-mediated killing of *S. aureus*, a triple mutant of *P. aeruginosa* PA14, the $\Delta pqsA \Delta pvdA \Delta pchE$ or $\Delta pqsL \Delta pvdA \Delta pchE$ strain, results in comparable survival of *S. aureus* 8325-4 in coculture and monoculture on CFBE monolayers and plastic (Fig. 4; see also Fig. S8 in the supplemental material).

The concentration of iron in the MEM (0.055 μM) is similar to that detected in bronchoalveolar lavage fluid from healthy patients (<0.1 μM) (66). Patients with CF have increased iron concentrations in the lower respiratory tract. The concentration of lower airway total iron varies between patients but has been detected around 8 μM on average (67). Although total iron can be easily measured, the concentration of iron readily available for

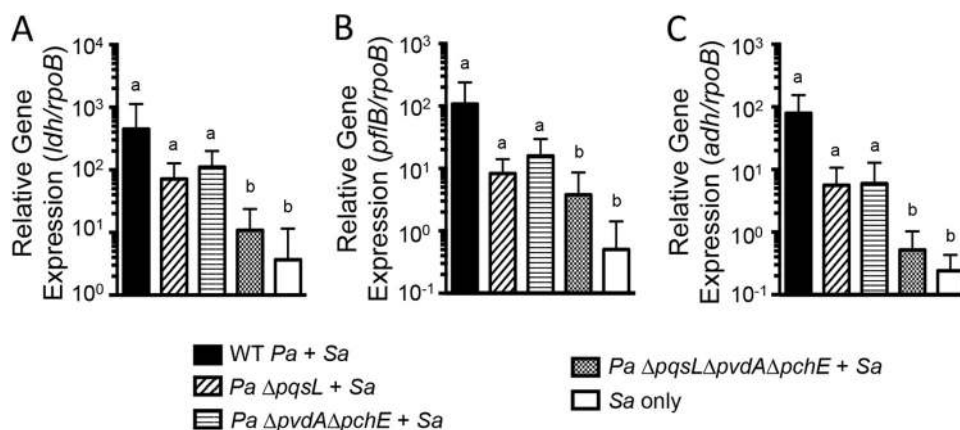


FIG 5 *P. aeruginosa*-produced HQNO and siderophores are required to induce *S. aureus* fermentation gene expression. *S. aureus* 8325-4 biofilms were grown on CFBE monolayers. After 6 h, expression of marker genes from lactate (*ldh*) (A), formate (*pflB*) (B), and ethanol (*adh*) (C) fermentation pathways in *S. aureus* was measured using qRT-PCR and was normalized to the expression of an *S. aureus* control gene (*rpoB*). Relative fermentation gene expression in *S. aureus* was compared between *S. aureus* monoculture and coculture with WT or specified deletion mutants of *P. aeruginosa* PA14. Gene expression levels from 7 replicate experiments were compared using the Friedman rank test and Dunn's multiple-comparison posttest. Differential *S. aureus* gene expression when in coculture with *P. aeruginosa* was compared to that in *S. aureus* monoculture (a, $P < 0.05$). Differential *S. aureus* gene expression from *S. aureus* monoculture or *S. aureus* in coculture with a *P. aeruginosa* mutant was compared to that in *S. aureus* coculture with WT *P. aeruginosa* (b, $P < 0.05$). Error bars represent standard errors of the means. *Sa*, *S. aureus*; *Pa*, *P. aeruginosa*.

microbial use is less clear. Under coculture conditions supplemented with 8 μM ferric chloride (FeCl_3), WT *P. aeruginosa* PA14 killed *S. aureus* 8325-4 more efficiently than in low-iron medium (see Fig. S9 in the supplemental material). Additionally, when supplemented with iron, *P. aeruginosa* PA14 $\Delta pvdA$ or $\Delta pchE$ single mutant strains did not experience a defect in *S. aureus* 8325-4 killing. *P. aeruginosa* PA14 did still require siderophores and HQNO for efficient killing of *S. aureus* 8325-4 under 8 μM iron conditions; however, loss of these components (as displayed through coculture with the $\Delta pvdA \Delta pchE$, $\Delta pqsL$, or $\Delta pqsL \Delta pvdA \Delta pchE$ mutant of *P. aeruginosa* PA14) did not yield restoration of growth/survival of *S. aureus* 8325-4 to levels equivalent to those of monoculture. In the presence of additional iron, *P. aeruginosa* siderophores, in particular, played a reduced role in *P. aeruginosa*-mediated *S. aureus* killing compared to that under low-iron (un-supplemented) conditions. Further, the lack of complete recovery of *S. aureus* growth/survival in coculture with *P. aeruginosa* PA14 $\Delta pqsL \Delta pvdA \Delta pchE$ in 8 μM iron medium indicates a role for additional *Pseudomonas aeruginosa* factors or nutrient competition in mediating *P. aeruginosa*-*S. aureus* interbacterial interactions. Taken together, the production of HQNO and both siderophores is required for efficient killing of *S. aureus* by *P. aeruginosa*.

HQNO and *P. aeruginosa* siderophores induce mixed acid fermentation gene expression in *S. aureus*. Based on the RNA expression results, HPLC metabolite quantification, and mutant *P. aeruginosa* coculture analyses, we hypothesized that molecules produced by *P. aeruginosa* lead to induction of fermentation pathway expression in *S. aureus*. To test this hypothesis, RNA expression of fermentation pathways was compared by qRT-PCR in *S. aureus* 8325-4 monoculture with that in *S. aureus* 8325-4-*P. aeruginosa* PA14 coculture biofilms on CFBE monolayers using key mutants identified from the late-stage killing phenotype (Fig. 5). Markers of gene expression of the three main fermentation pathways are the same as with those investigated in the plastic-grown coculture biofilms in Fig. 2: lactate fermentation (*ldh*, SAOUHSC_00206), formate fermentation (*pflB*, SAOUHSC_

00187), and alcohol fermentation (*adh*, SAOUHSC_00113). Similarly to plastic-grown cocultures, *S. aureus* 8325-4 *ldh* expression was strongly induced when in coculture with WT *P. aeruginosa* PA14 on CFBE cells with >120-fold-increased expression compared to *S. aureus* 8325-4 monoculture (Fig. 5A). Interestingly, coculture with either the *P. aeruginosa* PA14 $\Delta pqsL$ strain, which does not produce HQNO, or the *P. aeruginosa* PA14 $\Delta pvdA \Delta pchE$ double mutant, lacking two major siderophores, resulted in a trend toward decreased induction of *ldh*, compared to the coculture with WT *P. aeruginosa* PA14 (about 20-fold- and 30-fold-increased expression, respectively, compared to *S. aureus* 8325-4 monoculture). Coculture with the triple mutant *P. aeruginosa* PA14 $\Delta pqsL \Delta pvdA \Delta pchE$, lacking HQNO and siderophore production, greatly decreased the induction of *S. aureus* 8325-4 *ldh* expression in coculture to a nonsignificant increase of about 3-fold compared to *S. aureus* 8325-4 monoculture. Thus, both HQNO and *P. aeruginosa* siderophores appear to contribute to the induction of the lactate fermentation pathway expression when *S. aureus* 8325-4 is in coculture with *P. aeruginosa* PA14 and do so in an additive manner. Similarly, induction of *pflB* and *adh* in *S. aureus* 8325-4 was dependent on the production of both HQNO and *P. aeruginosa* siderophores (Fig. 5B and C). This additive impact of *P. aeruginosa*-produced HQNO and siderophores on *S. aureus* fermentation gene induction in CFBE-grown coculture biofilms was also consistent in a second tested strain, *S. aureus* USA300 (see Fig. S10A to C in the supplemental material). Finally, similar to what was observed in CFBE-grown coculture biofilms, an additive role of HQNO and *P. aeruginosa* siderophores in the induction of *S. aureus* 8325-4 *ldh*, *pflB*, and *adh* expression was detected in coculture biofilms grown on plastic (see Fig. S10D to F in the supplemental material).

While HQNO and *P. aeruginosa* siderophores significantly contribute to the induction of fermentation gene expression, over replicate experiments, coculture of *S. aureus* 8325-4 with *P. aeruginosa* PA14 $\Delta pqsL \Delta pvdA \Delta pchE$ displayed a nonsignificant trend toward elevated expression of *ldh*, *pflB*, and *adh* compared

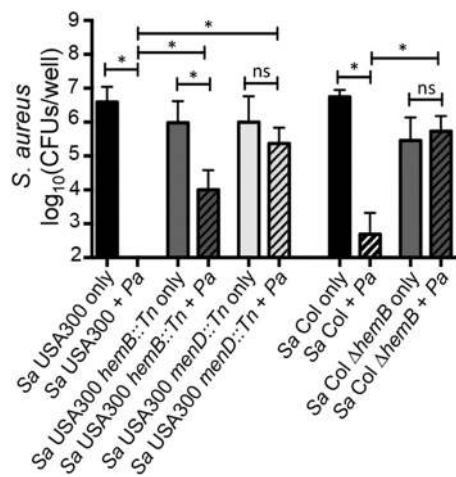


FIG 6 *S. aureus* SCV strains have increased resistance to killing by *P. aeruginosa*. Plastic-grown biofilm coculture assays were performed with *P. aeruginosa* PA14 and *S. aureus* USA300 and Col parent strains or genetic mutants with stable SCV phenotypes. *S. aureus* viability was measured at 16 h. Differential survival rates of *S. aureus* SCV strains were compared in coculture with *P. aeruginosa* versus monoculture and compared to parent *S. aureus* strain coculture viability using ANOVA and the Sidák multiple-comparison test for six replicate samples (*, $P < 0.05$; ns, not significant). Error bars represent standard errors of the means. Sa, *S. aureus*; Pa, *P. aeruginosa*.

to *S. aureus* 8325-4 monoculture. Thus, it is likely that other factors associated with coculture of *S. aureus* and *P. aeruginosa* contribute to *S. aureus* 8325-4 fermentation pathway induction. Namely, competition for oxygen likely makes the coculture environment less oxic as indicated by a >10-fold reduction in catalase expression by *S. aureus* 8325-4 when in coculture with *P. aeruginosa* PA14 and a >10-fold increase in expression of *nrdG*, a class III ribonucleotide reductase upregulated under anaerobic conditions (68) (see Table S5 in the supplemental material). Overall, *P. aeruginosa* production of HQNO and siderophores results in induction of lactate, formate, and alcohol fermentation pathway expression in *S. aureus*.

***S. aureus* small-colony variants are partially protected from *P. aeruginosa* killing.** Respiratory inhibitors like HQNO and pyocyanin were previously reported to select for *S. aureus* SCVs (50, 51). SCVs characteristically have a deficient electron transport chain and provide a survival mechanism that may help *S. aureus* persist in the presence of *P. aeruginosa* (50). We introduced *S. aureus* SCV strains with stable mutations in the electron transport chain into the coculture assay mixture on plastic to assess SCV susceptibility to *P. aeruginosa*-mediated killing of *S. aureus* in our biofilm model. Survival rates of *S. aureus* USA300 *hemB* and *menD* transposon mutants and an *S. aureus* Col *hemB* mutant, which generate SCVs, were compared to those of their respective WT parent strains in the presence of *P. aeruginosa* PA14 or in monoculture (Fig. 6). *S. aureus* USA300 *hemB*::Tn and *menD*::Tn mutants survived significantly better than WT *S. aureus* USA300 in the presence of *P. aeruginosa* PA14. However, protection from killing was partial as survival in coculture was 10- to 150-fold reduced compared to *S. aureus* monoculture. Interestingly, the *S. aureus* Col *hemB* mutant was completely protected from killing by *P. aeruginosa* PA14. Thus, growth as an SCV appears to provide a mechanism for *S. aureus* persistence in coculture; however, there

is heterogeneity in the degree of protection from *P. aeruginosa* PA14 between *S. aureus* strains.

DISCUSSION

It is well known in reports of the respiratory microbiota of patients with CF that *Pseudomonas* is a low-abundance, sometimes transient, organism during childhood. In pediatric patients, *S. aureus* is the primary pathogen, and it is not until late adolescence and adulthood that *P. aeruginosa* becomes a prevalent pathogen (20). Two main interaction phenotypes seen in these CF patients, the period of coexistence early in life and later-stage displacement of *Staphylococcus* by *Pseudomonas*, appear to be broadly modeled in our simplified, *in vitro* CFBE- and plastic-grown cocultures. While these *in vitro* coculture models clearly have limitations, they can be easily modified to address important questions pertaining to CF microbial interactions. Here, we showed the ability to study differential interactions as they occur over time in a single assay, including host cell colonization, biofilm formation, biofilm dispersion, and planktonic interactions. While not addressed here, we have also employed this coculture model to investigate the impacts of antibiotics and nutrient availability on interbacterial interactions, as well as the differential impact of multiple versus single bacterial species on host epithelial cell cytokine response (L. M. Filkins and G. A. O'Toole, unpublished data). Further, this simple model sets the foundation for building more complex *in vitro* polymicrobial communities with multiple bacterial species and additional host factors.

Using our CFBE-grown and plastic-grown coculture systems, we were able to further elucidate the multifactorial mechanism of *S. aureus* killing by *P. aeruginosa*, which we propose by the model presented in Fig. 7. We propose that *P. aeruginosa* secretes exoproducts, including siderophores and HQNO, and competes for oxygen, all of which negatively impact *S. aureus* growth and survival. Specifically, production of these exoproducts and oxygen utilization by *P. aeruginosa* inhibit the electron transport chain of *S. aureus* by sequestering iron that is a necessary heme cofactor of cytochromes, by inhibiting the oxidation of cytochrome *b* and reduction of cytochrome *aa₃* (65, 69), and by competing for the terminal electron acceptor. *S. aureus* responds to inhibition of the electron transport chain by increasing gene expression of fermentation pathways (formate, lactate, and ethanol). As a result, *S. aureus* metabolism is shifted away from acetate production and toward lactate fermentation. *P. aeruginosa* can preferentially consume *S. aureus*-produced lactate. We propose that over time the combination of a poisoned electron transport chain, and thus markedly slower growth (70, 71), competition for micronutrients, and additional antimicrobial products produced by *P. aeruginosa* leads to *S. aureus* cell death. As previously reported, *P. aeruginosa*-induced *S. aureus* cell lysis causes the release of iron, which can be further utilized by *P. aeruginosa* (43) and thus provide an additional competitive advantage to *P. aeruginosa* over *S. aureus*. The accompanying paper by Nguyen et al. (72) further explores the role of iron in the transition from *S. aureus* to *P. aeruginosa* predominance.

The RNA expression analyses performed here strongly indicate a shift toward fermentative metabolism for *S. aureus* in the presence of *P. aeruginosa*. This observation was further confirmed by the accumulation of lactate and decrease in acetate accumulation in *S. aureus* culture supernatants in the presence of *P. aeruginosa* exoproducts. Although *S. aureus* was previously demonstrated to

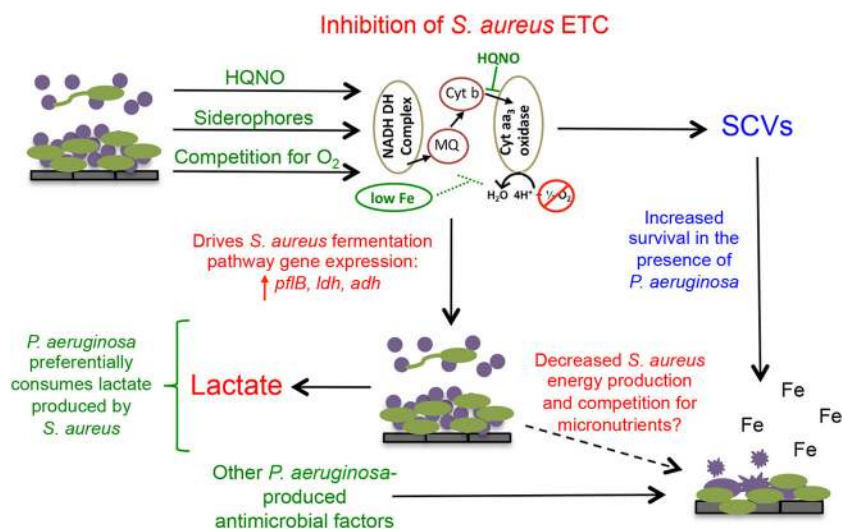


FIG 7 *P. aeruginosa*-*S. aureus* interaction model. *P. aeruginosa* produces HQNO and siderophores and competes for oxygen which directly or indirectly inhibits the electron transport chain (ETC) of *S. aureus*. Inhibition of the electron transport chain induces fermentation gene expression in *S. aureus* and increases production of lactate. *S. aureus*-produced lactate is preferentially consumed by *P. aeruginosa* over medium-provided glucose. We propose that, over time, inhibition of the electron transport chain leads to decreased energy production in *S. aureus*, which in combination with competition for micronutrients and additional antimicrobial factors produced by *P. aeruginosa* leads to *S. aureus* killing. *S. aureus* lysis further benefits *P. aeruginosa* by providing an additional iron source (43). Selection of *S. aureus* SCVs in the presence of *P. aeruginosa* allows a subset of *S. aureus* cells to persist in the hostile coculture environment. NADH DH, NADH dehydrogenase; MQ, menaquinone; Cyt aa₃, cytochrome aa₃; Cyt b, cytochrome b.

also produce ethanol and formate under anaerobic conditions (73), these additional metabolites were produced only to levels near the limit of detection under the culture conditions here, despite strong upregulation of genes from their respective fermentation pathway genes (*adh* and *pflB*). The gene expression and metabolite profile that we observed here when *S. aureus* was grown with *P. aeruginosa* are similar to those of an *S. aureus* Col *hemB* mutant, previously reported by Kohler et al. (61). In the Col *hemB* mutant, lactate was the primary fermentation metabolite detected, while no detectable formate accumulated and very low levels of ethanol were produced. Kohler et al. further determined that, although formate pyruvate lyase is expressed and translated at very high levels, the protein is inactive in the *hemB* mutant, explaining the lack of formate production. In our *in vitro* *S. aureus*-*P. aeruginosa* coculture model and sequential supernatant-treated monocultures, *S. aureus* metabolism mimics that seen in a stable, genetic mutant of the electron transport chain.

Although *P. aeruginosa* displaces *S. aureus* as the predominant organism and pathogen during adulthood, *S. aureus* remains a minor community member in some patients. Additionally, the prevalence of *S. aureus* SCVs correlates with worse disease state and increases with age (21, 74). The *in vitro* coculture interactions reported previously (50) and described here indicate that mutations in the electron transport chain (characteristic of *S. aureus* SCVs) partially protect *S. aureus* from *P. aeruginosa* killing (Fig. 7). Similarly, *S. aureus* SCVs may be selected for in respiratory communities where *P. aeruginosa* is abundant, contributing to their persistence in a hostile environment.

The precise mechanism of *S. aureus* cell death remains an open question. Based on the work here, it is evident that *S. aureus* cell death in this coculture model involves a shift to fermentation; however, *S. aureus* is equipped to grow under anaerobic conditions; thus, it is unlikely that the shift to lactate metabolism is the direct cause of death. Further, while *P. aeruginosa* exoproducts

were sufficient to induce the shift in *S. aureus* metabolism, treating *S. aureus* monocultures with *P. aeruginosa* culture supernatants did not efficiently kill *S. aureus* and *S. aureus* actively metabolizes in the presence of *P. aeruginosa* supernatants (Fig. 3B and C). One possible indication from the requirement of viable *P. aeruginosa* for efficient killing of *S. aureus* is the role of oxygen consumption and competition in *S. aureus* killing. Some aerotolerant anaerobes can grow in oxic environments if cocultured with rapidly respiring aerobic organisms (75), such as *P. aeruginosa*. It is hypothesized that *P. aeruginosa* consumption of oxygen is rapid enough to maintain sufficiently low levels for the growth of such anaerobes. Thus, while competition for oxygen is evidently not sufficient to decrease *S. aureus* viability alone, in combination with HQNO and *Pseudomonas aeruginosa* siderophores, reduced oxygen availability may put *S. aureus* at a significant coculture disadvantage.

In addition to the proposed role of oxygen competition, there are several possible mechanisms by which the shift to lactate fermentation promotes *S. aureus* cell death in the presence of *P. aeruginosa* cells. One model is that reduced energy production by fermentation, compared to respiration, puts *S. aureus* at a competitive growth disadvantage in coculture. Alternatively, the switch of *S. aureus* to a fermentative lifestyle might increase *S. aureus* susceptibility to other antimicrobial factors produced by *P. aeruginosa*; however, the increased persistence of *S. aureus* SCVs, which also rely on fermentation for their primary energy generation, in coculture with *P. aeruginosa* argues against this simple model. Thus, the SCV phenotype may indicate a key role for *S. aureus* being properly adapted to a fermentative lifestyle before encountering *P. aeruginosa* in coculture in order for *S. aureus* to persist. Another possible model is that *P. aeruginosa* competes for/depletes a micronutrient required by *S. aureus* specifically under conditions of fermentative growth. Finally, *S. aureus* cell death may be impacted by pH balance, likely perturbed by increased lactate accumulation and accumulation of reactive oxygen spe-

cies. At this time, it is not possible to distinguish among these (or alternative) models for *P. aeruginosa*-mediated killing of *S. aureus*. The difficulty of determining a precise mechanism of killing is not an unprecedented challenge. Analogous to our question of *P. aeruginosa*-mediated killing, the mechanism of antibiotic-mediated killing has been poorly understood for years and was recently brought back into focus for continued debate (76–79).

The respiratory microbial communities of CF patients are complex and dynamic throughout childhood and adolescence. Despite a strong focus on *P. aeruginosa* and *S. aureus* in CF lung infections, the driving factors that result in *P. aeruginosa* predominance during adulthood are not well understood. Here, we determined several possible mechanisms by which *P. aeruginosa* may displace *S. aureus* in respiratory communities. However, the immune response, host factors, and the other microbial community members undeniably also impact the transition from *S. aureus* to *P. aeruginosa* being the predominant pathogen in CF and the associated impact on patient outcome.

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REFERENCES

- Madan JC, Koestler DC, Stanton BA, Davidson L, Moulton LA, Housman ML, Moore JH, Guill MF, Morrison HG, Sogin ML, Hampton TH, Karagas MR, Palumbo PE, Foster JA, Hibberd PL, O'Toole GA. 2012. Serial analysis of the gut and respiratory microbiome in cystic fibrosis in infancy: interaction between intestinal and respiratory tracts and impact of nutritional exposures. *mBio* 3(4):e00251-12. <http://dx.doi.org/10.1128/mBio.00251-12>.
- Delhaes L, Monchy S, Frealle E, Hubans C, Salleron J, Leroy S, Prevotat A, Wallet F, Wallaert B, Dei-Cas E, Sime-Ngando T, Chabe M, Viscogliosi E. 2012. The airway microbiota in cystic fibrosis: a complex fungal and bacterial community—implications for therapeutic management. *PLoS One* 7:e36313. <http://dx.doi.org/10.1371/journal.pone.0036313>.
- Filkins LM, Hampton TH, Gifford AH, Gross MJ, Hogan DA, Sogin ML, Morrison HG, Paster BJ, O'Toole GA. 2012. Prevalence of streptococci and increased polymicrobial diversity associated with cystic fibrosis patient stability. *J Bacteriol* 194:4709–4717. <http://dx.doi.org/10.1128/JB.00566-12>.
- Rogers GB, Hart CA, Mason JR, Hughes M, Walshaw MJ, Bruce KD. 2003. Bacterial diversity in cases of lung infection in cystic fibrosis patients: 16S ribosomal DNA (rDNA) length heterogeneity PCR and 16S rDNA terminal restriction fragment length polymorphism profiling. *J Clin Microbiol* 41:3548–3558. <http://dx.doi.org/10.1128/JCM.41.8.3548-3558.2003>.
- Willner D, Furlan M, Haynes M, Schmieder R, Angly FE, Silva J, Tammadoni S, Nosrat B, Conrad D, Rohwer F. 2009. Metagenomic analysis of respiratory tract DNA viral communities in cystic fibrosis and non-cystic fibrosis individuals. *PLoS One* 4:e7370. <http://dx.doi.org/10.1371/journal.pone.0007370>.
- Zemanick ET, Harris JK, Wagner BD, Robertson CE, Sagel SD, Stevens MJ, Accurso FJ, Laguna TA. 2013. Inflammation and airway microbiota during cystic fibrosis pulmonary exacerbations. *PLoS One* 8:e62917. <http://dx.doi.org/10.1371/journal.pone.0062917>.
- Zhao J, Schloss PD, Kalikin LM, Carmody LA, Foster BK, Petrosino JF, Cavalcoli JD, VanDevanter DR, Murray S, Li JZ, Young VB, LiPuma JJ. 2012. Decade-long bacterial community dynamics in cystic fibrosis airways. *Proc Natl Acad Sci U S A* 109:5809–5814. <http://dx.doi.org/10.1073/pnas.1120577109>.
- Willger SD, Grim SL, Dolben EL, Shipunova A, Hampton TH, Morrison HG, Filkins LM, O'Toole GA, Moulton LA, Ashare A, Sogin ML, Hogan DA. 2014. Characterization and quantification of the fungal microbiome in serial samples from individuals with cystic fibrosis. *Microbiome* 2:40. <http://dx.doi.org/10.1186/2049-2618-2-40>.
- Boucher RC. 2007. Airway surface dehydration in cystic fibrosis: pathogenesis and therapy. *Annu Rev Med* 58:157–170. <http://dx.doi.org/10.1146/annurev.med.58.071905.105316>.
- Matsui H, Grubb BR, Tarran R, Randell SH, Gatzky JT, Davis CW, Boucher RC. 1998. Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell* 95:1005–1015. [http://dx.doi.org/10.1016/S0092-8674\(00\)81724-9](http://dx.doi.org/10.1016/S0092-8674(00)81724-9).
- Matsui H, Wagner VE, Hill DB, Schwab UE, Rogers TD, Button B, Taylor RM, II, Superfine R, Rubinstein M, Iglewski BH, Boucher RC. 2006. A physical linkage between cystic fibrosis airway surface dehydration and *Pseudomonas aeruginosa* biofilms. *Proc Natl Acad Sci U S A* 103:18131–18136. <http://dx.doi.org/10.1073/pnas.0606428103>.
- Cox MJ, Allgaier M, Taylor B, Baek MS, Huang YJ, Daly RA, Karaoz U, Andersen GL, Brown R, Fujimura KE, Wu B, Tran D, Koff J, Kleinhenz ME, Nielson D, Brodie EL, Lynch SV. 2010. Airway microbiota and pathogen abundance in age-stratified cystic fibrosis patients. *PLoS One* 5:e11044. <http://dx.doi.org/10.1371/journal.pone.0011044>.
- Fodor AA, Klem ER, Gilpin DF, Elborn JS, Boucher RC, Tunney MM, Wolfgang MC. 2012. The adult cystic fibrosis airway microbiota is stable over time and infection type, and highly resilient to antibiotic treatment of exacerbations. *PLoS One* 7:e45001. <http://dx.doi.org/10.1371/journal.pone.0045001>.
- Price KE, Hampton TH, Gifford AH, Dolben EL, Hogan DA, Morrison HG, Sogin ML, O'Toole GA. 2013. Unique microbial communities persist in individual cystic fibrosis patients throughout a clinical exacerbation. *Microbiome* 1:27. <http://dx.doi.org/10.1186/2049-2618-1-27>.
- Stressmann FA, Rogers GB, van der Gast CJ, Marsh P, Vermeer LS, Carroll MP, Hoffman L, Daniels TW, Patel N, Forbes B, Bruce KD. 2012. Long-term cultivation-independent microbial diversity analysis demonstrates that bacterial communities infecting the adult cystic fibrosis lung show stability and resilience. *Thorax* 67:867–873. <http://dx.doi.org/10.1136/thoraxjnl-2011-200932>.
- van der Gast CJ, Walker AW, Stressmann FA, Rogers GB, Scott P, Daniels TW, Carroll MP, Parkhill J, Bruce KD. 2011. Partitioning core and satellite taxa from within cystic fibrosis lung bacterial communities. *ISME J* 5:780–791. <http://dx.doi.org/10.1038/ismej.2010.175>.
- Mahenthiralingam E. 2014. Emerging cystic fibrosis pathogens and the microbiome. *Paediatr Respir Rev* <http://dx.doi.org/10.1016/j.prrv.2014.04.006>.
- Hill UG, Floto RA, Haworth CS. 2012. Non-tuberculous mycobacteria in cystic fibrosis. *J R Soc Med* 105(Suppl 2):S14–S18. <http://dx.doi.org/10.1258/jrsm.2012.12s003>.
- Sibley CD, Parkins MD, Rabin HR, Duan K, Norgaard JC, Surette MG. 2008. A polymicrobial perspective of pulmonary infections exposes an enigmatic pathogen in cystic fibrosis patients. *Proc Natl Acad Sci U S A* 105:15070–15075. <http://dx.doi.org/10.1073/pnas.0804326105>.
- Cystic Fibrosis Foundation. 2011. Cystic Fibrosis Foundation patient registry 2010 annual data report. Cystic Fibrosis Foundation, Bethesda, MD.
- Wolter DJ, Emerson JC, McNamara S, Buccat AM, Qin X, Cochrane E, Houston LS, Rogers GB, Marsh P, Prehar K, Pope CE, Blackledge M, Deziel E, Bruce KD, Ramsey BW, Gibson RL, Burns JL, Hoffman LR. 2013. *Staphylococcus aureus* small-colony variants are independently associated with worse lung disease in children with cystic fibrosis. *Clin Infect Dis* 57:384–391. <http://dx.doi.org/10.1093/cid/cit270>.
- Wong JK, Ranganathan SC, Hart E. 2013. *Staphylococcus aureus* in early cystic fibrosis lung disease. *Pediatr Pulmonol* 48:1151–1159. <http://dx.doi.org/10.1002/ppul.22863>.
- Hirschhausen N, Block D, Bianconi I, Bragonzi A, Birtel J, Lee JC, Dubbers A, Kuster P, Kahl J, Peters G, Kahl BC. 2013. Extended *Staphylococcus aureus* persistence in cystic fibrosis is associated with bacterial adaptation. *Int J Med Microbiol* 303:685–692. <http://dx.doi.org/10.1016/j.ijmm.2013.09.012>.
- McNamara PJ, Proctor RA. 2000. *Staphylococcus aureus* small colony variants, electron transport and persistent infections. *Int J Antimicrob Agents* 14:117–122. [http://dx.doi.org/10.1016/S0924-8579\(99\)00170-3](http://dx.doi.org/10.1016/S0924-8579(99)00170-3).
- Com G, Carroll JL, Castro MM, Tang X, Jambhekar S, Berlinski A.

2014. Predictors and outcome of low initial forced expiratory volume in 1 second measurement in children with cystic fibrosis. *J Pediatr* 164:832–838. <http://dx.doi.org/10.1016/j.jpeds.2013.11.064>.
26. Sagel SD, Gibson RL, Emerson J, McNamara S, Burns JL, Wagener JS, Ramsey BW. 2009. Impact of *Pseudomonas* and *Staphylococcus* infection on inflammation and clinical status in young children with cystic fibrosis. *J Pediatr* 154:183–188. <http://dx.doi.org/10.1016/j.jpeds.2008.08.001>.
27. Emerson J, Rosenfeld M, McNamara S, Ramsey B, Gibson RL. 2002. *Pseudomonas aeruginosa* and other predictors of mortality and morbidity in young children with cystic fibrosis. *Pediatr Pulmonol* 34:91–100. <http://dx.doi.org/10.1002/ppul.10127>.
28. Zemanick ET, Emerson J, Thompson V, McNamara S, Morgan W, Gibson RL, Rosenfeld M. 2014. Clinical outcomes after initial *Pseudomonas* acquisition in cystic fibrosis. *Pediatr Pulmonol* <http://dx.doi.org/10.1002/ppul.23036>.
29. Manos J, Hu H, Rose BR, Wainwright CE, Zablotzka IB, Cheney J, Turnbull L, Whitchurch CB, Grimwood K, Harmer C, Anuj SN, Harbour C. 2013. Virulence factor expression patterns in *Pseudomonas aeruginosa* strains from infants with cystic fibrosis. *Eur J Clin Microbiol Infect Dis* 32:1583–1592. <http://dx.doi.org/10.1007/s10096-013-1916-7>.
30. Deretic V, Govan JR, Konyecsni WM, Martin DW. 1990. Mucoicid *Pseudomonas aeruginosa* in cystic fibrosis: mutations in the muc loci affect transcription of the *algR* and *algD* genes in response to environmental stimuli. *Mol Microbiol* 4:189–196. <http://dx.doi.org/10.1111/j.1365-2958.1990.tb00586.x>.
31. Govan JR, Fyfe JA. 1978. Mucoicid *Pseudomonas aeruginosa* and cystic fibrosis: resistance of the mucoicid from carbenicillin, flucloxacillin and tobramycin and the isolation of mucoicid variants in vitro. *J Antimicrob Chemother* 4:233–240. <http://dx.doi.org/10.1093/jac/4.3.233>.
32. Macia MD, Blanquer D, Togores B, Saulea J, Perez JL, Oliver A. 2005. Hypermutation is a key factor in development of multiple-antimicrobial resistance in *Pseudomonas aeruginosa* strains causing chronic lung infections. *Antimicrob Agents Chemother* 49:3382–3386. <http://dx.doi.org/10.1128/AAC.49.8.3382-3386.2005>.
33. Oliver A, Mena A. 2010. Bacterial hypermutation in cystic fibrosis, not only for antibiotic resistance. *Clin Microbiol Infect* 16:798–808. <http://dx.doi.org/10.1111/j.1469-0691.2010.03250.x>.
34. Harmer C, Alnassafi K, Hu H, Elkins M, Bye P, Rose B, Cordwell S, Triccas JA, Harbour C, Manos J. 2013. Modulation of gene expression by *Pseudomonas aeruginosa* during chronic infection in the adult cystic fibrosis lung. *Microbiology* 159:2354–2363. <http://dx.doi.org/10.1099/mic.0.066985-0>.
35. Rajan S, Saiman L. 2002. Pulmonary infections in patients with cystic fibrosis. *Semin Respir Infect* 17:47–56. <http://dx.doi.org/10.1053/srin.2002.31690>.
36. Haley CL, Colmer-Hamood JA, Hamood AN. 2012. Characterization of biofilm-like structures formed by *Pseudomonas aeruginosa* in a synthetic mucus medium. *BMC Microbiol* 12:181. <http://dx.doi.org/10.1186/1471-2180-12-181>.
37. Kluge S, Hoffmann M, Benndorf D, Rapp E, Reichl U. 2012. Proteomic tracking and analysis of a bacterial mixed culture. *Proteomics* 12:1893–1901. <http://dx.doi.org/10.1002/ptmc.201100362>.
38. Ruger M, Ackermann M, Reichl U. 2014. Species-specific viability analysis of *Pseudomonas aeruginosa*, *Burkholderia cepacia* and *Staphylococcus aureus* in mixed culture by flow cytometry. *BMC Microbiol* 14:56. <http://dx.doi.org/10.1186/1471-2180-14-56>.
39. Yang L, Liu Y, Markussen T, Hoiby N, Tolker-Nielsen T, Molin S. 2011. Pattern differentiation in co-culture biofilms formed by *Staphylococcus aureus* and *Pseudomonas aeruginosa*. *FEMS Immunol Med Microbiol* 62:339–347. <http://dx.doi.org/10.1111/j.1574-695X.2011.00820.x>.
40. Baldan R, Cigana C, Testa F, Bianconi I, De Simone M, Pellin D, Di Serio C, Bragonzi A, Cirillo DM. 2014. Adaptation of *Pseudomonas aeruginosa* in cystic fibrosis airways influences virulence of *Staphylococcus aureus* in vitro and murine models of co-infection. *PLoS One* 9:e89614. <http://dx.doi.org/10.1371/journal.pone.0089614>.
41. Fugere A, Lalonde Seguin D, Mitchell G, Deziel E, Dekimpe V, Cantin AM, Frost E, Malouin F. 2014. Interspecific small molecule interactions between clinical isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus* from adult cystic fibrosis patients. *PLoS One* 9:e86705. <http://dx.doi.org/10.1371/journal.pone.0086705>.
42. Park JH, Lee JH, Cho MH, Herzberg M, Lee J. 2012. Acceleration of protease effect on *Staphylococcus aureus* biofilm dispersal. *FEMS Microbiol Lett* 335:31–38. <http://dx.doi.org/10.1111/j.1574-6968.2012.02635.x>.
43. Mashburn LM, Jett AM, Akins DR, Whiteley M. 2005. *Staphylococcus aureus* serves as an iron source for *Pseudomonas aeruginosa* during in vivo coculture. *J Bacteriol* 187:554–566. <http://dx.doi.org/10.1128/JB.187.2.554-566.2005>.
44. Mitchell G, Seguin DL, Asselin AE, Deziel E, Cantin AM, Frost EH, Michaud S, Malouin F. 2010. *Staphylococcus aureus* sigma B-dependent emergence of small-colony variants and biofilm production following exposure to *Pseudomonas aeruginosa* 4-hydroxy-2-heptylquinoline-N-oxide. *BMC Microbiol* 10:33. <http://dx.doi.org/10.1186/1471-2180-10-33>.
45. Kessler E, Safrin M, Olson JC, Ohman DE. 1993. Secreted LasA of *Pseudomonas aeruginosa* is a staphylolytic protease. *J Biol Chem* 268:7503–7508.
46. Castric PA. 1975. Hydrogen cyanide, a secondary metabolite of *Pseudomonas aeruginosa*. *Can J Microbiol* 21:613–618. <http://dx.doi.org/10.1139/m75-088>.
47. Hassan HM, Fridovich I. 1980. Mechanism of the antibiotic action pyocyanine. *J Bacteriol* 141:156–163.
48. Machan ZA, Taylor GW, Pitt TL, Cole PJ, Wilson R. 1992. 2-Heptyl-4-hydroxyquinoline N-oxide, an antistaphylococcal agent produced by *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 30:615–623. <http://dx.doi.org/10.1093/jac/30.5.615>.
49. Korgaonkar A, Trivedi U, Rumbaugh KP, Whiteley M. 2013. Community surveillance enhances *Pseudomonas aeruginosa* virulence during polymicrobial infection. *Proc Natl Acad Sci U S A* 110:1059–1064. <http://dx.doi.org/10.1073/pnas.1214550110>.
50. Biswas L, Biswas R, Schlag M, Bertram R, Gotz F. 2009. Small-colony variant selection as a survival strategy for *Staphylococcus aureus* in the presence of *Pseudomonas aeruginosa*. *Appl Environ Microbiol* 75:6910–6912. <http://dx.doi.org/10.1128/AEM.01211-09>.
51. Hoffman LR, Deziel E, D'Argenio DA, Lepine F, Emerson J, McNamara S, Gibson RL, Ramsey BW, Miller SI. 2006. Selection for *Staphylococcus aureus* small-colony variants due to growth in the presence of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* 103:19890–19895. <http://dx.doi.org/10.1073/pnas.0606756104>.
52. Pernet E, Guillemot L, Burgel PR, Martin C, Lambeau G, Sermet-Gaudelus I, Sands D, Leduc D, Morand PC, Jeammet L, Chignard M, Wu Y, Touqui L. 2014. *Pseudomonas aeruginosa* eradicates *Staphylococcus aureus* by manipulating the host immunity. *Nat Commun* 5:5105. <http://dx.doi.org/10.1038/ncomms6105>.
53. Anderson GG, Moreau-Marquis S, Stanton BA, O'Toole GA. 2008. In vitro analysis of tobramycin-treated *Pseudomonas aeruginosa* biofilms on cystic fibrosis-derived airway epithelial cells. *Infect Immun* 76:1423–1433. <http://dx.doi.org/10.1128/IAI.01373-07>.
54. Moreau-Marquis S, Redelman CV, Stanton BA, Anderson GG. 2010. Co-culture models of *Pseudomonas aeruginosa* biofilms grown on live human airway cells. *J Vis Exp* 2010(44):2186. <http://dx.doi.org/10.3791/2186>.
55. Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26:139–140. <http://dx.doi.org/10.1093/bioinformatics/btp616>.
56. Sihto HM, Tasara T, Stephan R, Johler S. 2014. Validation of reference genes for normalization of qPCR mRNA expression levels in *Staphylococcus aureus* exposed to osmotic and lactic acid stress conditions encountered during food production and preservation. *FEMS Microbiol Lett* 356:134–140. <http://dx.doi.org/10.1111/1574-6968.12491>.
57. Zhang YH, Lynd LR. 2005. Regulation of cellulase synthesis in batch and continuous cultures of *Clostridium thermocellum*. *J Bacteriol* 187:99–106. <http://dx.doi.org/10.1128/JB.187.1.99-106.2005>.
58. Hampton TH, Green DM, Cutting GR, Morrison HG, Sogin ML, Gifford AH, Stanton BA, O'Toole GA. 2014. The microbiome in pediatric cystic fibrosis patients: the role of shared environment suggests a window of intervention. *Microbiome* 2:14. <http://dx.doi.org/10.1186/2049-2618-2-14>.
59. Gardner JF, Lascelles J. 1962. The requirement for acetate of a streptomycin-resistant strain of *Staphylococcus aureus*. *J Gen Microbiol* 29:157–164. <http://dx.doi.org/10.1099/00221287-29-1-157>.
60. Strasters KC, Winkler KC. 1963. Carbohydrate metabolism of *Staphylococcus aureus*. *J Gen Microbiol* 33:213–229. <http://dx.doi.org/10.1099/00221287-33-2-213>.
61. Kohler C, von Eiff C, Peters G, Proctor RA, Hecker M, Engelmann S. 2003. Physiological characterization of a heme-deficient mutant of *Staph-*

- Staphylococcus aureus* by a proteomic approach. *J Bacteriol* 185:6928–6937. <http://dx.doi.org/10.1128/JB.185.23.6928-6937.2003>.
62. Hunt JC, Phibbs PV, Jr. 1983. Regulation of alternate peripheral pathways of glucose catabolism during aerobic and anaerobic growth of *Pseudomonas aeruginosa*. *J Bacteriol* 154:793–802.
 63. van der Kooij D, Oranje JP, Hijnen WA. 1982. Growth of *Pseudomonas aeruginosa* in tap water in relation to utilization of substrates at concentrations of a few micrograms per liter. *Appl Environ Microbiol* 44:1086–1095.
 64. Buch A, Archana G, Naresh Kumar G. 2008. Metabolic channeling of glucose towards gluconate in phosphate-solubilizing *Pseudomonas aeruginosa* P4 under phosphorus deficiency. *Res Microbiol* 159:635–642. <http://dx.doi.org/10.1016/j.resmic.2008.09.012>.
 65. Voggu L, Schlag S, Biswas R, Rosenstein R, Rausch C, Gotz F. 2006. Microevolution of cytochrome bd oxidase in *Staphylococcus aureus* and its implication in resistance to respiratory toxins released by *Pseudomonas*. *J Bacteriol* 188:8079–8086. <http://dx.doi.org/10.1128/JB.00858-06>.
 66. Moreau-Marquis S, Bomberger JM, Anderson GG, Swiatecka-Urban A, Ye S, O'Toole GA, Stanton BA. 2008. The DeltaF508-CFTR mutation results in increased biofilm formation by *Pseudomonas aeruginosa* by increasing iron availability. *Am J Physiol Lung Cell Mol Physiol* 295:L25–L37. <http://dx.doi.org/10.1152/ajplung.00391.2007>.
 67. Stites SW, Plautz MW, Bailey K, O'Brien-Ladner AR, Wesselius LJ. 1999. Increased concentrations of iron and isoferritins in the lower respiratory tract of patients with stable cystic fibrosis. *Am J Respir Crit Care Med* 160:796–801. <http://dx.doi.org/10.1164/ajrccm.160.3.9811018>.
 68. Masalha M, Borovok I, Schreiber R, Aharonowitz Y, Cohen G. 2001. Analysis of transcription of the *Staphylococcus aureus* aerobic class Ib and anaerobic class III ribonucleotide reductase genes in response to oxygen. *J Bacteriol* 183:7260–7272. <http://dx.doi.org/10.1128/JB.183.24.7260-7272.2001>.
 69. Kogut M, Lightbown JW. 1962. Selective inhibition by 2-heptyl-4-hydroxyquinoline N-oxide of certain oxidation-reduction reactions. *Biochem J* 84:368–382.
 70. Belay N, Rasooly A. 2002. *Staphylococcus aureus* growth and enterotoxin A production in an anaerobic environment. *J Food Prot* 65:199–204.
 71. Ferreira MT, Manso AS, Gaspar P, Pinho MG, Neves AR. 2013. Effect of oxygen on glucose metabolism: utilization of lactate in *Staphylococcus aureus* as revealed by in vivo NMR studies. *PLoS One* 8:e58277. <http://dx.doi.org/10.1371/journal.pone.0058277>.
 72. Nguyen AT, Jones JW, Ruge MA, Kane MA, Oglesby-Sherrouse AG. 2015. Iron depletion enhances production of antimicrobials by *Pseudomonas aeruginosa*. *J Bacteriol* 197:2265–2275. <http://dx.doi.org/10.1128/JB.00072-15>.
 73. Sun JL, Zhang SK, Chen JY, Han BZ. 2012. Metabolic profiling of *Staphylococcus aureus* cultivated under aerobic and anaerobic conditions with (1)H NMR-based nontargeted analysis. *Can J Microbiol* 58:709–718. <http://dx.doi.org/10.1139/w2012-046>.
 74. Besier S, Smaczny C, von Mallinckrodt C, Krahl A, Ackermann H, Brade V, Wichelhaus TA. 2007. Prevalence and clinical significance of *Staphylococcus aureus* small-colony variants in cystic fibrosis lung disease. *J Clin Microbiol* 45:168–172. <http://dx.doi.org/10.1128/JCM.01510-06>.
 75. Gerritse J, Schut F, Gottschal JC. 1992. Modelling of mixed chemostat cultures of an aerobic bacterium, *Comamonas testosteroni*, and an anaerobic bacterium, *Veillonella alcalescens*: comparison with experimental data. *Appl Environ Microbiol* 58:1466–1476.
 76. Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ. 2007. A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* 130:797–810. <http://dx.doi.org/10.1016/j.cell.2007.06.049>.
 77. Liu Y, Imlay JA. 2013. Cell death from antibiotics without the involvement of reactive oxygen species. *Science* 339:1210–1213. <http://dx.doi.org/10.1126/science.1232751>.
 78. Keren I, Wu Y, Inocencio J, Mulcahy LR, Lewis K. 2013. Killing by bactericidal antibiotics does not depend on reactive oxygen species. *Science* 339:1213–1216. <http://dx.doi.org/10.1126/science.1232688>.
 79. Ezraty B, Vergnes A, Banzhaf M, Duverger Y, Huguenot A, Brochado AR, Su SY, Espinosa L, Loiseau L, Py B, Typas A, Barras F. 2013. Fe-S cluster biosynthesis controls uptake of aminoglycosides in a ROS-less death pathway. *Science* 340:1583–1587. <http://dx.doi.org/10.1126/science.1238328>.