The putative bacterial oxygen sensor Pseudomonas prolyl hydroxylase (PPHD) suppresses antibiotic resistance and pathogenicity in Pseudomonas aeruginosa

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**Pseudomonas aeruginosa** is an extracellular opportunistic bacterial pathogen commonly associated with infectious complications in susceptible individuals, such as those with underlying diseases including HIV/AIDS and cystic fibrosis. Antibiotic resistance in multiple strains of *P. aeruginosa* is a rapidly developing clinical problem. We have previously demonstrated that the oxygen levels at the site of *P. aeruginosa* infection can strongly influence virulence and antibiotic resistance in this pathogen, although the oxygen-sensing and -signaling mechanisms underpinning this response have remained unknown. In this study, we investigated the potential role of the putative oxygen sensor Pseudomonas prolyl hydroxylase (PPHD) in the control of virulence and antibiotic resistance in *P. aeruginosa*. We found that a *P. aeruginosa* strain lacking PPHD (PAO310) exhibits increased virulence associated with increased bacterial motility. Furthermore, PPHD-deficient *P. aeruginosa* displayed enhanced antibiotic resistance against tetracycline through increased expression of the xenobiotic transporters mexEF-oprN and MexXY. Of note, the effect of the PPHD knockout on antibiotic resistance was phenocopied in bacteria exposed to atmospheric hypoxia. We conclude that PPHD is a putative bacterial oxygen sensor that may link microenvironmental oxygen levels to virulence and antibiotic resistance in *P. aeruginosa*.

Humans exist in a state of communion with commensal microbial organisms including thousands of species of bacteria that provide essential support for the growth and development of organs including the skin and intestine and the maturation of the host immune system. These bacteria comprise the human microbiome. In the vast majority of cases, interactions between humans and bacteria result in a neutral or beneficial outcome for the host. However, in a limited number of cases, microbes display the capacity for virulence and pathogenicity, and this can lead to tissue damage in the host, resulting in infectious disease (1). Our capacity to clinically manage opportunistic bacterial infection is rapidly diminishing because many of the causative pathogens develop antibiotic resistance.

*Pseudomonas aeruginosa*, a motile, Gram-negative facultative anaerobe, is an opportunistic pathogen that is responsible for 17% of cases of nosocomial pneumonia and is a primary cause of morbidity and mortality in cystic fibrosis patients (2, 3). *P. aeruginosa* infection is also frequently associated with infections in individuals with HIV/AIDS, urinary tract infections, ventilator-associated lung infection, endocarditis, meningitis, ocular infection, ear infection, and wound/burn/skin infections (2).

*P. aeruginosa* is an extracellular pathogen that produces toxins (e.g. exotoxin A, which causes direct tissue damage) and proteases (e.g. alkaline protease) that degrade neutrophil-derived elastin and consequently help the bacterium to avoid host immunity. *P. aeruginosa* expresses a type III secretion system that allows the direct injection of bacterial toxins into host cells, siderophores that promote the chelation of iron and virulence factors such as pyocyanin and pyoverdine, which support bacterial metabolism and the production of exotoxins, respectively. Together, these virulence mechanisms promote bacterial colonization of tissues and the development of infectious disease. A key mechanism underpinning the development of virulence in *P. aeruginosa* is quorum-sensing, which refers to the intercellular communication between individual bacteria in the niche of the infected tissue (4). Several important factors that mediate the dialog between bacteria during quorum sensing are the products of bacterial metabolism creating another link between the bacterial metabolic strategy and the capacity of the pathogen for virulence.

Antimicrobial resistance is a common and developing problem in *P. aeruginosa* infection caused in part by high expression levels of a number of xenobiotic efflux pumps (5, 6). Typically, when it colonizes a host, *P. aeruginosa* grows in a biofilm that provides a physical barrier to antimicrobial access. In summary, *P. aeruginosa* is a formidable opportunistic pathogen that is armed with an array of features that support its virulence in human infection.

Hypoxia is an environmental feature that is frequently encountered at sites of infection (5). For example, the cystic fibrosis lung, commonly infected with *P. aeruginosa*, has been shown to be hypoxic (7). Furthermore, *Mycobacterium tuberculosis* was shown to reside in a hypoxic environment within tuberculous granulomas in rabbits (8). Biopsies from patients...
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with skin infection showed an elevated level of hypoxia-inducible factor (HIF) 1α, a marker of tissue hypoxia (9). We previously demonstrated the impact of hypoxia on *P. aeruginosa* antibiotic resistance, virulence, and infection (6, 10, 11). However, the nature of the oxygen-sensing pathways that determine these responses remains unknown and is the topic of the current study.

Prolyl hydroxylases (PHDs)² are oxygen sensors in metazoa. They are Fe(II)- and 2-oxoglutarate–dependent dioxygenases whose activity depends on molecular oxygen. When sufficient oxygen is available (normoxia), PHDs hydroxylate the hypoxia-inducible factor (HIF) α subunit. The von Hippel–Lindau tumor suppressor protein polyubiquitinates the hydroxylated HIFα and thus marks it for proteosomal degradation. Conversely, when oxygen is limited, HIFα accumulates and translocates to the nucleus where it transcriptionally activates genes involved in erythropoiesis and angiogenesis (12).

PHDs are therefore primary oxygen sensors in metazoa (13, 14). Recently a homolog to the human oxygen sensing enzyme PHD2 was identified in *P. aeruginosa* and termed *Pseudomonas* prolyl hydroxylase (PPHD) (13). Subsequently, another bacterial PHD enzyme was discovered in *Bacillus anthracis* (15). PPHD has a close structural homology to human PHD2 in that it contains an active 2OG oxygenase domain as demonstrated by biochemical and structural analysis (13). Furthermore, the elongation factor EF-Tu has been identified as a substrate for PPHD. However, cross-reactivity with substrates does not occur (i.e. EF-Tu is not a substrate for PHD2, and the oxygen-dependent degradation domain of HIF-1α is not a substrate for PPHD). Because of the role of PHDs in mammalian oxygen sensing, it was hypothesized that PPHD might play a role in oxygen sensing in *P. aeruginosa* (13, 16). More recently we and others have shown that PPHD influences *P. aeruginosa* virulence; however, the mechanisms involved remain to be defined (11, 16). In this study, we investigated the role of PPHD as a possible prokaryotic oxygen sensor linking microenvironmental hypoxia to virulence and antibiotic resistance in *P. aeruginosa*.

**Results**

**PPHD suppresses virulence in *P. aeruginosa***

We have previously demonstrated that microenvironmental hypoxia alters virulence in *P. aeruginosa* (10, 11). To determine whether the putative oxygen sensor PPHD may play a role in controlling virulence and antibiotic resistance, we compared WT (PAO1) with a *P. aeruginosa* strain deficient in PPHD (PAO310). We first confirmed the absence of PPHD mRNA expression in PAO310 by PCR (Fig. 1A). We next investigated the impact of PPHD knockout on bacterial virulence using *Galleria mellonella* larvae as a model of host infection (17). PAO310 was more virulent in *G. mellonella* than PAO1 as demonstrated both qualitatively and quantitatively (Fig. 1B and C, respectively and Fig. S2). A representative image of *G. mellonella* injected with *P. aeruginosa* at 24 h is shown in which lighter-colored larvae are viable and darker-colored larvae were melanized, which immediately precedes death. The data in Fig. 1C are presented as LD₅₀ (the number of bacteria required to kill 50% of the larvae). In summary, PPHD knockout results in increased lethality in *G. mellonella*, indicating that PPHD suppresses bacterial virulence in *P. aeruginosa*.

We next investigated the influence of PPHD expression on key determinants of virulence in *P. aeruginosa*. We first compared bacterial motility and quantified biofilm formation in PAO1 and PAO310 strains. Consistent with the increased virulence observed in Fig. 1, motility of *P. aeruginosa* (both swimming (Fig. 2A) and swarming (Fig. 2B)) was increased when PPHD is not present. Furthermore, biofilm mass was also increased in PAO310 (Fig. 2C). Taken together, these data support a role for PPHD in suppressing key determinants of *P. aeruginosa* virulence.

**PPHD influences antibiotic susceptibility of tetracycline antibiotics**

We previously demonstrated that microenvironmental hypoxia plays a determining role in antibiotic resistance in *P. aeruginosa* (6). Therefore, we next investigated the impact of PPHD knockout on antibiotic resistance. Minimal inhibitory concentration of antibiotics from a broad range of antibiotic classes were tested in PAO1 and PAO310 strains of *P. aeruginosa*. The minimal inhibitory concentration (MIC) values for tetracycline antibiotics (tetracycline, doxycycline, minocycline, flucloxacillin, ceftriaxone, and chloramphenicol) in PAO1 and PAO310 strains were compared. The results showed that PPHD knockout increased the MIC of these antibiotics in PAO310 compared to PAO1 (Fig. 2D). These data suggest that PPHD knockout enhances antibiotic resistance in *P. aeruginosa*.

²The abbreviations used are: PHD, prolyl hydroxylase; PPHD, *Pseudomonas* prolyl hydroxylase; MIC, minimal inhibitory concentration; HIF, hypoxia-inducible factor; RND, resistance-nodulation-division.
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![Figure 2. PPHD reduces motility and biofilm formation in *P. aeruginosa*.](image)

*Figure 2. PPHD reduces motility and biofilm formation in *P. aeruginosa*. A and B, motility measurements of WT (PAO1) and PPHD mutant (PAO310) *P. aeruginosa*. All data in A and B are presented as means ± S.E. of six or seven independent experiments. Right panels, representative images of motility of indicated strains. C, biofilm quantification of indicated strains. The data represent means ± S.E. of six independent experiments. *, *p < 0.05; **, *p < 0.01, two-tailed unpaired Student's t-test. PAO1 is the WT strain, and PAO310 is the PPHD knockout strain.*

and tigecycline) were selectively increased in *P. aeruginosa* lacking the PPHD gene, whereas susceptibility of other classes of antibiotics was not influenced (Table 1).

Exposing PAO1 to tetracycline (512 μg/ml) prior to injection into larvae increased its LD$_{50}$ by 2972-fold (Fig. 3). In contrast, for PAO310, tetracycline pretreatment did not affect its LD$_{50}$ further suggesting that the *P. aeruginosa* lacking PPHD was more resistant to the antibiotic. Taken together, these data indicate that PPHD selectively suppresses tetracycline antibiotic resistance in *P. aeruginosa*.

**Efflux pump mexEF–OprN is up-regulated in the absence of PPHD**

We next investigated the possible mechanisms underpinning the effects of PPHD knockout on antibiotic resistance. A key determinant of antibiotic resistance in *P. aeruginosa* is the level of antibiotic efflux via RND efflux pumps (18, 19). Indeed, we previously demonstrated that the expression of these pumps is increased in hypoxia, thereby contributing to hypoxia-induced antibiotic resistance (6). We next investigated whether RND efflux pumps contribute to increased tetracycline resistance in PAO310. To do this we determined the MIC of tetracycline in the presence of an efflux pump inhibitor (Phe–Arg–β-naphthylamide dihydrochloride). Blocking the RND efflux pumps made PAO310 more susceptible to tetracycline (MIC was lowered from 256 to 64 μg/ml), indicating that the absence of PPHD contributes to antibiotic resistance by increasing tetracycline extrusion via efflux pumps (Fig. 4A). We quantified the expression of the linker protein gene of four RND efflux pumps. MexE (part of mexEF–OprN) was increased in PAO310 (PPHD knockout mutant), whereas mexA and mexC were unchanged (Fig. 4B and Fig. S1). Of note, the induction of mexX with a subinhibitory concentration of tetracycline in PAO1 (WT *P. aeruginosa*) was lost in PAO310 (PPHD knockout strain) (Fig. S1). These data suggest that the absence of PPHD increases mexEF–OprN expression in *P. aeruginosa*.

**Hypoxia increases resistance to tetracycline antibiotics in *P. aeruginosa***

PPHD is a prolyl hydroxylase, homolog to human PHD2. PHD2 hydroxylates the target HIF-1α protein in metazoan cells in an oxygen-dependent manner. The hydroxylation activity of PHD2 can therefore be inhibited by low oxygen concentrations (hypoxia) (20). To investigate whether hypoxia mimics the effects of PPHD knockout on tetracycline antibiotic susceptibility, we tested whether exposure to hypoxia (1% oxygen) altered antibiotic resistance in a similar way to PPHD knockout. Similar to PPHD knockout (albeit to a lesser degree), hypoxia increased tetracycline, doxycycline, and minocycline MICs in PAO1 by ~2-fold (tetracycline, 32–64 μg/ml; doxycycline and minocycline, 16–32 μg/ml), respectively (Fig. 5A). To test the potential clinical relevance of our observations, we also investigated tetracycline susceptibility in clinical *P. aeruginosa* isolates (derived from patient infections) under hypoxic conditions. Consistent with the effects observed in PAO1, two of three acute clinical strains demonstrated a 2-fold increase in MIC in hypoxia (Fig. 5B). In summary, exposure to hypoxia phenocopies the impact of PPHD knockout on tetracycline resistance in *P. aeruginosa*, thereby supporting the potential role for PPHD as a bacterial oxygen sensor.

**Discussion**

Hypoxia is frequently a prominent microenvironmental feature at sites of infection. We have previously shown that exposure of the opportunistic pathogen *P. aeruginosa* to a hypoxic microenvironment is a key determinant of pathogen virulence and antibiotic resistance. The mechanism whereby hypoxia mediates its influence on these parameters of pathogen behavior is the topic of this study. Developing our understanding of how the microenvironment at the site of infection affects pathogen virulence and antibiotic resistance will help identify new avenues of therapeutic intervention in *P. aeruginosa* infection.

Recently the prolyl hydroxylase PPHD was identified in *P. aeruginosa* and hypothesized to play a role in oxygen sensing (13). PPHD is a structural homolog of human PHD2, which is an important regulator of the hypoxic response in mammals (12). PPHD was shown to hydroxylate the prokaryotic elongation factor EF-Tu on a proline residue (13).

For this study we used the PPHD knockout mutant PAO310 to determine its influence on virulence and antibiotic resistance. PAO310 was more virulent than the WT (PAO1) in the *G. mellonella* model (Fig. 1). This is consistent with a previous
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Table 1

| Antibiotic class | Antibiotic                  | MIC PAO1 WT (µg/ml) | MIC PAO310 PPHD KO (µg/ml) | Method | No. of independent experiments |
|------------------|-----------------------------|---------------------|----------------------------|--------|-----------------------------|
| β-Lactams        | Piperacillin/tazobactam     | 8                   | 8                          | VITEK2 | n = 3                       |
|                  | Aztreonam                   | 4                   | 4                          | E-test | n = 1                       |
| Aminoglycosides  | Amikacin                    | ≤2                  | ≤2                         | VITEK2 | n = 3                       |
|                  | Gentamicin                   | ≤1                  | ≤1                         | VITEK2 | n = 3                       |
|                  | Tobramycin                   | ≤1                  | ≤1                         | VITEK2 | n = 3                       |
| Carbapenems      | Meropenem                    | 2                   | 2                          | VITEK2 | n = 3                       |
|                  | Imipenem                     | 8                   | 8                          | VITEK2 | n = 3                       |
|                  | Ertapenem                    | ≥8                  | ≥8                         | MBD (GNX2F) | n = 3 |
|                  | Doripenem                    | 1                   | 1                          | MBD (GNX2F) | n = 3 |
| Cephalosporins   | Cefotaxime                   | 16                  | 16                         | VITEK2 | n = 3                       |
|                  | Cefepime                     | 2                   | 2                          | VITEK2 | n = 3                       |
| Quinolones       | Ciprofloxacin                | ≤0.25               | ≤0.25                      | VITEK2 | n = 3                       |
|                  | Levofloxacin                 | 0.5                 | 0.5                        | VITEK2 | n = 3                       |
| Penicillins      | Ticarcillin                  | 32                  | 32                         | VITEK2 | n = 3                       |
|                  | Ticarcillin/clavulanic acid  | 16                  | 16                         | VITEK2 | n = 3                       |
| Polymyxins       | Colistin                     | 1                   | 1                          | MBD (GNX2F) | n = 3 |
|                  | Polymyxin B                  | 1                   | 1                          | MBD (GNX2F) | n = 3 |
| Tetracyclines    | Tetracycline                 | 32                  | 256                        | MBD   | n = 4*                      |
|                  | Doxycycline                  | 16                  | 64                         | MBD   | n = 3                       |
|                  | Minocycline                  | 16                  | 32                         | MBD   | n = 3                       |
|                  | Tigecycline (glycylcycline)  | 8                   | 16                         | MBD   | n = 3                       |
| Others           | Trimethoprim/sulfamethoxazole| 4                   | ≥8                         | MBD (GNX2F) | n = 3 |

* p < 0.05 by Mann-Whitney test.

Figure 3. High dose of tetracycline does not decrease virulence of PPHD knockout mutant. LD₅₀ values of indicated strains and treatment are shown. Tet indicates 512 µg/ml tetracycline. PAO1 is the WT strain, and PAO310 is the PPHD knockout strain. The data represent means ± S.E. of three to eight independent experiments. *, p < 0.05, one-way analysis of variance with Tukey’s multiple comparison test.

Figure 4. PPHD regulates selectively antibiotic sensitivity to tetracycline antibiotics by altering RND efflux pump mexEF–OprN expression. A, MIC determination in presence or absence of efflux pump inhibitor (EPI) Phe–Arg–β-naphtylamide dihydrochloride (20 µg/µl), individual data points of three independent experiments, and median are shown. *, p < 0.05 Kruskal–Wallis method with Dunne’s multiple comparison test. B, fold change of mexE mRNA of indicated strains. **, p < 0.01, two-tailed unpaired Student’s t test, The data represent means ± S.E. of five independent experiments. PAO1 is the WT strain, and PAO310 is the PPHD knockout strain.

P. aeruginosa and how their hydroxylation affects pathogenicity and antibiotic resistance. Our current studies aim to identify the target(s) for PPHD in P. aeruginosa, and we hope in the future to be able to answer this vitally important question.

Resistance to tetracycline antibiotics was selectively increased in PAO310. This was associated with a significant increase in mexE mRNA (Table 1 and Figs. 3 and 4). MexE is part of the RND efflux pump mexEF–oprN. An increase in tetracycline resistance caused by overexpression of mexEF–oprN was reported before (21), although it should be noted that whether tetracycline is a major substrate of mexEF–oprN remains controversial (22, 23). We conclude that PPHD influences efflux pump expression and thus changes antibiotic susceptibility of P. aeruginosa for tetracycline antibiotics.
PHD2, the mammalian homolog of PPHD is a key oxygen sensor linked to the HIF pathway (24). We tested whether PPHD inhibition by hypoxia in WT *P. aeruginosa* (PAO1) has influence on antibiotic susceptibility toward tetracycline antibiotics in a similar way to PPHD knockout. Resistance to tetracycline antibiotics was 2-fold higher in PAO1 at 1% O2 when compared with 21% O2 (Fig. 5). This is similar to the effect seen when PPHD is absent, albeit to a lesser extent. In conclusion, we provide evidence that PPHD activity decreases virulence of *P. aeruginosa* and increases antibiotic susceptibility to tetracycline antibiotics by altering the expression of the RND efflux pumps mexEF–oprN and MexXY. Therefore, *P. aeruginosa* PPHD may represent a new therapeutic target in reducing virulence and antibiotic resistance in *P. aeruginosa* infection.

Because PPHD mutation correlates with some of the effects we have reported hypoxia to elicit in *P. aeruginosa*, these data raise the intriguing possibility that PPHD may provide a molecular link between tissue oxygen levels and bacterial pathogenicity in infectious disease. However, further evidence is required to assess both the oxygen-sensing activity of PPHD and its molecular targets in linking microenvironmental oxygen levels and bacterial pathogenesis and antibiotic resistance.

**Experimental procedures**

**Determination of virulence using the in vivo *G. mellonella* model**

Sixth-instar larvae of *G. mellonella* (Livefoods direct) were used for the *in vivo* virulence determination. Bacteria were grown to an A<sub>600</sub> of 0.6 and diluted to A<sub>600</sub> of 0.1 in PBS (Fisher Chemical). Serial dilutions up to 10<sup>−7</sup> in PBS were prepared and plated on agar plates in triplicates to determine the bio burden. 20 μl of each dilution was injected into the hemocoel through the last pro leg (BD microfine U100 insulin syringe; Becton, Dickinson and Company). For each dilution 10 larvae were used and incubated in Petri dishes (Greiner Bio-One) on filter paper (Fisher). 10 larvae were injected with PBS as control. After 24-h incubation, larval death was assessed by the lack of movement upon stimulation. At least three independent experiments were conducted. The data are presented as LD<sub>50</sub> values.

**Bacterial Strains**

PAO310 was extracted from a saturating library of sequence-defined transposon insertion mutants. This approach generated over 30,000 defined *P. aeruginosa* mutants, of which PAO310 was one (28). To our knowledge, only PPHD is different between PAO310 and PAO1.

**Bacterial culture conditions**

*P. aeruginosa* was grown in Luria broth (Sigma) or on tryptic soy agar (Sigma). For hypoxic culture, broth was preincubated at 1% oxygen in a hypoxia chamber (Coy Laboratory) prior to inoculation.

**Motility**

Motility was assessed by investigating swimming and swarming. Motility plates were inoculated with a sterile pipette tip (1–10 μl; Star-lab) in the center. After 24 h of incubation at 37 °C, the zone of motility was measured.

**Swimming**

10-cm plates with 1% trypthone (Foremedium), 0.5% NaCl (Fisher chemical), and 0.3% agarose (Sigma) were used for the swimming assay. The plates were wrapped with parafilm to avoid dehydration.

**Swarming**

For swarming 10-cm plates with 0.5% agar (Sigma), 0.5% glucose (Sigma) in Luria broth (0.8 g/100 ml) were prepared.

**Biofilm**

Overnight cultures were diluted to A<sub>600</sub> of 0.05. Per condition 1 ml of diluted bacteria suspension was incubated in duplicate in a 12-well plate (Greiner Bio-One) for 24 h. For quantification the biofilm was washed two times with 1 ml of PBS, stained with 0.5 ml of 0.1% crystal violet solution (Sigma) for 15 min at room temperature, and washed again three times with 1 ml of PBS. Crystal violet stain was eluted from biofilm bacteria with 1 ml of ethanol (96%, v/v, Sigma), and absorbance at 595 nm was measured with the CLARIO star spectrometer (BMG LabTech).

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**Figure 5. Hypoxia increases MIC of tetracycline antibiotics**

A, MIC of indicated antibiotic and treatment in PAO1. B, tetracycline MIC of indicated acute clinical *P. aeruginosa* strains and treatment. Normoxia: room air O<sub>2</sub>, hypoxia: 1% O<sub>2</sub>. Median with range of three to five independent experiments is shown.
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Labtech). Dishes with medium only were treated the same way and served as negative control.

\textbf{Antibiotic susceptibility testing}

MIC of \textit{P. aeruginosa} was determined with several methods. For MIC testing with the VITEK 2 system (Bio Merieux), AST-N352 cards were used according to the manufacturer’s instructions. E-test (Bio Merieux) were used according to the manufacturer’s instructions. Micro broth dilutions were performed using Sensititre GNX2F susceptibility plate (Thermo Scientific) and with freshly prepared plates using tetracycline hydrochloride (Sigma), doxycycline hydrochloride (Sigma), minocycline hydrochloride (Sigma), and tigecycline hydrate (Sigma). Therefore, serial dilutions of the respective antibiotic in cation adjusted Mueller Hinton broth (Becton, Dickinson and Company) double-concentrated were prepared, and 50 \(\mu\)l were transferred into a 96-well plate (Greiner Bio-One). \textit{P. aeruginosa} from a freshly grown tryptic soy agar plate were used to prepare a suspension with the same turbidity as a 0.5 McFarland standard. 100 \(\mu\)l of this suspension was added to 11.5 ml of Mueller Hinton broth (Becton, Dickinson and Company). 50 \(\mu\)l were added to the wells with the double-concentrated antibiotics and incubated for 18–24 h at 37 °C. Broth without antibiotic served as growth control. To determine the influence of RND efflux pumps on antibiotic susceptibility, Phe–Arg–β-naphthylamide dihydrochloride (20 \(\mu\)g/ml, Sigma) was added. Furthermore, antibiotic susceptibility testing was performed at 1% oxygen.

\textbf{DNA and RNA extraction}

DNA was extracted from overnight cultures with the Wizard genomic DNA purification kit (Promega) according to the manufacturer’s instructions. For RNA extraction overnight cultures of \textit{P. aeruginosa} were diluted to an \(A_{500}\) of 0.001 and grown statically for 24 h. The bacteria were harvested and processed as described elsewhere (25). 1 \(\mu\)g of RNA was reversed transcribed to cDNA with Superscript II (Invitrogen).

\textbf{Polymerase chain reaction}

To test for the presence of the \textit{PPHD} gene, \textit{PPHD} was amplified of genomic DNA with the following primer: forward, 5'-'TGA AAA ACG GCC AGT AGC GGG CAT TGA TAC TCC TT-3', and reverse, 5'-'TGA ACG GAA ACA GCT ATG ACC CAC GAT CAA GGT CTG GGG TC-3'. 16S rRNA was amplified as control. Primers for 16S were published previously (26).

\textbf{Quantitative PCR}

To determine gene expression levels, quantitative PCR was carried out as described elsewhere (25). Genes were normalized to \textit{P. aeruginosa} 16S rRNA. Primers for 16S and \textit{mexE} were published previously (26, 27). \textit{mexX} primer were designed with primer 3 (forward, 5'-'TGGTTCGAGATGCAGCCTGC-3'; and reverse, 5'-'CTTCGGTGTAATCGGTTCC-3').

\textbf{Statistical analysis}

The data are presented as means ± S.E. for parametric data and as median for nonparametric data for at least three independent experiments. Statistical analysis was carried out with two-tailed unpaired Student’s \(t\) test, one-way analysis of variance with Tukey’s multiple comparison test, the Mann Whitney test or the Kruskal–Wallis method with Dunne’s multiple comparison test. \(p\) values of <0.05 were considered statistically significant.

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