Nutrient Availability as a Mechanism for Selection of Antibiotic Tolerant Pseudomonas aeruginosa within the CF Airway

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Abstract

Microbes are subjected to selective pressures during chronic infections of host tissues. Pseudomonas aeruginosa isolates with inactivating mutations in the transcriptional regulator LasR are frequently selected within the airways of people with cystic fibrosis (CF), and infection with these isolates has been associated with poorer lung function outcomes. The mechanisms underlying selection for lasR mutation are unknown but have been postulated to involve the abundance of specific nutrients within CF airway secretions. We characterized lasR mutant P. aeruginosa strains and isolates to identify conditions found in CF airways that select for growth of lasR mutants. Relative to wild-type P. aeruginosa, lasR mutants exhibited a dramatic metabolic shift, including decreased oxygen consumption and increased nitrate utilization, that is predicted to confer increased fitness within the nutrient conditions known to occur in CF airways. This metabolic shift exhibited by lasR mutants conferred resistance to two antibiotics used frequently in CF care, tobramycin and ciprofloxacin, even under oxygen-dependent growth conditions, yet selection for these mutants in vitro did not require preceding antibiotic exposure. The selection for loss of LasR function in vivo, and the associated adverse clinical impact, could be due to increased bacterial growth in the oxygen-poor and nitrate-rich CF airway, and from the resulting resistance to therapeutic antibiotics. The metabolic similarities among diverse chronic infection-adapted bacteria suggest a common mode of adaptation and antibiotic resistance during chronic infection that is primarily driven by bacterial metabolic shifts in response to nutrient availability within host tissues.

Introduction

Microbes are subjected to selection in host environments during the course of chronic infections [1,2,3]. The characteristics selected may have profound impacts on disease outcomes, particularly if they confer increased microbial fitness or resistance to therapy. One example of this phenomenon is the adaptation of Pseudomonas aeruginosa within the airways of people with cystic fibrosis (CF). Diverse phenotypic changes have been observed among CF chronic P. aeruginosa infection isolates, including changes in several surface antigens [4,5], altered antibiotic susceptibilities [6], and overproduction of the mucoid exopolysaccharide alginate [3]. P. aeruginosa CF adaptive changes have been associated with poor clinical outcomes [7,8] and, in the case of mucoidy, a diminished likelihood of eradication by antibiotics [9].

Recently, several groups have described P. aeruginosa CF isolates with inactivating mutations in the gene lasR [2,8,10,11,12]. Genetic analyses demonstrated that these mutants emerged from existing, chronically-infecting lineages, as opposed to representing new infections, and that multiple lineages with independent lasR mutations occurred within individual patients, indicative of strong selective pressure against LasR function [2,11]. lasR encodes a central regulator of the bacterial intercellular signaling system known as quorum sensing that requires the synthesis and recognition of P. aeruginosa small molecule products, including acyl-homoserine lactones (AHL). lasR mutant isolates occur in at least one-third of P. aeruginosa culture-positive individuals younger than 15 years attending CF clinics in Seattle [2,3]. Among this population, lasR mutant isolates emerged relatively early during CF airway infection (on average 2 years before mucoidy), and were associated with worse lung function [8]. LasR inactivation conferred distinct phenotypic consequences, including distinctive colony morphology (autolysis and surface iridescent sheen) that facilitates the identification of mutant isolates, a growth advantage...
Chronic infections are distinguished from many other infections in that they are difficult to eradicate with antibiotics. Thus, the microbes that cause chronic infections persist within host tissues for long periods despite our best treatment efforts. During the course of these chronic infections, the causative microbes often change genetically. For example, a bacterium that commonly infects the lungs of people with the genetic disease cystic fibrosis (CF) undergoes several known changes that affect the growth of this pathogen. However, the causes and clinical impact of the changes undergone by this and other chronically infecting microbes are unclear. We show that a common, early mutation found in bacteria isolated from chronically infected CF airways renders these bacteria better able to grow in the nutrients found in CF lung secretions. Interestingly, these same changes also confer resistance to several antibiotics used commonly to treat CF patients. Many of the characteristics conferred by this mutation are exhibited by other microbes found in chronic infections, suggesting that adaptation of these microbes to host tissue nutrient environments may be a common mechanism of antibiotic resistance in chronic infections.

### Results

**lasR mutants have a growth advantage in NO\textsubscript{3}\textsuperscript{−} and accumulate the toxic metabolite nitric oxide (NO\textsuperscript{−})**

Given the evidence that lasR mutants may have a growth advantage in NO\textsubscript{3}\textsuperscript{−} [11], we compared the growth of P. aeruginosa laboratory strain PA14 and clinical isolates carrying wild-type lasR alleles with their derived lasR mutant strains in the presence of various concentrations of NO\textsubscript{3}\textsuperscript{−}. lasR mutants exhibited a substantial growth advantage in minimal medium with added NO\textsubscript{3}\textsuperscript{−}. As shown in Fig. 1B for a lasR mutant with a gentamicin insertion cassette derived from PA14 (PA14-lasR::Gm), a growth advantage was detected in NO\textsubscript{3}\textsuperscript{−} concentrations as low as 125 \(\mu\text{M}\), well below the average NO\textsubscript{3}\textsuperscript{−} concentrations recently measured in CF airway secretions [17,18], and the advantage was more pronounced at higher NO\textsubscript{3}\textsuperscript{−} concentrations (not shown).

The average rate of lasR mutant growth (calculated as the slopes of lines fit to the datasets shown between 8 and 16 minutes) in 50 mM NO\textsubscript{3}\textsuperscript{−} was increased ~5-fold relative to wild-type. Similar results were obtained using Luria Broth, with PA14 with an unmarked las deletion (PA14ΔlasR), and with paired lasR wild-type and mutant clinical isolates (not shown). This analysis confirms and extends our previous finding that lasR mutations confer a growth advantage with nitrogen sources that are abundant in the CF airway, including NO\textsubscript{3}\textsuperscript{−}, as well as with aromatic amino acids [11].

lasR mutant strains and isolates converted NO\textsubscript{3}\textsuperscript{−} to NO\textsubscript{2}\textsuperscript{−}, and degraded NO\textsubscript{2}\textsuperscript{−}, at significantly higher rates than did wild-type strains and isolates (Fig. 1C). For example, the average rate of NO\textsubscript{3}\textsuperscript{−} production by lasR mutant strains was ~4.4-fold greater than by wild-type. In contrast, lasR mutant strains and isolates demonstrated a relatively modest spontaneous increase in NO\textsubscript{−} reduction relative to wild-type (Fig. 1D); slopes for lines fit to each dataset in Fig. 1D between 25 and 100 seconds demonstrated that lasR mutant cells had an NO\textsubscript{−} degradation rate only ~1.8-fold greater than wild-type cells. These activities resulted in dramatically higher levels of NO\textsuperscript{−} (Fig. 1E) in lasR mutant cultures that could not be explained by any concurrent difference in growth rates between lasR mutants and wild-type in added NO\textsubscript{3} (compare Figs. 1B and 1E). The accumulation of NO\textsuperscript{−}, a potent microbicide [24], in lasR mutant cultures would be predicted to result in cell death at very high cell densities (as observed with P. aeruginosa cells with mutations in the quorum sensing regulator rhlR [16]) and in increased susceptibility to exogenous NO\textsuperscript{−} sources.

**lasR mutation confers increased susceptibility to nitrosative stress, and selection requires a membrane-bound NO\textsubscript{3}\textsuperscript{−} reductase**

Because lasR mutant P. aeruginosa produces elevated levels of endogenous NO\textsuperscript{−}, and bacterial cells possess a finite capacity for detoxifying NO\textsuperscript{−} that can be exceeded by exposure to exogenous reactive nitrogen species (RNS) [25], we predicted that lasR mutants would also be more susceptible to the exogenous nitrosative stress presented by either NO\textsuperscript{−} donors (which have relatively short aqueous half-lives [26]) or acidified NO\textsubscript{3}\textsuperscript{−} (with substantially greater aqueous half-life [27]). Therefore, lasR mutant strains and isolates were tested for these susceptibility phenotypes. lasR mutants were more susceptible to growth inhibition by the addition of NO\textsuperscript{−} donor compounds to liquid cultures (Fig. 2A), and by NO\textsubscript{3}\textsuperscript{−} disks during growth on acidified agar medium (Fig. 2B). These results are in agreement with our previous phenotype array findings that lasR inactivation in clinical P. aeruginosa isolates confers increased susceptibility to high concentrations of NO\textsubscript{3}\textsuperscript{−} in unbuffered liquid minimal medium.
Furthermore, analysis of clinical isolate pairs demonstrated that the impact of lasR mutation on NO₂⁻ susceptibility was similar to the effect demonstrated previously for mucoidy [27], as shown in Fig. 2B for one isolate pair (NCAMT0101-2 and -3).

P. aeruginosa encodes two NO₃⁻ reductases, one in the bacterial inner membrane and the other in the periplasm. It was found previously that, of these two, only the membrane-bound enzyme was required for anaerobic growth of P. aeruginosa [17]. Interestingly, we found that spontaneous lasR mutants did not emerge during extended growth on agar medium from strains with transposon insertions in genes encoding subunits of the membrane-bound NO₃⁻ reductase (narJ and narK2), while sectors displaying the characteristic lasR phenotype arose frequently among strains with similar mutations in genes encoding the periplasmic enzyme (PA1173 and napA) [Fig. 2C]. Furthermore, the growth advantage in NO₃⁻ conferred by lasR mutation (Fig. 1B) was not observed in the absence of narK genes (narK1narK2lasR, data not shown). Thus, the membrane-bound NO₃⁻ reductase was required for both the growth advantage of lasR mutants in added NO₃⁻ and for rapid lasR mutant emergence in vitro. These results functionally link the growth advantage in NO₃⁻ conferred by a lasR mutation with the selection of these mutants, at least in vitro, and perhaps also in the NO₃⁻-rich CF airway [17,18].

Factors that detoxify NO⁻ increase P. aeruginosa lasR mutant growth

The findings that lasR mutants overproduce the potent microbicide NO⁻ (Fig. 1E), that they undergo autolysis at high cell density [11], and that lasR mutants exhibit increased growth inhibition by exogenous sources of NO⁻ (Fig. 2A) suggested that factors that detoxify NO⁻ could enrich for lasR mutant growth. This hypothesis is supported by the observation that the cell death observed when RhlR mutants are grown anaerobically as biofilms can be prevented by addition of an NO⁻ scavenger [16]. Similarly,
we found that P. aeruginosa lasR mutants growing near disks containing hemoglobin, which scavenges NO\textsuperscript{•} stoichiometrically, also grew better than did cells farther away from the disk (Fig. 3A). These results suggest that the presence of an NO\textsuperscript{•} “sink” such as hemoglobin increased growth of lasR mutant P. aeruginosa.

Some bacteria, including the gram-positive CF bacterial pathogen Staphylococcus aureus, are known to be relatively resistant to the effects of NO\textsuperscript{•} as a result of efficient cellular detoxification mechanisms [24]. Furthermore, we found previously that the presence of live, but not dead, S. aureus decreased expression of a P. aeruginosa gene (fhp [25]) involved in NO\textsuperscript{•} degradation [28], suggesting that S. aureus may detoxify NO\textsuperscript{•} produced by P. aeruginosa.

The catalytic effect of growing S. aureus cells would be predicted to be even more robust than that of the stoichiometric agent hemoglobin. Therefore, we compared the growth of lasR mutants and wild type bacteria in the presence and absence of S. aureus.

The CF pathogen S. aureus increases the growth of P. aeruginosa lasR mutants, apparently through NO\textsuperscript{•} detoxification

When grown near S. aureus, lasR mutants exhibited wild-type growth phenotypes, as manifested by thicker colonies, using either clinical isolates or laboratory strains of each species (Figs. 3B–C).
This phenotypic change did not require contact with \textit{S. aureus}. Cell-free culture medium, cell sonicates, and organic extracts of \textit{S. aureus} cultures did not exhibit the activity of \textit{S. aureus} colonies, suggesting that \textit{S. aureus} cell activity was required for this phenotypic change.

To further characterize the growth of \textit{lasR} mutants and its modification by \textit{S. aureus}, we inoculated static, liquid cultures with equal numbers of \textit{P. aeruginosa} wild-type and \textit{lasR} mutant \textit{S. aureus} partially defective for NO$^\cdot$ degradation (\textit{hmp} mutants [24]), and measured the growth of each strain after incubation. As in previous experiments [e.g., Fig. 2A] [11], \textit{P. aeruginosa} \textit{lasR} mutants grown alone did not have a growth defect relative to wild-type strains and isolates in these nutrient conditions (not shown). We found that \textit{lasR} mutant growth was enhanced by co-culture with wild-type \textit{S. aureus}, but not by \textit{hmp} mutant \textit{S. aureus} (Fig. 3D). In addition, \textit{lasR} mutant colonies growing on LB agar near colonies of \textit{hmp} mutant \textit{S. aureus} displayed substantially more autolysis than did \textit{lasR} mutants growing near wild-type \textit{S. aureus} (not shown), supporting the notion that \textit{S. aureus} NO-detoxification is required to impede \textit{lasR} \textit{P. aeruginosa} colony autolysis. These results suggest that the presence of \textit{S. aureus}, which commonly co-infects CF airways with \textit{P. aeruginosa} [29], encourages the growth of \textit{lasR} mutant \textit{P. aeruginosa} by detoxifying NO$^\cdot$. This effect of \textit{S. aureus} and other microbes could contribute to the relatively low tensions of NO$^\cdot$ observed within CF airways [19], which would be predicted to further
encourage the growth of lasR mutant *P. aeruginosa* by providing a mechanism to mitigate the toxic effects resulting from the shift to nitrate metabolism.

**Oxygen utilization is diminished in lasR mutants, resulting in resistance to oxidative stress**

Low molecular oxygen tension and abundant nitrogen oxides have been observed in CF secretions [18,20]. Furthermore, deficiency in las signaling has been shown to result in decreased expression of cytochromes central to oxygen utilization [30]. Therefore, *P. aeruginosa* lasR mutants could have decreased utilization of oxygen as an electron acceptor. To test this hypothesis, we examined rates of oxygen utilization in liquid (Fig. 4A) and agar-grown (not shown) *P. aeruginosa* cultures. lasR mutant cultures exhibited oxygen consumption rates at approximately 40–50% those of wild-type cultures (determined by comparing slopes of lines fit to each dataset from 1–5 minutes in Fig. 4A). Aerobic metabolism generates toxic reactive oxygen species (ROS), including superoxide (O$_2^-$) [31]. As lasR mutant cells exhibit decreased rates of oxygen utilization relative to wild-type cells (Fig. 4A), lasR mutant cells could consequently contain lower endogenous levels of ROS. Hydroethidine is a specific probe of superoxide concentration [32].

![Figure 4](image_url)

**Figure 4. lasR mutant *P. aeruginosa* strains and isolates exhibit lower rates of oxygen utilization and resistance to paraquat, tobramycin and ciprofloxacin.** (A) Change in oxygen concentration during stirred incubation of washed cells of the indicated strains resuspended at equivalent cell densities in LB with 400 µM KNO$_3$ at 37°C. Average of 3 experiments ± s.d.; results representative of 3 separate experiments. Slopes for lines fit to each dataset between 1 and 5 minutes were significantly different (p<0.04). Complementation of lasR mutants with a wild-type copy of lasR on a plasmid restored wild-type phenotypes (not shown). The difference was no longer statistically significant in the absence of added KNO$_3$ (LB was shown previously to contain approximately 23 µM NO$_3^-$ [71]; not shown). (B) Fluorescence yields generated by adding a saturated DMSO solution of hydroethidine (HE), a probe of superoxide concentration [32], for 5 minutes on lawns of the indicated strains (where p*lasR* indicates complementation with a wild-type copy of lasR on a plasmid) grown on LB agar. Average ± s.d. of triplicates and representative of five separate experiments; similar results were obtained in liquid cultures and with clinical isolate pairs for Patient 1 (not shown). (C) Zone diameters of growth inhibition for the indicated clinical isolates and strains by disks containing 1 µmol of paraquat after 24 hours' incubation in air at 37°C on LB agar with 400 µM KNO$_3$. Results shown are average ± s.d. for triplicates and are representative of >10 separate experiments. Complementation with a copy of lasR on a plasmid restored wild-type phenotypes to lasR mutants (data not shown). (D) As in (C), except with disks containing 3.75 µg of ciprofloxacin or 3 µg of tobramycin on MH agar and 400 µM KNO$_3$ (the lasR mutant strain tested for tobramycin susceptibility was PA14-L1, which does not contain an engineered aminoglycoside resistance gene). Average ± s.d. for triplicates. *, p<0.001 compared both with wild-type and the complemented mutant. No decreases in susceptibility were noted with disks of control antibiotics: carbenicillin, tetracycline, aztreonam, and polymyxin. Results with the unmarked deletion strain PA14ΔlasR were similar to those with the lasR mutants shown for both (C) and (D). (E) Tobramycin disk diffusion diameters for experiments as in 5d except with the indicated strains. Experiment at right compares the oxyRkatA-lasR mutant carrying an empty plasmid vector with the same strain carrying the same plasmid but with a wild-type copy of lasR, and on agar media containing 300 µg/mL carbenicillin for plasmid maintenance. Similar results were observed for disks of ciprofloxacin (not shown). Results shown are averages ± s.d. for triplicates. 

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fluorescent indicator of intracellular \( O_2^- \) [32]. Hydroethidine addition to air-grown agar (Fig. 4B) or liquid (not shown) cultures of lasR mutants yielded much lower cell fluorescence than did its addition to wild-type cultures. We demonstrated that cell permeability was equivalent in wild-type PA14 and lasR mutant cells using two established methods: one measuring uptake of ethidium bromide during efflux pump chemical blockade [33] and another based on the uptake of the fluorescent molecule NPN without efflux pump inactivation [34] (data not shown). These results indicate that intracellular \( O_2^- \) concentrations are lower in lasR mutant cells than in wild-type cells. By analogy to the increased susceptibility of lasR mutants to exogenous nitrosative stress associated with higher endogenous NO\(^-\) production (Figs. 1E and 2A–B), these results suggest that lasR mutant cells would be more resistant to exogenous sources of oxidative stress, including redox-cycling agents [31,35].

To test this hypothesis, we measured the response of \( P. aeruginosa \) cultures to the redox-cycling agent paraquat, which reacts with intracellular oxygen to generate \( O_2^- \) [35]. As shown in Fig. 4C, cultures of lasR mutants (in both laboratory strain and clinical isolate backgrounds) were more resistant to paraquat and, as with nitrite susceptibility (Fig. 2B, far right), this effect was present in lasR mutant clinical isolates after several years of infection (Fig. 4C, far right). Differences in susceptibility to exogenous hydrogen peroxide exhibited the same trend, but to a lesser extent (not shown). Thus, the susceptibility of lasR mutant \( P. aeruginosa \) to exogenous oxidative stress is altered, apparently due to lower endogenous production of ROS and higher residual capacity for detoxification. Polyacrylamide gel enzymatic activity assays [31] demonstrated that lasR mutant cells and their wild-type counterparts exhibited similar activities of superoxide dismutases, enzymes that degrade \( O_2^- \) (data not shown), supporting the concept that the differences in endogenous \( O_2^- \) levels, and susceptibility to paraquat, were due to differences in \( O_2^- \) production rather than differences in \( O_2^- \) degradation.

**lasR mutants have a growth advantage under conditions of oxidative stress**

The lower endogenous \( O_2^- \) concentrations of lasR mutants indicate that they might have a growth advantage compared with wild-type cells when grown under conditions of oxidative stress. To test this hypothesis, agar-suspended cultures were inoculated with equal numbers of lasR and wild-type cells in the presence of paraquat, and then the density of each strain was determined in serial, thin culture slices. Using an oxygen microprobe, oxygen concentration within these cultures became undetectable within approximately 2 mm of depth below the surface after 24 hours of incubation (data not shown). This growth medium is a viscous gel, limiting the motility and sedimentation of cells and thus preserving two-dimensional culture structure, resulting in the establishment of a stable oxygen gradient. In this way, this culture may reproduce some aspects of CF respiratory secretions, which are relatively viscous compared to liquid cultures and exhibit oxygen gradients [20]. Furthermore, as ROS such as \( O_2^- \) are side-products of oxygen-based respiration, ROS are produced at decreasing amounts with increased depth within the cultures. As shown in Fig. 5A, lasR cells greatly outcompeted wild-type cells at more superficial depths, where oxygen was detectable and \( O_2^- \) could be produced upon paraquat exposure. This effect diminished with increasing depth and, therefore, with lower oxygen concentration. Thus, under growth conditions in which superoxide is generated, lasR mutants have a relative fitness advantage.

The shift to increased nitrate based metabolism by lasR mutants confers tolerance to antibiotics commonly used in CF treatment

One condition under which ROS are generated within bacterial cells is upon exposure to bactericidal antibiotics, including fluoroquinolones and aminoglycosides, under aerobic conditions [22,36]. Bacterial killing by both classes of antibiotics has been shown to be attributable in part to induction of superoxide production [37]. In addition, efficient aminoglycoside uptake (and thus bacterial killing) requires aerobic electron transport [23]. Furthermore, a las-regulated \( P. aeruginosa \) exoproduct, the Pseudo-F monos quinolone signal, induces an oxidative stress response, increased cellular ROS, and increased susceptibility to fluoroquinolones in \( P. aeruginosa \) [38], functionally linking response to oxidative stress and susceptibility to fluoroquinolones. A relationship between fluoroquinolone susceptibility and oxidative stress is supported by work in other bacterial species [39], including the observation that spontaneous mutants in superoxide response regulator genes have been selected by exposure of both \( E. coli \) and \( S. enteritidis \) to fluoroquinolones [40]. Thus, we predicted that the lower oxygen utilization rates and increased resistance to sources of superoxide exhibited by lasR mutants would result in decreased susceptibility to the fluoroquinolone ciprofloxacin and the aminoglycoside tobramycin, both of which are used frequently to treat CF patients [41]. As shown in Fig. 4D, surface cultures on nitrate-containing agar medium of lasR mutants were less susceptible to disks containing these drugs. Agar-suspended cultures in the same medium demonstrated that these differences were oxygen-dependent (Figs. 5B–C), as with paraquat (Fig. 5A). These results suggest that, under these culture conditions, inactivating lasR mutation confers resistance to two of the antibiotics used most frequently in CF care, tobramycin and ciprofloxacin. To further investigate the relationship between oxidative stress and antibiotic resistance, we compared the susceptibilities to oxidative stress and antibiotics of strains of \( P. aeruginosa \) carrying the double mutation oxyRkatA, or the triple mutation oxyRkatAlasR. Strains null for oxyR and katA are defective for the defensive response to oxidative stress [36]; accordingly, the oxyRkatA mutant exhibited increased susceptibility to paraquat compared with wild-type (Fig. S1). However, the oxyRkatAlasR triple mutant was even more resistant to paraquat than was wild-type, confirming and extending the observation that a lasR mutation confers resistance to ROS (not shown). Similarly, the oxyRkatAlasR mutant was more susceptible to tobramycin (Fig. 4E) (as shown for the oxyR single mutant and the aminoglycoside gentamicin [36]) and to ciprofloxacin (not shown) than was wild-type; with paraquat, the oxyRkatAlasR triple mutant exhibited resistance to each of these drugs, an effect that was reversed by complementation with a wild-type copy of lasR on a plasmid (Fig. 4E and data not shown). These results indicate that lasR mutation confers resistance to these two antibiotics through its effects on respiratory activity and oxidative stress response. As lasR mutant strains and isolates also exhibit increased tolerance to some \( \beta \)-lactams due to increased \( \beta \)-lactamase activity [11], these results suggest that the emergence of lasR mutant isolates during chronic infections could adversely impact the clinical response to all three of the antibiotic classes used most commonly during standard CF treatment (\( \beta \)-lactams, fluoroquinolones, and aminoglycosides).

The recent discovery [42] that increased bacterial production of NO\(^-\) (which is increased by LasR inactivation, Fig. 1E) confers additional protection against a wide variety of antibiotics, including \( \beta \)-lactams, quinolones, and aminoglycosides, further supports this possibility.
Discussion

In this work, *P. aeruginosa* isolates with inactivating mutations in the AHL-responsive transcriptional regulator LasR exhibited a profound growth advantage with nitrogen substrates found in the CF airway. These differences are attributable to *lasR*-dependent increased utilization of nitrogen oxides and decreased utilization of oxygen. This metabolic shift results in an increase in the production of the RNS NO, and a corresponding decrease in the ROS O2, the latter of which is associated with decreased susceptibility in our conditions to at least two antibiotics used frequently in treating CF lung infections. This growth advantage in conditions characteristic of CF airways, and the resulting antibiotic resistance, may explain the observed high prevalence of...
LasR mutants and the associated worse lung function of CF patients whose airways contain these mutants [9].

The metabolic changes that occur upon lasR inactivation would be predicted to favor growth of lasR mutants arising spontaneously in the CF airway due to the confluence of selective forces encountered in this environment. For example, the abundant NO$_3^-$ and NO$_2^-$ [17,18] and low oxygen tensions [20] found in CF secretions, as well as the relatively low NO$_2^-$ levels [19], would provide optimal metabolic conditions for lasR mutant selection. As suggested previously [43], P. aeruginosa likely adapts to a continuum of different oxygen tensions, with variation in the relative ratio of oxygen and nitrate utilization. Inactivating mutations in lasR may confer advantages in a variety of these microenvironments found in the CF lung. Also contributing to the beneficial nature of this environment for lasR mutant growth is the presence of NO$_2^-$-detoxifying microbes, such as S. aureus and perhaps anaerobic bacteria, the latter of which were recently found to occupy CF secretions at high densities [44]; it should be noted that, while contact of lasR mutant P. aeruginosa with wild-type P. aeruginosa was also shown previously to reverse autolysis and sheen [11], it is not yet clear whether the mechanism of this effect is similar to that of S. aureus. The availability of amino acids as nutrient sources in CF secretions [13] would provide an additional selective pressure for lasR mutant growth [11]. Similarly, lasR mutants are relatively resistant to sources of oxidative stress, including tobramycin and ciprofloxacin (Figs. 4D–E), two antibiotics that, along with ceftazidime (to which lasR mutants are also relatively tolerant due to augmented β-lactamase activity [11]), are among the antibiotics used most commonly in CF treatment [41]. Although other sources of ROS are present in CF airways, such as H$_2$O$_2$ from host cells [36], whether exogenously adding these molecules to P. aeruginosa effectively confers intracellular oxidative stress is not as clear as is the effect of the above antibiotics [22,23]. While the results presented here demonstrate that nutrient conditions (particularly relating to oxygen and nitrogen oxides) are sufficient to enrich for lasR mutant growth in vitro, the frequent treatment of CF patients with the above antibiotics likely provides additional selection for these mutants, resulting in a complex dynamic between the CF airway nutrient environment, P. aeruginosa adaptation, therapy, and pathophysiology. These ideas are summarized in the model in Fig. 6.

There are multiple therapeutic and pathophysiologic implications of the model in Fig. 6. For example, assuming that P. aeruginosa infection leads to airway inflammation, and thus to obstructive lung disease, as suggested by current models of CF pathogenesis [41], the growth advantage of lasR mutant cells within the CF airway would be predicted to render such mutants more pathogenic to CF patients by virtue of higher cell density and greater consequent inflammation. [It should be noted that while lasR mutant P. aeruginosa strains were shown to be less pathogenic in animal models of short-term respiratory infection [45], those models may not accurately reflect the pathogenic mechanisms of chronic CF airway infection, during which many “acute” virulence factors are not expressed [46]. This effect may contribute to the observed association between lasR mutant CF airway infection and worse lung function [8]. Furthermore, the clinical response to standard antibiotic therapy in patients infected with lasR mutants would be predicted to be poor relative to patients with wild type isolates, perhaps further contributing to the clinical impact and rendering eradication increasingly difficult [8]. Thus, the presence of lasR mutants in CF respiratory cultures may be of prognostic value, and aggressive, directed treatment of these mutants upon isolation (i.e., through the expanded use of monobactams, tetracyclines, or polymyxin in the case of lasR mutant infection) or with regimens that do not select for their growth may lead to improved outcomes.

While recent publications have shown that quorum sensing regulates the expression of demethylation genes [14,15,16] and oxygen metabolic genes [30] at the transcriptional level, the mechanism of the distinct metabolic behaviors of lasR mutant and wild-type cells is likely to be as complex as the quorum sensing system itself. In P. aeruginosa, quorum sensing involves at least three parallel signaling systems, at least four different signal receptors, and regulation by diverse environmental cues [14,47,48]. However, some mechanistic clues are evident from our results. Previously, we showed that the two-component metabolic regulatory system CbrAB contributes to the metabolic phenotypes of lasR mutant clinical isolates of P. aeruginosa [11]; mutants in this system have decreased capacities to use amino acids as nitrogen sources [49], and lasR mutant isolates have upregulated expression of the transcriptional metabolism regulator cbrB [11]. The current results also suggest an additional mechanism for the growth advantage of lasR mutant P. aeruginosa in specific amino acids (most markedly with phenylalanine, but also with other aromatic and branched-chain amino acids [11]). Many enzymes that metabolize amino acids are inactivated by reactive oxygen species (ROS),

**Figure 6. A model for metabolic changes in CF-adapted lasR mutant isolates of P. aeruginosa.** According to the model, patients are initially infected with environmental isolates carrying wild-type copies of the lasR gene (left). These isolates have relatively high utilization of oxygen (activity indicated by the sizes of the green arrows) and lower utilization of nitrogen oxides (NO$_3^-$). Selective pressures encountered in the host, including abundant host NO$_3^-$ and amino acids (AA), low host NO$_2^-$, the presence of other bacterial species that metabolize NO$_3^-$, reduced O$_2$ concentrations, and treatment with β-lactams or antibiotics that generate ROS, favor the emergence of lasR mutant isolates with higher utilization of nitrates and lower utilization of oxygen. This metabolic shift confers a growth advantage in the nutrient conditions in the CF airway, including abundant NO$_3^-$, and relative resistance to the antibiotics used most frequently to treat CF patients.

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including the first enzyme in the phenylalanine catabolic pathway, phenylalanine hydroxylase [50,51]. Therefore, cells with lower intracellular concentrations of ROS, such as lasR mutants (Fig 4B), would be predicted to be better able to utilize amino acids such as phenylalanine as nutrient sources. Additionally, the las system is involved in regulating the levels and timing of production of a family of hydroxalkylquinoline (HAQ) molecules [52], including the compounds 4-hydroxy-2-heptylquinoline (HHQ), the overproduction of which generates the sheen characteristic of lasR mutant colonies [11]; its N-oxide HQNO, which is a redox-cycling agent [29]; and the Pseudomonas quinolone signal (PQS) [52]. Exposure to PQS was shown to modify P. aeruginosa responses to reactive oxygen species and ciprofloxacin [38], suggesting a functional linkage between HAQs, oxidative stress responses, and susceptibility to fluoroquinolones. Therefore, these quinolines may regulate metabolic properties in both source and neighboring cells, and temporal differences in their production resulting from LasR inactivation may contribute to the observed metabolic changes.

Numerous explanations have been offered for the identification of lasR mutant P. aeruginosa in diverse clinical and experimental conditions [10,11,12,53,54,55,56,57,58,59,60]. For example, in experimental growth medium in which P. aeruginosa growth requires the production of lasR-regulated protease, lasR mutants emerge that “cheat” from the protease produced by wild-type strains [59,60]. However, CF sputum is abundant in free amino acids [13] (upon which P. aeruginosa lasR mutants can grow without requiring protease [11,59]), and it has been shown that both laboratory strains [61] and clinical isolates [12] may produce protease in the absence of a functional las system. Furthermore, lasR mutants are frequently isolated from CF sputum without detectable wild-type co-isolates [2,11]. These findings suggest that cheating alone does not explain the high prevalence of lasR mutants among people with CF. Alternatively, it has been suggested that lasR mutants emerge due to physiological characteristics that confer relative fitness advantages in specific growth conditions [11,56]. The current results support the hypothesis that lasR mutant P. aeruginosa have a growth advantage in nutrient and antibiotic conditions found in the CF airway (as summarized in Fig. 6). While it is unclear which of these forces, antibiotics or nutrients, predominates in vivo in selecting for inactivating lasR mutations, their combination would be predicted to exert powerful pressure against LasR function.

The hypothesis that P. aeruginosa adaptation to the CF airway is driven in large part by metabolic forces found in CF airway secretions is supported by findings with other adapted mutants from chronic infections. For example, mucoid P. aeruginosa strains and isolates also exhibit upregulated NO\textsubscript{3}\textsuperscript{−} metabolism relative to non-mucoid P. aeruginosa and, as a result, are more susceptible than nonmucoid isolates to acidified NO\textsubscript{2}\textsuperscript{−} [27]. Furthermore, the mucoid phenotype is promoted by hypoxia [20]. Similarly, P. aeruginosa isolates with another CF adaptation, mutations that upregulate the glucose-6-phosphate dehydrogenase gene zsf, confer resistance to oxidative stress and paraquat [62,63]. The enrichment for lasR mutant P. aeruginosa, with growth advantages in CF airway conditions, by S. aureus is also reminiscent of the reverse interaction: the selection of S. aureus metabolic mutants, known as small-colony variants (SCVs), due to co-culture with wild-type P. aeruginosa [29]. S. aureus SCVs are defective for aerobic growth, are resistant to aminoglycoside antibiotics such as tobramycin, and frequently exhibit both increased expression of denitrification genes [64] and associated increased susceptibility to NO\textsubscript{2}\textsuperscript{−} [65], much like lasR mutant P. aeruginosa. The symmetry of this S. aureus-P. aeruginosa relationship, in each direction favoring the growth of antibiotic-resistant, metabolic mutants with decreased aerobic activity, further suggests a common mechanism for selection during chronic CF infections, and perhaps during many other chronic infections, driven by the metabolic forces present in host tissues. In support of this hypothesis, the likelihood of persistent, latent infection by the respiratory pathogen Mycobacterium tuberculosis is thought to be determined in large part by the lung metabolic milieu, particularly the relative ambient concentrations of nitrogen and oxygen species [66]. Similarly, the pathogenic fungus Cryptococcus neoformans exhibits early metabolic adaptations in animal models of chronic pulmonary infection, including altered responses to nitrosative stress and superoxide [67]. As with M. tuberculosis [68], these findings support the concept that chronic CF airway infections with P. aeruginosa could be amenable to therapies that increase airway nitrosative stress. Such therapies could include inhaled NO\textsubscript{2} [27] or L-arginine [19], two treatments already being examined as candidate CF treatments. Our results support the utility of these treatments both in preventing P. aeruginosa adaptive changes associated with advanced lung function decline [7,8] and that may be attributable to current antibiotic regimens (Fig. 6), as well as in treating patients with advanced infection in which these adaptations have already occurred.

In summary, the nutrient conditions characteristic of the CF airway select for growth of lasR mutant P. aeruginosa, resulting in decreased susceptibility to antibiotics without the need for antibiotic exposure. Adaptation of many microbes to new environments during chronic infections may commonly result in metabolic changes that impact response to antibiotics. This scenario may be particularly relevant for opportunistic pathogens such as P. aeruginosa, many of which naturally occupy competitive and nutrient-poor environmental niches like soil and water, as they adapt to the specific nutrient conditions found in host environments such as the nitrogen-rich CF airway.

Materials and Methods

Bacteria

Table 1 lists the bacterial strains and isolates used in this work, except for the strains carrying transposon insertion mutations in nitrate metabolic genes, which were obtained from the PA14 transposon insertion mutant library [69]. The origins of all strains and isolates are described in the references provided in Table 1, except for the narK1K2 and narK1K2lasR mutants, described below.

Mutant construction and plasmids

Each deletion in the lasR, narK1K2 and narK1K2lasR mutants was generated using allelic exchange with sacB-containing counterselectable gene replacement vectors using sucrose counterselection essentially as described [70]. Briefly, the lasR gene was entirely deleted from the chromosome except for the start and stop codon, using the plasmid sacB-based pEX18Gm for integration and excision. The narK1-narK2 genes, which are organized tandemly as an operon, were deleted as a one continuous stretch of DNA using identical methods, both in wild-type PA14 as well as the lasR mutant background. The deletion removed the narK coding sequence beginning from the 30\textsuperscript{th} codon of narK1 until the 462\textsuperscript{nd} codon of narK2, leaving the first 29 codons of narK1 and the last 7 codons of narK2 intact.

The plasmid pUCPSK-lasR was the kind gift of Eric Déziel and was used for complementation of lasR deficient strains and isolates as described [61].

Growth conditions and chemicals

Except where indicated, all cultures were inoculated from LB overnight cultures of bacteria or cells suspended from LB agar
cultures. Liquid static cultures were grown in LB with 400 μM KNO₃ (Sigma) except where indicated otherwise. Phosphate buffered LB agar was prepared as described [27], and consists of a phosphate buffer supplemented with a carbon source (glucose), nitrogen and sulfur sources [(NH₄)₂SO₄ and (NH₄)NO₃], amino acids, nucleic acid bases, and vitamins (thiamine, niacin, biotin, and pantothenic acid).

### Chemicals

Hemoglobin, hydroethidine, tobramycin, paraquat (methylviologen dichloride hydrate), potassium nitrate, and sodium nitrite were obtained from Sigma. NO donors DEANO (DEA-NON-Oate) and ProlinNO (Proli-NONOate) were purchased from AG Scientific (San Diego, CA) and SperNO was obtained from CalBiochem (San Diego, CA). Ciprofloxacin was from Biochem-Scientific (San Diego, CA) and SperNO was obtained from AG Scientific (San Diego, CA). Prepared antibiotic disks with tobramycin, kanamycin, gentamicin, carbenicillin, tetracycline, aztreonam, cefazidime, and polymyxin B were from Becton Dickinson. Growth media and agar were from Becton Dickinson & Co.

### Growth assays

Growth of cells in the indicated liquid media was measured optically using a BioScreen C Microbiology Microplate reader (Growth Curves USA, Piscataway, NJ) without shaking (except immediately prior to readings), a condition that limits oxygen mass-transfer. Assays to look for mutant sectors were performed by inoculating 10 μl drops of 1:10-diluted overnight cultures on LB with 400 μM KNO₃, followed by incubation at 37°C for 24 hours and then at room temperature for up to approximately 1 month thereafter.

### Table 1. List of strains used in the described experiments.

| Strain name | Description | References |
|-------------|-------------|------------|
| **P. aeruginosa** | Laboratory strain with phenotypic and genotypic features that resemble many clinical isolates | [11] |
| PA14        | Engineered mutant of PA14 with a gentamicin resistance cassette inserted into the lasR gene | [11] |
| PA14-lasR::Gm | Spontaneous lasR mutant of PA14 without an antibiotic resistance marker | [11] |
| PA14ΔlasR   | PA14 with an unmarked deletion in lasR | This study |
| AMT0023-30  | CF clinical isolate with wild-type lasR allele from a young patient (previously referred to as Patient 1 Early) | [2,11] |
| AMT0023-30-L1 | Spontaneous lasR mutant of AMT0023-30 (previously referred to as Patient 1 Late) | [11] |
| NC-AMT0101-3 | CF clinical isolate with wild-type lasR allele from a second young patient | [2,11] |
| NC-AMT0101-2 | CF clinical isolate collected from the same patient as NC-AMT0101-3 but 8-6 years later with wild-type lasR allele and mucoid phenotype | [2,11] |
| NC-AMT0101-1 | CF clinical isolate from the same culture as NC-AMT0101-2 with naturally-occurring mutant lasR allele, nonmucoid | [2,11] |
| PAO1        | Laboratory strain with an unmarked deletion in oxyR | [36] |
| oxyR        | PAO1 with unmarked deletion in oxyR | [36] |
| oxyRkatA    | Above oxyR mutant with katA::Gm | [36] |
| oxyRkatA ΔlasR | lasR mutant that emerged spontaneously during growth for 3 days on LB agar from oxyRkatA with the mutation G191V | This study |
| narK1narK2-ΔlasR | Strains with clean deletions in narK1 and narK2 with and without clean deletions in lasR | This study |

| **S. aureus** | Laboratory strain of S. aureus | [24] |
| hmp         | Derived mutant S. aureus Newman with the gene that encodes the NO⁺ detoxifying flavohemoprotein deleted | [24] |
| AMT0064-6   | Clinical CF isolate of S. aureus | [29] |

### Assays for denitrification activity and NO₂⁻ susceptibility

NO⁻ was quantified using an ISO-NOPMC Mark II electrode (WPI Instruments, Fl) and dissolved oxygen was measured in parallel using a Clark-type electrode MLT1120 (ADI Instruments) with standard curves as per manufacturer instruction. Data from both probes were analyzed through an Analog Adapter MLT1122 (ADI Instruments). NO₂⁻ disk diffusion on acidified, buffered LB agar was performed as described [27], except that all incubations were performed with aerobic growth.

### Assay for oxygen utilization

Respiration rates in liquid cultures were measured by resuspending PBS-washed cells in prewarmed LB with 400 μM KNO₃ in a microrespiration system (Unisense AS, Denmark). Calibrations were performed according to manufacturer’s instructions using air-purged and argon-purged growth medium.

### Hydroethidine assay

Fluorescence after hydroethidine addition to lawns of cells during growth on LB agar (similar results were obtained with and without added NO₃⁻) was measured using excitation/emission wavelengths of 396/570 nm [32], followed by photography and quantitation using NIH ImageJ software (NIH, Bethesda, Md, http://rsb.info.nih.gov/ij/).

### Agar growth assay

Agar-suspended cultures were grown in 0.9% LB agar inoculated with equal cell numbers of all cell types- approximately 10⁵ CFU of the indicated strains (resulting in a final cell density of approximately 2×10⁵ CFU/mL), except when indicated otherwise.
Nitrogen metabolic assays

NO\textsubscript{2} production was measured using the Griess Reagent System kit (Promega, Madison WI). Nitrate was quantitated enzymatically using a commercially available reagent set (R-Biopharm, Marshall, MI). Rates of NO\textsubscript{2} degradation were determined as previously described [24]; briefly, five milliliters cultures in PY medium were grown by shaking at 37°C to an OD\textsubscript{660}=0.4. Cells were then resuspended to 1×10\textsuperscript{8} cells ml\textsuperscript{-1} in 8 ml final volume. A two-hole rubber stopper sealed with Parafilm enclosed the cell suspension in an 8 ml glass vial with no gaseous headspace. Cells were stirred vigorously at 37°C as ProlineNO was added through one open port to 1 headspace. Cells were stirred vigorously at 37°C to establish the metabolic state of the incubation before addition of test compounds. After incubation, the plunger of the syringe was depressed slowly, ejecting a cylinder of culture. Serial, 1.5 mm slices of culture were removed and added to 1 ml each of sterile PBS, and vortexed for 30 seconds before enumeration of cells from the resulting solution by plating.

Oxygen metabolism in deep-agar cultures

Deep-agar cultures inoculated with serial dilutions of P. aeruginosa las\textsuperscript{R} and wild-type cells in LB-0.9% agar with and without 400 μM KNO\textsubscript{3} and with and without pararquat were grown overnight at 37°C. Oxygen concentrations were subsequently recorded using a microsensor setup (Ox 10 oxygen microsensor, PA 2000 picocoumeter, both from Unisense AS, Denmark) at 37°C in a pre-conditioned water bath. Data were recorded using SensorTrace Basic software (Unisense). The probe was advanced into the agar, and measurements taken, in 50 μm increments.

Statistics

Differences between experimental measurements were computed using unpaired, two-tailed Student’s t-tests.

Supporting Information

Figure S1 las\textsuperscript{R} inactivating mutation decreases susceptibility to pararquat. Experiment performed as described in Fig. 4C, except with the indicated strains. Results shown are averages ±s.d. for three replicates and are representative of two separate experiments. Found at: doi:10.1371/journal.ppat.1000712.s001 (0.13 MB TIF)

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Author Contributions

Conceived and designed the experiments: LRH WMH DJH. Performed the experiments: LRH ARR HDK MJ B DJH FCF SIM. Analyzed the data: LRH ARR ISH WMH MK DJH. Contributed reagents/materials/analysis tools: LRH ARR HDK WMH MK DJH. Performed the experiments: LRH ARR HDK MK DJH. Wrote the paper: LRH ARR HDK DJH MJ B DJH FCF SIM.

References

1. Giannakis M, Chen SL, Karam SM, Engrand L, Gordon JI (2008) Helicobacter pylori evolution during progression from chronic atrophic gastritis to gastric cancer and its impact on gastric stem cells. Proc Natl Acad Sci U S A 105: 4358–4363.
2. Smith EE, Buckley DG, Wu Z, Saezphimrakhan C, Hoffman LR, et al. (2006) Genetic adaptation by Pseudomonas aeruginosa to the airways of cystic fibrosis patients. Proc Natl Acad Sci U S A 103: 8407–8492.
3. Gowan JR, Deriche V (1996) Microbial pathogenesis in cystic fibrosis: mucoid Pseudomonas aeruginosa and Burkholderia cepacia. Microbiol Rev 60: 539–574.
4. Mahenthiralingam E, Campbell ME, Speert DP (1994) Nonmotility and virulence and antimicrobial resistance. J Bacteriol 187: 4908–4920.
5. Ernst RK, Adams KN, Moskowitz SM, Kraig GM, Kawasaki K, et al. (2006) The Pseudomonas aeruginosa lipid A deacetylase: selection for expression and loss within the cystic fibrosis airway. J Bacteriol 188: 191–201.
6. Burns JL, Van Dalen JM, Shawar RM, Otto KL, Garber RL, et al. (1999) Effect of chronic intermittent administration of inhaled tobramycin on respiratory microbial flora in patients with cystic fibrosis. J Infect Dis 179: 1190–1196.
7. Li Z, Kosorok MR, Farrell PM, Laxova A, West SE, et al. (2005) Longitudinal development of mucoid Pseudomonas aeruginosa infection and lung disease progression in children with cystic fibrosis. JAMA 293: 581–589.
8. Hoffman LR, Kulaksara HD, Emerson J, Houston LS, Burns JL, et al. (2009) Pseudomonas aeruginosa las\textsuperscript{R} mutants are associated with cystic fibrosis lung disease progression. J Cyst Fibros 8: 66–70.
9. Gibson RL, Emerson J, Mayer-Hamblett N, Burns JL, McNamara S, et al. (2007) Duration of treatment effect after tobramycin solution for inhalation in young children with cystic fibrosis. Pediatr Pulmonol 42: 610–623.
10. Sahukde P, Smart CH, Morgan JA, Panagia S, Walshaw MJ, et al. (2005) A cystic fibrosis epidemic strain of Pseudomonas aeruginosa displays enhanced virulence and antimicrobial resistance. J Bacteriol 187: 4908–4920.
11. D’Argenio DA, Wu M, Hoffman LR, Kulaksara HD, Deziel E, et al. (2007) Growth phenotypes of Pseudomonas aeruginosa lasR mutants adapted to the airways of cystic fibrosis patients. Mol Microbiol 64: 512–533.
12. Tingey P, Smith L, Rose B, Zhu H, Goubear T, et al. (2007) Phenotypic characterization of clonal and non-clonal Pseudomonas aeruginosa strains isolated from lungs of adults with cystic fibrosis. J Clin Microbiol 45: 1697–1704.
13. Barth AL, Pitt TL (1996) The high amino-acid content of sputum from cystic fibrosis patients promotes growth of auxotrophic Pseudomonas aeruginosa. J Med Microbiol 45: 110–119.
14. Wagner VE, Bushnell D, Passador I, Brooks AI, Igleswki BH (2003) Microarray analysis of Pseudomonas aeruginosa quorum-sensing regulons: effects of growth phase and environment. J Bacteriol 185: 2090–2095.
15. Toyofuku M, Nomura N, Fuji T, Takaya N, Maseda H, et al. (2007) Quorum sensing regulates denitrification in Pseudomonas aeruginosa PA01. J Bacteriol 189: 4969–4972.
16. Yoon SS, Hennigan RF, Hilliard GM, Ochiner UA, Parvizyak K, et al. (2002) Pseudomonas aeruginosa anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. Dev Cell 3: 593–603.
17. Palmer KL, Brown SA, Whiteley M (2007) Membrane-bound nitrate reductase is required for anaerobic growth in cystic fibrosis sputum. J Bacteriol 189: 4449–4455.
18. Grasemann H, Ioannidou I, Tomkiewicz RP, de Groot H, Rubik BK, et al. (1998) Nitric oxide metabolites in cystic fibrosis lung disease. Arch Dis Child 78: 49–53.
19. Grasemann H, Kurtz F, Katjen F (2006) Inhaled L-arginine improves nitric oxide and pulmonary function in patients with cystic fibrosis. Am J Respir Crit Care Med 174: 208–212.
20. Worrallsch T, Tarran R, Uhrich M, Schwab U, Ceci M, et al. (2002) Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients. J Clin Invest 109: 317–325.
21. Chen F, Xia Q, Ju LK (2006) Competition between oxygen and nitrate respirations in continuous culture of Pseudomonas aeruginosa performing aerobic denitrification. Biotechnol Bioeng 93: 1069–1078.
22. Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collin JJ (2007) A common mechanism of cellular death induced by bactericidal antibiotics. Cell 130: 797–810.
23. Bryan LE (1985) Antibiotic uptake and the cytoplasmic membrane. Annu Rev Microbiol 39: 105–110.
24. Richardson AR, Dunman PM, Fang FC (2006) The nitrosative stress response of Staphylococcus aureus is required for resistance to innate immunity. Mol Microbiol 61: 927–939.

25. Aratı H, Hayashi M, Kuri A, Ishii M, Igarashi Y (2005) Transcriptional regulation of the flavohemoglobin gene for aerobic nitric oxide detoxification by the second nitric oxide-responsive regulator of Pseudomonas aeruginosa. J Bacteriol 187: 3960–3968.

26. Richardson AR, Libby SJ, Fang FC (2006) A nitric oxide-inducible lactate dehydrogenase enables Staphylococcus aureus to resist innate immunity. Science 319: 1672–1676.

27. Yoon SS, Coakley R, Lau GW, Lymar SV, Gaston B, et al. (2006) Anaerobic infections with cystic fibrosis. Am J Respir Crit Care Med 177: 995–1001.

28. Hassett DJ, Alsabbagh E, Parvatiyar K, Howell ML, Wilmott RW, et al. (2000) Haussler S, Becker T (2008) The pseudomonas quinolone signal (PQS) balances phase is essential for aerobic survival of a protease-resistant catalase, KatA, released upon cell lysis during stationary phase is required for resistance to innate immunity. J Infect Dis 187: 1699–1705.

29. Hassett DJ, Schweizer HP, Ohman DE (1995) Response of Pseudomonas aeruginosa to pyocyanin: mechanisms of resistance, antioxidant defenses, and detection of a manganese-cofactored superoxide dismutase. Antimicrob Agents Chemother 39: 871–879.

30. Schuster M, Lostroh CP, Ogi T, Greenberg EP (2003) Identification, timing, and reversibility of Pseudomonas aeruginosa quorum-controlled genes: a transcriptome analysis. J Bacteriol 185: 2066–2079.

31. Hassett DJ, Charniga L, Bean K, Ohman DE (1992) Response of Pseudomonas aeruginosa to pyocyanin: a manganese-cofactored superoxide dismutase. Infect Immun 60: 328–336.

32. Yoon SS, Coakley R, Lau GW, Lymar SV, Gaston B, et al. (2006) Anaerobic Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. Am J Respir Crit Care Med 168: 1380–1384.

33. Hu G, Cheng PY, Sham A, Perfect JR, Kronstad JW (2008) Metabolic respiratory strain. Proc Natl Acad Sci U S A 103: 19890–19895.

34. Hassett DJ, Charniga L, Bean K, Ohman DE (1992) Response of Pseudomonas aeruginosa to pyocyanin: a manganese-cofactored superoxide dismutase. Infect Immun 60: 328–336.

35. Hassett DJ, Schweizer HP, Ohman DE (1995) Response of Pseudomonas aeruginosa to pyocyanin: mechanisms of resistance, antioxidant defenses, and demonstration of a manganese-cofactored superoxide dismutase. Infect Immun 60: 328–336.

36. Hassett DJ, Alabagh E, Parvatiyar K, Howell ML, Wilmott RW, et al. (2000) A protease-resistant catalase, KatA, released upon cell lysis during stationary phase is essential for aerobic survival of a Pseudomonas aeruginosa mutant at low cell densities. J Bacteriol 182: 4557–4563.

37. Wang X, Zhao X (2009) Contribution of oxidative damage to antimicrobial lethality. Antimicrob Agents Chemother 53: 1395–1402.

38. Haussler S, Becker T (2000) The pseudomonas quinolone signal (PQS) balances life and death in Pseudomonas aeruginosa populations. PLoS Pathog 4: e1000166. doi:10.1371/journal.ppat.1000166.

39. Abbasia I, Becerra MG, Battan PC, Paez PL (2004) Oxidative stress involved in the antibacterial action of different antibiotics. Biochem Biophys Res Commun 317: 605–609.

40. O'Regan E, Quinn T, Pages JM, McCusker M, Piddock L, et al. (2009) Multiple mutations in menD induce small-colony-variant phenotype. J Bacteriol 188: 687–693.

41. Schuster M, Lostroh CP, Ogi T, Greenberg EP (2003) Identification, timing, and reversibility of Pseudomonas aeruginosa quorum-controlled genes: a transcriptome analysis. J Bacteriol 185: 2066–2079.

42. Haussler S, Becker T (2008) The pseudomonas quinolone signal (PQS) balances phase is essential for aerobic survival of a protease-resistant catalase, KatA, released upon cell lysis during stationary phase is required for resistance to innate immunity. J Infect Dis 187: 1699–1705.

43. Yoon SS, Coakley R, Lau GW, Lymar SV, Gaston B, et al. (2006) Anaerobic infections with cystic fibrosis. Am J Respir Crit Care Med 177: 995–1001.

44. Hassett DJ, Alabagh E, Parvatiyar K, Howell ML, Wilmott RW, et al. (2000) A protease-resistant catalase, KatA, released upon cell lysis during stationary phase is essential for aerobic survival of a Pseudomonas aeruginosa mutant at low cell densities. J Bacteriol 182: 4557–4563.

45. Wang X, Zhao X (2009) Contribution of oxidative damage to antimicrobial lethality. Antimicrob Agents Chemother 53: 1395–1402.

46. Hasselt DJ, Charniga L, Bean K, Ohman DE, Cohen MS (1992) Response of Pseudomonas aeruginosa to pyocyanin: mechanisms of resistance, antioxidant defenses, and detection of a manganese-cofactored superoxide dismutase. Infect Immun 60: 328–336.

47. Diggle SP, Griffin AS, Campbell GS, West SA (2007) Cooperation and conflict in quorum-sensing bacterial populations. Nature 450: 411–414.

48. Kabrol S, Oliver A, Pier GB, Andremont A, Ruym R (2003) Transcription of quorum-sensing system genes in clinical and environmental isolates of Pseudomonas aeruginosa. J Bacteriol 185: 7222–7230.

49. Sandou KM, Mizunberg SM, Schuster M (2007) Social cheating in Pseudomonas aeruginosa quorum sensing. Proc Natl Acad Sci U S A 104: 15876–15881.

50. Kohler C, von Eiff C, Liebeke M, McNamara PJ, Lalk M, et al. (2008) A defect in menadione biosynthesis induces global changes in gene expression in Pseudomonas aeruginosa. J Bacteriol 190: 6351–6364.

51. von Eiff C, McNamara P, Becker K, Bates D, Lei XH, et al. (2006) Phenotype microarray profiling of Salmonella enterica serovar Typhimurium strains with the small-colony-variant phenotype. J Bacteriol 188: 675–693.

52. Boshoff HI, Barry CE, 3rd (2005) Tuberculosis - metabolism and respiration in Escherichia coli. Int J Med Microbiol 296: 735–751.

53. Ruym R, Kabrol S, Oliver A, Pier GB, Andremont A, Ruym R (2003) Transcription of quorum-sensing system genes in clinical and environmental isolates of Pseudomonas aeruginosa. J Bacteriol 185: 7222–7230.

54. Kabrol S, Oliver A, Pier GB, Andremont A, Ruym R (2003) Transcription of quorum-sensing system genes in clinical and environmental isolates of Pseudomonas aeruginosa. J Bacteriol 185: 7222–7230.

55. Sandou KM, Mizunberg SM, Schuster M (2007) Social cheating in Pseudomonas aeruginosa quorum sensing. Proc Natl Acad Sci U S A 104: 15876–15881.

56. Kohler C, von Eiff C, Liebeke M, McNamara PJ, Lalk M, et al. (2008) A defect in menadione biosynthesis induces global changes in gene expression in Pseudomonas aeruginosa. J Bacteriol 190: 6351–6364.

57. von Eiff C, McNamara P, Becker K, Bates D, Lei XH, et al. (2006) Phenotype microarray profiling of Salmonella enterica serovar Typhimurium strains with the small-colony-variant phenotype. J Bacteriol 188: 675–693.

58. Boshoff HI, Barry CE, 3rd (2005) Tuberculosis - metabolism and respiration in Escherichia coli. Int J Med Microbiol 296: 735–751.

59. Ruym R, Kabrol S, Oliver A, Pier GB, Andremont A, Ruym R (2003) Transcription of quorum-sensing system genes in clinical and environmental isolates of Pseudomonas aeruginosa. J Bacteriol 185: 7222–7230.

60. Sandou KM, Mizunberg SM, Schuster M (2007) Social cheating in Pseudomonas aeruginosa quorum sensing. Proc Natl Acad Sci U S A 104: 15876–15881.

61. Kohler C, von Eiff C, Liebeke M, McNamara PJ, Lalk M, et al. (2008) A defect in menadione biosynthesis induces global changes in gene expression in Pseudomonas aeruginosa. J Bacteriol 190: 6351–6364.

62. von Eiff C, McNamara P, Becker K, Bates D, Lei XH, et al. (2006) Phenotype microarray profiling of Salmonella enterica serovar Typhimurium strains with the small-colony-variant phenotype. J Bacteriol 188: 675–693.

63. Boshoff HI, Barry CE, 3rd (2005) Tuberculosis - metabolism and respiration in Escherichia coli. Int J Med Microbiol 296: 735–751.

64. Ruym R, Kabrol S, Oliver A, Pier GB, Andremont A, Ruym R (2003) Transcription of quorum-sensing system genes in clinical and environmental isolates of Pseudomonas aeruginosa. J Bacteriol 185: 7222–7230.