# *Pseudomonas aeruginosa lasR* mutant fitness in microoxia is supported by an Anr-regulated oxygen-binding hemerythrin

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***Pseudomonas aeruginosa*** **strains with loss-of-function mutations in the transcription factor LasR are frequently encountered in the clinic and the environment. Among the characteristics common to LasR-defective (LasR−) strains is increased activity of the transcription factor Anr, relative to their LasR+ counterparts, in low-oxygen conditions. One of the Anr-regulated genes found to be highly induced in LasR− strains was *PA14_42860* (*PA1673*), which we named *mhr* for microoxic hemerythrin. Purified *P. aeruginosa* Mhr protein contained the predicted di-iron center and bound molecular oxygen with an apparent $K_d$ of ∼1 μM. Both Anr and Mhr were necessary for fitness in *lasR*+ and *lasR* mutant strains in colony biofilms grown in microoxic conditions, and the effects were more striking in the *lasR* mutant. Among genes in the Anr regulon, *mhr* was most closely coregulated with the Anr-controlled high-affinity cytochrome *c* oxidase genes. In the absence of high-affinity cytochrome *c* oxidases, deletion of *mhr* no longer caused a fitness disadvantage, suggesting that Mhr works in concert with microoxic respiration. We demonstrate that Anr and Mhr contribute to LasR− strain fitness even in biofilms grown in normoxic conditions. Furthermore, metabolomics data indicate that, in a *lasR* mutant, expression of Anr-regulated *mhr* leads to differences in metabolism in cells grown on lysogeny broth or artificial sputum medium. We propose that increased Anr activity leads to higher levels of the oxygen-binding protein Mhr, which confers an advantage to *lasR* mutants in microoxic conditions.**

*Pseudomonas aeruginosa* | hemerythrin | microoxic growth | *lasR* | Anr

*Pseudomonas aeruginosa* is a devastating pathogen for healthcare systems worldwide and causes opportunistic infections at multiple body sites that are extremely difficult to treat. *P. aeruginosa* is especially damaging to the lungs of individuals with the genetic disease cystic fibrosis (CF), where it establishes chronic infections of the airway and is a major predictor of morbidity and mortality (1, 2). The success of *P. aeruginosa* in disease is due to a confluence of factors, including intrinsic and acquired antibiotic resistance (3), production of a battery of secreted, virulence-associated molecules (4), the ability to form antibiotic- and immune cell-recalcitrant biofilms on biotic surfaces and implanted devices (5), and versatile metabolic capabilities (6, 7), such as a pronounced ability to grow in the hypoxic or anoxic conditions engendered by biofilms and chronic infections (8, 9).

*P. aeruginosa* utilizes quorum sensing (QS) to coordinate the expression of a broad set of genes involved in virulence and nutrient acquisition (10). The gene that encodes one of the key transcriptional regulators involved in QS, *lasR*, frequently sustains loss-of-function mutations. LasR-defective (LasR−) strains of *P. aeruginosa* are commonly isolated from the lungs of individuals with CF (11–13) and other pulmonary diseases (14, 15), from implanted device infections (5, 16), from acute corneal ulcers (17), and from the environment (17).

Several factors may drive or contribute to the selection for *P. aeruginosa lasR* mutants and their observed fitness relative to their wild-type counterparts including the advantages of social cheating (18, 19) and enhanced growth on specific carbon and nitrogen sources (10). In addition, both laboratory strains and clinical isolates with mutations in *lasR* display higher expression of the Anr regulon in microoxic environments than their *lasR*-intact counterparts (17, 20). Anr and its homologs have been well-characterized as oxygen-sensitive transcription factors (21–23). The *P. aeruginosa* Anr regulon includes genes encoding enzymes involved in microoxic and anoxic respiration, fermentation, microoxic ethanol oxidation, CupA fimbriae synthesis, and a number of hypothetical proteins including a putative hemerythrin PA14_42860 (PA1673) (7, 24–26).

Hemerythrins are typified by an antiparallel four-helix bundle with conserved histidine, glutamate, and aspartate residues in an H–HxxxE–HxxxH–HxxxxD motif that forms a di-iron active site (27, 28). These proteins were first described to bind and transport oxygen in the body fluids and tissues of invertebrate worms and leeches (29, 30). More recent genomic analyses show that hemerythrins are present in all domains of life (28, 31), although roles for bacterial hemerythrins are much less well understood than those of their eukaryotic homologs (31). A hemerythrin in

## Significance

***Pseudomonas aeruginosa*, a versatile bacterium that lives in environmental habitats and causes life-threatening opportunistic infections, uses quorum sensing to coordinate gene expression. The *lasR* gene, which encodes a quorum-sensing regulator, is often found to have loss-of-function mutations in clinical isolates. Interestingly, LasR− strains have high activity of the oxygen-sensitive transcription factor Anr in microoxic conditions. This report identifies and characterizes an Anr-regulated microoxic hemerythrin that binds oxygen. We show that both *anr* and *mhr* are critical to fitness in microoxia and that these genes uniquely benefit LasR− strains in biofilms grown in normoxia. Our data suggest that the success of *P. aeruginosa* in chronic infections is in part through its propensity to lose LasR functionality resulting in increased microoxic fitness.**

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*Campylobacter jejuni* protects oxygen-sensitive iron-sulfur cluster enzymes from oxidative damage as the cells experience fluctuations in oxygen concentrations (32). In *Methylococcus capsulatus*, a single-domain hemerythrin improves the activity of an inner-membrane methane monooxygenase (33). These previous studies are consistent with hemerythrin playing a role in regulating interactions between cellular enzymes and oxygen through binding oxygen or participating in its transport and delivery in support of oxic metabolism.

In this work, we demonstrate that in colony biofilms with the wild type, Δ*lasR* mutant fitness is dependent on Anr and the Anr-regulated $O_2$-binding hemerythrin Mhr. Mhr is encoded by *PA14_42860* (*PA1673*), and *mhr* transcript and protein levels were higher upon loss of LasR function in both laboratory strains and clinical isolates. Mhr was specifically important for microoxic fitness in the wild type and conferred a fitness advantage to the Δ*lasR* mutant in colony biofilms under both microoxic and normoxic conditions. The fitness benefit of Mhr microoxic fitness phenotype was dependent on the presence of the high-affinity cytochrome *c* oxidases, and metabolomics data support the model that Mhr influenced central metabolism as well as other cellular processes. The frequent natural occurrence of *lasR* mutants may be due in part to the microoxic fitness benefits of higher levels of Anr-regulated Mhr.

## Results

**Anr Contributes to Δ*lasR* Mutant Fitness in Microoxic Colony Biofilms.** We previously reported that Δ*lasR* mutants in laboratory strains PA14 and PAO1 as well as in diverse clinical isolates had higher activity of the transcription factor Anr than their LasR+ counterparts under microoxic conditions (17, 20). To determine if Anr activity contributes to the higher fitness of *lasR* mutant strains, we developed an assay for the assessment of fitness in colony biofilms grown under microoxic conditions (0.2%; 2.5 μM $O_2$). In competitive fitness assays, wherein different strains were competed against a PA14 strain tagged with a constitutively expressed *lacZ* gene, the two strains had access to a common pool of secreted products including enzymes (e.g., LasA and LasB proteases) and small molecules (such as redox-active phenazines), despite differences in exoproduct production between strains (Fig. 1*A* for experimental design). Using this assay, we observed that PA14 Δ*lasR* was more fit than the PA14 wild type under microoxic conditions (Fig. 1*B*). The increased fitness of Δ*lasR* could be complemented by restoring *lasR* to its native locus (Fig. 1*C*). In both PA14 and the PA14 Δ*lasR* derivative, deletion of *anr* led to a loss of fitness that was complemented by restoration of *anr* (Fig. 1*B*). The presence of *anr* was essential to the microoxic fitness advantage that the Δ*lasR* mutant had over the wild type (Fig. 1*B*).

**Anr Regulates a Putative Hemerythrin in *P. aeruginosa*.** The transcription factor Anr regulates dozens of genes involved in denitrification, fermentation, and microoxic metabolism. Multiple transcriptome studies comparing *P. aeruginosa* strains to Δ*anr* derivatives in laboratory strain and CF clinical isolate backgrounds revealed that expression levels of the uncharacterized gene *PA14_42860* (*PA1673* in strain PAO1) are severely reduced in the absence of Anr under microoxic and anoxic conditions (8, 20). Furthermore, analysis of pairs of genetically related isolates containing a *lasR* mutant and a *lasR*+ counterpart found this gene, named here *mhr* for microoxic hemerythrin, to be one of the most up-regulated genes in *lasR* mutants (20). An Anr consensus-binding motif (TTGATCGGCGTCAA) was found 81 bp from the translational start of Mhr (34). To test whether Anr was a positive regulator of *mhr*, we constructed a *mhr* promoter fusion to *lacZ* and integrated it at a neutral site on the chromosome in the wild type and Δ*anr* backgrounds. Under microoxic conditions, *mhr* promoter activity was 64-fold higher in the wild type than in the Δ*anr* mutant. Mutation of two nucleotides in the Anr consensus

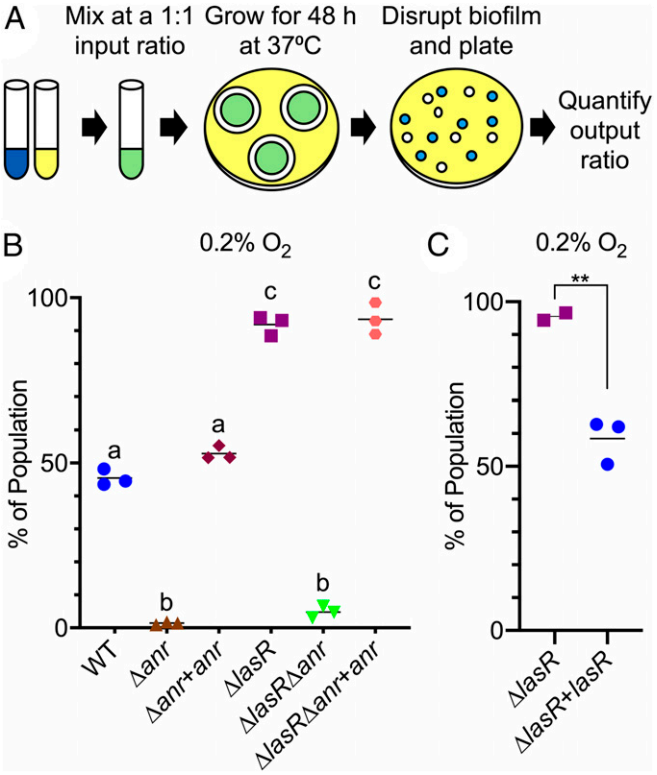


**Fig. 1.** Increased fitness of the Δ*lasR* mutant in microoxic conditions is dependent on *anr*. (*A*) Assay scheme for the competition of *P. aeruginosa* strains (yellow culture) against a *P. aeruginosa* strain tagged with a constitutively expressed *lacZ* (blue culture). (*B*) Fitness of wild type (WT), Δ*anr*, Δ*anr* + *anr*, Δ*lasR*, Δ*lasR*Δ*anr*, and Δ*lasR*Δ*anr* + *anr* relative to the *lacZ*-labeled WT on tryptone agar at 0.2% $O_2$ at 37 °C for 48 h. Using a one-way ANOVA with multiple comparisons test, a–b, a–c, and b–c are significantly different; $P < 0.0001$. (*C*) Relative fitness of Δ*lasR* and Δ*lasR* + *lasR* using the assay conditions described for assays in *B*. $^{**}P < 0.01$ by unpaired *t* test.

motif that are essential for Anr regulation at other promoters (35) led to a large and significant reduction in β-galactosidase production (Fig. 2*A*). Consistent with previous reports that *mhr* transcripts were more abundant in *lasR* loss-of-function mutants compared to their *lasR*-intact counterparts, *mhr* promoter activity was 1.9-fold higher in the Δ*lasR* mutant compared to the wild type (Fig. 2*B*). As in the wild type, promoter activity was Anr-dependent in the Δ*lasR* mutant (Fig. 2*B*).

To determine if Mhr protein levels were also higher in LasR− strains, we expressed and purified *P. aeruginosa* Mhr from *Escherichia coli* (*SI Appendix*, Fig. S1) and used it to raise an anti-Mhr polyclonal antibody. Western blot analysis detected purified Mhr at about 15 kDa, similar to its predicted molecular weight of 17.9 kDa, and a similar band was detected in *P. aeruginosa* whole-cell lysates from the wild type but not in the Δ*anr* mutants (Fig. 2*C*). Mhr protein levels in the Δ*lasR* strain were also dependent on *anr*. We quantified the relative amounts of Mhr using the Licor Odyssey near-infrared Western blotting system. The Mhr protein levels were, on average, 2.8-fold higher in the Δ*lasR* mutant than in the wild type (Fig. 2*D*). We also analyzed Mhr levels in a clinical isolate from a corneal eye infection (262 K) that was a natural *lasR* mutant (17) and a 262-K derivative in which the native allele was replaced with the PA14 allele, as well as two clinical isolates from the same subject with CF with one isolate bearing a *lasR* mutation. We found that, in the two clinical isolate backgrounds, the *lasR* mutant in each pair had significantly more Mhr than the corresponding LasR+ strain (Fig. 2 *E* and *F*).

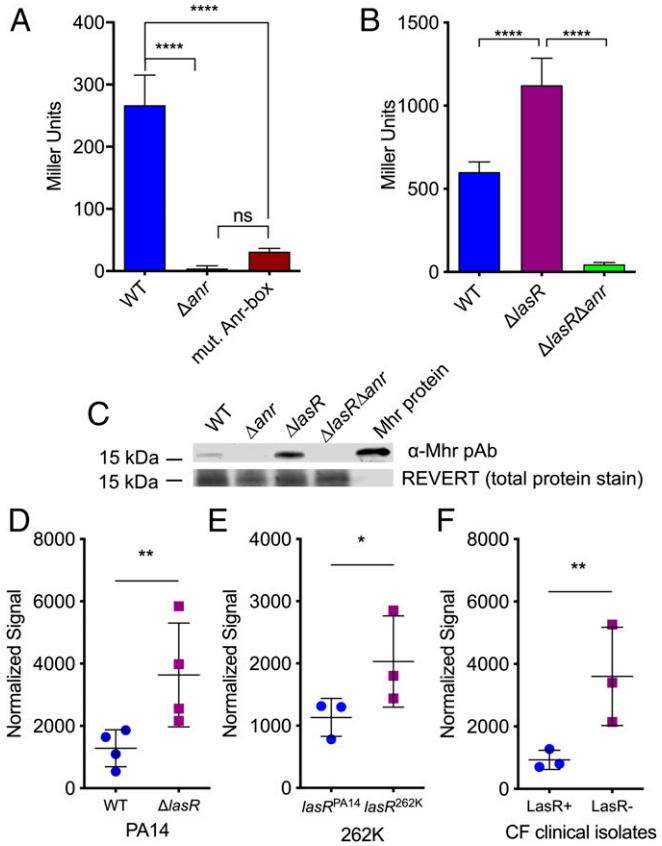


**Fig. 2.** *mhr* transcription and Mhr protein levels are Anr-regulated and higher in the *lasR* mutant strains. (*A* and *B*) β-Galactosidase activity in strains bearing an *mhr-lacZ* promoter fusion. ****$P < 0.0001$ by one-way ANOVA, multiple comparisons test; ns, not significant. (*A*) PA14 wild-type (WT), Δ*anr*, and WT bearing a promoter fusion variant in which the Anr-box was mutated as described in *Materials and Methods* (mut. Anr-box). (*B*) β-Galactosidase activity in WT, Δ*lasR*, and Δ*lasR*Δ*anr* containing the *mhr-lacZ* promoter fusion. (*C*) Western blot using a rabbit α-Mhr polyclonal antibody. Lanes from left to right: WT, Δ*anr*, Δ*lasR*, Δ*lasR*Δ*anr*, and 12.5 ng Mhr that was purified from *E. coli*. Total protein stained with REVERT, used for normalization, is shown. (*D*–*F*) Quantification of Mhr by Western blot for pairs of *lasR* mutant and *lasR*+ strains, normalized to total protein using the REVERT stain (Licor). Each point within a group represents band intensity data from a separate experiment on a different day. *$P < 0.05$, **$P < 0.01$ by ratio paired *t* test. (*D*) Mhr levels in PA14 WT and Δ*lasR*. (*E*) Mhr levels in the natively LasR– keratitis isolate 262K and a derivative in which its *lasR* allele was replaced with that from strain PA14. (*F*) Mhr levels for a LasR+ chronic CF isolate and its genetically related LasR– partner recovered from the same patient. Cells were grown as colony biofilms in 0.2% oxygen for 16 h on tryptone agar for all experiments in this figure.

**Mhr Is a Bacteriohemerythrin That Binds Oxygen Via a Di-Iron Center.** The Mhr sequence contains the conserved di-iron–binding motif H–HxxxE–HxxxH–HxxxxD (27, 28). When the *P. aeruginosa* Mhr sequence was threaded onto the crystal structure of *Methylococcus capsulatus* hemerythrin (Hr), all seven of the residues in the conserved motif were predicted to form the metal-binding active site (Fig. 3*A*) (36). In addition to its presence in other *Pseudomonas* spp., the closest Mhr homologs by sequence were found in other gamma proteobacteria (31) (*Stenotrophomonas maltophilia*, *Xanthomonas campestris*, *Acinetobacter baumannii*, *Dyella* spp.) and the nitrogen-fixing plant symbiont *Azotobacter chroococcum*, and the active-site residues are conserved across all of these homologous proteins (37) (*SI Appendix*, Fig. S2). Consistent with presence of the di-iron–binding motif, we detected iron in Mhr protein with a stoichiometry of two metal ions per protein.

*P. aeruginosa* Mhr purified from *E. coli* had an absorbance spectrum with maxima at 328 and 371 nm, which is similar to the absorbance spectra of other hemerythrin proteins (38). As in other hemerythrins, there were spectroscopic differences between the oxygen-bound (oxy-Mhr) and oxygen-free (deoxy-Mhr) species (Fig. 3*B* and *SI Appendix*, Fig. S3*A*). Analysis of spectroscopic changes upon gradual addition of an oxygen-containing solution yielded an apparent $K_d$ value of 0.74 μM (Fig. 3*C* and *SI Appendix*, Fig. S3*B*). Thus, Mhr was shown to bind iron and oxygen with an affinity that is relevant to microoxic conditions.

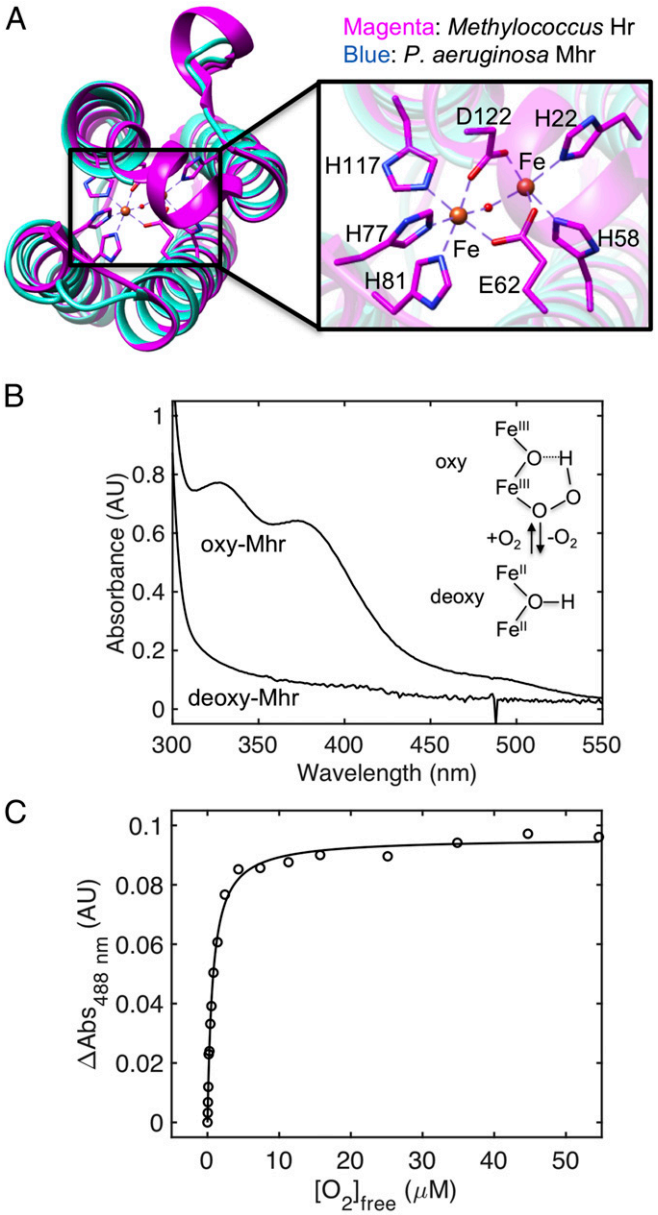


**Fig. 3.** Mhr is an oxygen-binding hemerythrin. (*A*) Structural model of *P. aeruginosa* Mhr based on the crystal structure for *M. capsulatus* hemerythrin protein (*Methylococcus* Hr) (Protein Data Bank ID 4XPX) (36). The region around the di-iron center was zoomed in to display the key oxygen-binding residues. (*B*) Electronic absorption spectra of deoxy-Mhr and oxy-Mhr in a 20-mM Tris·HCl buffer at pH 8. The broad peak around 488 nm is characteristic of the oxy-Mhr form. (*C*) Plot of the absorbance changes versus the concentration of free (unbound) oxygen. Fits of the absorbance changes versus the concentration of total oxygen in *SI Appendix*, Fig. S3, have yielded an apparent $K_d$ value of 0.74 μM.

**Mhr Plays a Role in Fitness in Microoxic Conditions but Not in Anoxic Conditions.** To test whether Mhr was important for growth under microoxic conditions, we constructed Δ*lasR*Δ*mhr* and Δ*mhr* strains and performed competition assays at 0.2% oxygen. Both the Δ*mhr* and the Δ*lasR*Δ*mhr* mutant were less fit than their parental strains under microoxic conditions (Fig. 4*A*). We were able to complement the fitness phenotypes of the Δ*mhr* strains by expressing *mhr* using the arabinose-inducible plasmid pMQ70 (Fig. 4 *B* and *C*). Under anoxic conditions in which nitrate is used an alternative electron acceptor the Δ*mhr* mutant had no fitness defect, suggesting that Mhr does not play a physiological role in the absence of oxygen (*SI Appendix*, Fig. S4).

**Mhr Plays a Role in Δ*lasR* Mutant Fitness in Normoxic Conditions.** Similar to its fitness phenotype under microoxic conditions, the Δ*lasR* mutant was more fit than the wild type in our colony biofilm competition assay under normoxic conditions (21% oxygen) (Fig. 4*D*). Deleting *anr* in the wild type did not affect fitness under normoxia, but it did result in a fitness defect in the Δ*lasR* background (Fig. 4*D*). Similarly, the Δ*mhr* mutant was as fit as the wild type under normoxic conditions (Fig. 4*E*), but loss of *mhr* led to reduced fitness in the Δ*lasR* mutant background. Together, these data suggest that Anr, in part due to its regulation of Mhr, contributes to Δ*lasR* fitness even in colony biofilms grown in normoxia.

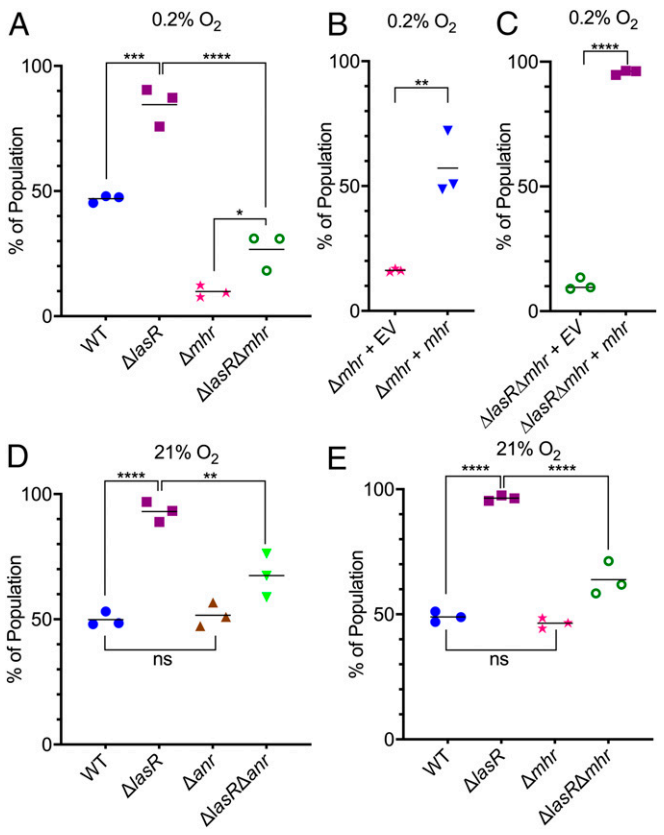


**Fig. 4.** Fitness of the Δ*lasR* mutant in microoxic and normoxic conditions is dependent on *mhr*. (*A*) Microoxic fitness of the wild type (WT), Δ*lasR*, Δ*mhr*, and Δ*lasR*Δ*mhr* relative to the *lacZ*-labeled WT under 0.2% $O_2$. (*B*) Microoxic fitness of Δ*mhr* carrying the empty vector pMQ70 (EV) or a pMQ70-mhr (*mhr*) against the *lacZ*-labeled WT carrying pMQ70. (*C*) Microoxic fitness of the Δ*lasR*Δ*mhr* + EV or Δ*lasR*Δ*mhr* + *mhr*. In *B* and *C*, 0.2% arabinose and carbenicillin (300 μg/mL) was added to the agar medium. (*D*) Normoxic (21% $O_2$) fitness of WT, Δ*lasR*, Δ*anr*, and Δ*lasR*Δ*anr*. (*E*) Normoxic fitness of Δ*lasR*, Δ*mhr*, and Δ*lasR*Δ*mhr*. All assays were performed grown on tryptone agar at 37 °C for 48 h. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.00001$ by one-way ANOVA, multiple comparisons (*A*, *D*, and *E*) or *t* test (*B* and *C*). ns, not significant.

The role for Mhr in PA14 Δ*lasR* but not wild type in normoxic colonies raised the question of whether Anr activity is more strongly induced in Δ*lasR* mutants as oxygen becomes depleted. Using a liquid culture assay in which increasing volumes of medium were used to decrease the surface area for oxygen transfer, we found that, in both strains, *mhr* promoter induction was low with high aeration (1- and 5- mL culture volumes) and increased with culture volume, consistent with Anr activity inversely correlating with oxygen availability (*SI Appendix*, Fig. S5*A*). At the 10- and 20-mL volumes, the Δ*lasR* mutant had significantly higher levels of *mhr* promoter activity than the wild type despite similar culture densities (*SI Appendix*, Fig. S5 *A* and *B*). Using the 10-mL culture volume, we found that, like Δ*lasR*, the Δ*lasI* strain which cannot synthesize the LasR ligand 3-oxo-C12-homoserine lactone (3OC12HSL) also had significantly higher levels of *mhr* promoter activity and the elevated activity could be complemented by exogenous 3OC12HSL (*SI Appendix*, Fig. S5*C*).

***mhr* Is Coregulated with and Epistatic to Genes Involved in Microoxic Respiration.** To gain more insight into the mechanisms by which Mhr increases microoxic fitness, we analyzed its expression pattern relative to other Anr-regulated genes known to be involved in metabolism. To do so, we used eADAGE, a tool that enables the visualization of transcriptional pattern relationships deduced from the analysis of 1,056 publicly available samples (39). Using the Anr-regulated genes shown in color in Fig. 5*A* as input, we found that *mhr* showed an expression pattern that closely mirrored that of the *ccoNOPQ-2* genes that encode components of one of the two high-affinity cytochrome $cbb_3$ oxidases in *P. aeruginosa*. The deletion of *ccoNOPQ-2* has little effect on the microoxic growth because of the enzymes encoded by the remaining *ccoNOPQ-1* operon, which have partially redundant activity (8). A strain lacking both loci, *ccoNOPQ-1* and *ccoNOPQ-2* (Δ*cco*), has a significant microoxic growth defect (8), and thus we used this strain to determine the interaction between Mhr and $cbb_3$ oxidases. When the Δ*cco* mutant was competed against the constitutively labeled wild type in microoxia, it was recovered at 0 to 0.012% of the total population. In light of this drastic fitness difference, we competed a *lacZ*-tagged Δ*cco* strain against either an untagged version of itself or the untagged Δ*cco*Δ*mhr* mutant to determine if the Δ*mhr* phenotype required the presence of the high-affinity cytochrome $cbb_3$ oxidases. The Δ*cco* and Δ*cco*Δ*mhr* mutants had similar microoxic fitness profiles ($P = 0.2$) (Fig. 5*B*), suggesting that Mhr may improve microoxic fitness through direct or indirect interaction with $cbb_3$ oxidases. Because the microoxic defect of the Δ*cco* mutant is much more severe than that of the Δ*mhr* mutant, we conclude that, while the $cbb_3$ oxidases may benefit from the cellular function of Mhr, these enzymes do not require Mhr to function.

**Metabolomics Analysis Indicates that Mhr Influences the Metabolism of a Δ*lasR* Mutant.** In light of the contribution of Mhr to the fitness of Δ*lasR* under normoxic conditions, we assessed the effects of Mhr on the metabolism of the Δ*lasR* mutant by performing a metabolomics comparison of Δ*lasR* and Δ*lasR*Δ*mhr* colony biofilms grown with atmospheric oxygen. We analyzed cells grown on either lysogeny broth (LB) or artificial sputum medium (ASM) (40) designed to mimic the sputum conditions found in the lungs of individuals with CF. To identify metabolites that were different upon the loss of *mhr* in both datasets, we used a linear model with two factors (genotype and medium). Our analysis found that comparison of Δ*lasR* to the Δ*lasR*Δ*mhr* mutant across both media revealed 60 metabolites with significant ($P < 0.05$) differences between these two strains and a $\log_2$-transformed fold change >0.5 in either LB or ASM (Fig. 5*C* and Dataset S1). Among the largest differences between Δ*lasR* and Δ*lasR*Δ*mhr* were those metabolites

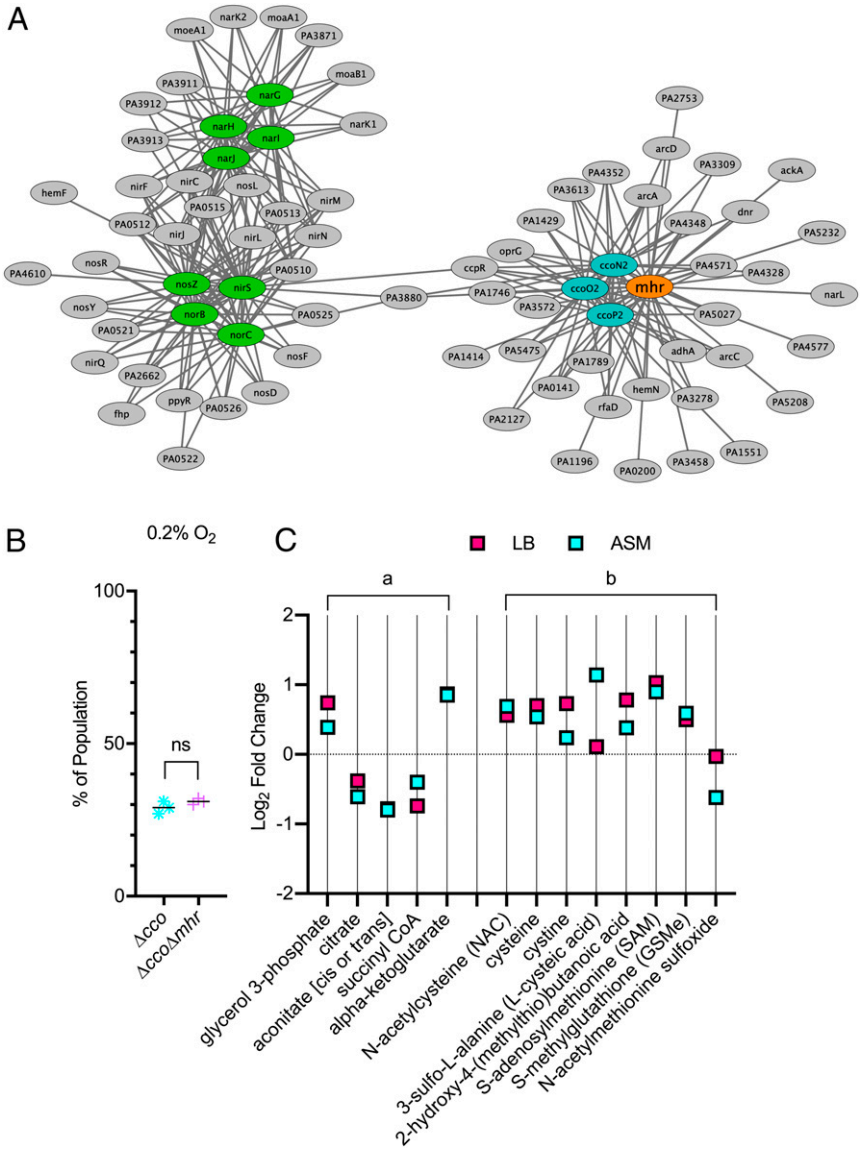


**Fig. 5.** *mhr* is coregulated with *ccoNOPQ-2*, the Δ*mhr* microoxic fitness phenotype requires high-affinity cytochrome *c* oxidases, and Mhr affects cell metabolism. (*A*) Similarity in expression pattern for genes within the Anr regulon in a publicly available data compendium determined using a machine-learning–derived model for gene expression, eADAGE. Similarities of expression pattern for Anr-regulated genes (ovals) are depicted as edge length. High-affinity cytochrome C oxidases subunits *ccoN2*, *ccoO2*, and *ccoP2* are shown in blue, and denitrification structural genes are shown in green. (*B*) Microoxic (0.2% $O_2$) fitness comparison for Δ*cco*1Δ*cco*2 (Δ*cco*) and Δ*cco*1Δ*cco*2Δ*mhr* (Δ*cco*Δ*mhr*) was determined by competition against the constitutively *lacZ*-labeled Δ*cco*1Δ*cco*2 (Δ*cco* att::*lacZ*). Assay was performed on tryptone agar at 37 °C for 4 d. (*C*) Metabolomics analysis of 16-h colony biofilms on either LB agar or ASM agar under 21% oxygen. Plotted $\log_2$-transformed fold difference (Δ*lasR*/Δ*lasR*Δ*mhr*) met the following criteria: 1) $P < 0.05$ by false discovery rate analysis and 2) $\log_2$-transformed fold difference magnitude at least 0.5 on LB or ASM. Plotted metabolites were grouped by pathway: (a) energy generation and (b) sulfur metabolism. Each point represents the average of five replicates. See Dataset S1 for additional information.

involved in energy generation, including metabolites in the tricarboxylic acid (TCA) cycle. Amino acid metabolic pathways, particularly those involved in the metabolism of sulfur-containing and aromatic amino acids, were also affected by Mhr. Other pathways included those related to the metabolism of nucleotides and lipids, as well as the synthesis of LPS, cell wall, and siderophores. The differences detected in these pathways are consistent with Mhr affecting cellular metabolism and being important for oxic respiration and intracellular redox.

## Discussion

Findings from this study and others demonstrate that *P. aeruginosa* LasR− strains have high Anr activity (17, 20) and activity of Anr-regulated pathways (41) in microoxic environments. In this study, we showed that Δ*lasR* mutants had increased fitness in the colony biofilms grown in microoxic and normoxic atmospheres and that this increase in fitness was dependent on *anr* and Anr-regulated *mhr*. Furthermore, the relative fitness defect incurred by loss of either *anr* or *mhr* was greater in a Δ*lasR* mutant background than in the wild type. Compared to other Anr regulated genes, expression of *mhr* and the Anr-regulated *ccoNOPQ-2* encoding a high-affinity terminal oxidase followed similar patterns across a large data compendium, and the loss of Mhr did not cause a further fitness defect in a strain lacking high-affinity cytochrome *c* oxidases, suggesting that Mhr and these enzymes may work together. Both proteomic and transcriptomic studies provided evidence for the

coordinated induction of Mhr and these high-affinity cytochrome *c* oxidases in synthetic cystic fibrosis medium under microoxic conditions compared to normoxic conditions (42) and in CF sputum (42, 43).

There are several possible models for how Mhr improves microoxic fitness. Hemerythrins have been shown to play roles in oxygen transport and delivery (44), and our observation that *mhr* is tightly coexpressed with *ccoNOPQ*-2 suggests that Mhr could play some mechanistic role in improving access of $cbb_3$ oxidases to oxygen beyond what would be achievable via passive diffusion when extracellular oxygen levels are low. In *M. capsulatus* (33), a homologous hemerythrin has been suggested to deliver oxygen to the membrane-bound methane monooxygenase. Mhr may also indirectly alter oxygen availability to oxygenases or confer protection to oxygen-sensitive enzymes. Our metabolomics data suggest that Mhr influences metabolites in the TCA cycle consistent with a role in substrate oxidation or respiration. Comparison of the metabolome of the Δ*lasR* and Δ*lasR*Δ*mhr* mutants on two different media also revealed striking effects on sulfur-containing amino acid catabolism which may be related to changes in intracellular redox state and might affect the activity of Fe-S cluster-containing proteins (32). Any of these proposed roles of Mhr are not mutually exclusive.

The elevated Anr activity in *lasR* mutants was not abolished in coculture by the presence of factors produced by the wild type. The model that Δ*lasR* mutants have a fitness advantage due to elevated Anr activity, and therefore high Mhr, is not incompatible with models for increased Δ*lasR* mutant fitness relative to *lasR*+ strains due to the benefits of social cheating and the cost-less exploitation of public goods (18). Other studies have shown that LasR− strains have increased resistance to lysis in alkaline conditions (45) and enhanced growth on particular carbon sources (10) which likely also promote fitness. Thus, we posit that oxygen limitation can act as one positive selection pressure against LasR signaling. We do not yet fully understand how an increase in Anr activity is incurred by *lasR* deficiency, and further studies will explore the molecular mechanism behind this phenomenon.

## Materials and Methods

Materials and methods describing the growth conditions, plasmid construction, competition experiments, promoter fusion experiments, protein expression and purification, Western blots, generating absorption spectra for deoxy- and oxy-Mhr, characterization of oxygen binding by Mhr, sequence alignment of Mhr homologs, iron content assay, structural homology modeling, eADAGE analysis, and metabolomics analysis are described in detail in the *SI Appendix, SI Materials and Methods*.

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