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Nitric-oxide-driven oxygen release in anoxic *Pseudomonas aeruginosa*

Putative nitric oxide dismutation

NO₂⁻ + NO → O₂ + N₂

**Highlights**

*Pseudomonas aeruginosa* was found to release O₂ at the onset of anoxia.

Peaks of O₂ were amplified in a nitric oxide reductase (NOR) mutant.

The O₂ release was mediated by nitric oxide (NO).
Nitric-oxide-driven oxygen release in anoxic Pseudomonas aeruginosa

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SUMMARY
Denitrification supports anoxic growth of Pseudomonas aeruginosa in infections. Moreover, denitrification may provide oxygen (O₂) resulting from dismutation of the denitrification intermediate nitric oxide (NO) as seen in Methylomirabilis oxyfera. To examine the prevalence of NO dismutation we studied O₂ release by P. aeruginosa in airtight vials. P. aeruginosa rapidly depleted O₂ but NO supplementation generated peaks of O₂ at the onset of anoxia, and we demonstrate a direct role of NO in the O₂ release. However, we were not able to detect genetic evidence for putative NO dismutases.

The supply of endogenous O₂ at the onset of anoxia could play an adaptive role when P. aeruginosa enters anaerobiosis. Furthermore, O₂ generation by NO dismutation may be more widespread than indicated by the reports on the distribution of homologues genes. In general, NO dismutation may allow removal of nitrate by denitrification without release of the very potent greenhouse gas, nitrous oxide.

INTRODUCTION
Denitrification adds flexibility to the bacterial metabolism, by providing alternative electron acceptors for electron transport phosphorylation, when molecular oxygen (O₂) is missing (Zumft, 1997). The denitrification pathway is comprised of stepwise reductions of nitrate (NO₃⁻) to nitrite (NO₂⁻), nitric oxide (NO), nitrous oxide (N₂O), and finally dinitrogen (N₂) by committed reductases (Figure 1)(Line et al., 2014). Denitrification is widespread among bacteria with no distinct pattern of distribution (Zumft, 1997) and is present even in pathogens (Philippot, 2005). By measuring production and consumption of N₂O, Pseudomonas aeruginosa is the first pathogen in which ongoing denitrification during infection has been demonstrated (Kolpen et al., 2014a).

Recent examinations based on molecular identification have reclassified the gram-negative denitrifier P. aeruginosa from being ubiquitous to be predominantly confined to hospitals settings (Crone et al., 2020). P. aeruginosa is of major clinical concern in chronic wounds, foreign-body-associated infections, cystic fibrosis (CF) lung infections, and immunocompromised patients (Alhede et al., 2014; Ciofu et al., 2017; Haiby et al., 2010; Moser et al., 2017). The chronic infections are often characterized by biofilm formation of aggregated P. aeruginosa cells surrounded by host cells, primarily neutrophils that display a state of chronic activation, depleting O₂ for the generation of the toxic reactive oxygen species superoxide (O₂⁻) and nitric oxide (NO) (Kolpen et al., 2010, 2014b). Therefore, growth by aerobic respiration is limited (Kolpen et al., 2010), which is in accordance with the slow growth of P. aeruginosa observed in CF lung infections (Kragn et al., 2014).

Such growth of P. aeruginosa during anoxia can be achieved by denitrification, whereby the terminal oxidases (TOX) of the aerobic respiratory pathway are replaced by the nitrogen oxide reductases performing a stepwise reduction of NO₃⁻ to N₂ mediated by the nitrate (Nar), nitrite (Nir), nitric oxide (Nor), and nitrous oxide (Nos) reductases (Zumft, 1997) (Figure 1). In this process, nitrogen oxides replace O₂ as the final electron acceptors (Chen and Strous, 2013). The proton motive force generated by denitrification is lower than that of aerobic respiration, but we have demonstrated that physiological concentrations of NO₃⁻ (<1 mM NO₃⁻) are able to support growth of P. aeruginosa comparable with the slow rates observed during chronic
CF lung infection (Kragh et al., 2014; Line et al., 2014). Likewise, physiological concentrations of NO\textsubscript{2} can be used as alternative electron acceptor for anaerobic respiration by denitrification to support anaerobic growth (Grasemann et al., 1998; Jones et al., 2000; Kolpen et al., 2014a; Linnane et al., 1998; Major et al., 2010; Zumft, 1997), generating NO in a one-step reduction (Rinaldo et al., 2011).

The important intermediate of denitrification, NO, is implicated in many cellular processes and signaling pathways (Barraud et al., 2009; Cutruzzola and Frankenberg-Dinkel, 2016; Hossain and Boon, 2017) as well as displaying toxicity due to its high reactivity such as in nitrosylation of nucleic acids, proteins, and interaction with enzyme cofactors (Bowman et al., 2011; Hyduke et al., 2007; Poole and Hughes, 2000). Accordingly, the production and removal of NO is tightly regulated primarily by the regulator transcription factors Anr and Dnr (Arai et al., 1997; Castiglione et al., 2009; Schreiber et al., 2007; Trunk et al., 2010; Zumft, 1997).

\textit{P. aeruginosa} expresses two known enzymatic NO detoxification mechanisms, with NO being reduced to N\textsubscript{2}O by Nor in the denitrification pathway under anoxic conditions (Kumita et al., 2004) and by the nitric oxide dioxygenase activity of flavohemoglobin (Fhp) generating N\textsubscript{2}O\textsubscript{3} from O\textsubscript{2} and NO at aerobic conditions (Gardner and Gardner, 2002; Gardner et al., 1998). A third bacterial mechanism for removal of NO has recently been discovered in \textit{Methylomirabilis oxyfera}, where dismutation of NO, nitric oxide dismutase (Nod), results in the generation of O\textsubscript{2} and N\textsubscript{2} (Ettwig et al., 2010). Homologues genes for Nod appears to be widespread in environmental samples (Zhu et al., 2017), but the presence of Nod has not yet been reported for pathogenic bacteria. Therefore, we aimed to obtain evidence for the existence of Nod in \textit{P. aeruginosa} by employing microrespirometry of appropriate precursors and knockout mutants according to a proposed NO cycle in \textit{P. aeruginosa} in the presence of putative dismutation of NO (Figure 2).

**RESULTS**

\textbf{Oxygen release in nitric oxide reductase mutant \textit{P. aeruginosa}}

Initial observation of O\textsubscript{2} concentration in airtight liquid cultures initiated in normoxic conditions (~200 \(\mu\text{M} \) O\textsubscript{2}) of \textit{P. aeruginosa} WT and \(\Delta\text{norB}\) displayed rapid depletion of O\textsubscript{2} as expected due to the energetic preference of aerobic respiration in \textit{P. aeruginosa} (Chen and Strous, 2013). Unexpectedly, however, small peaks of O\textsubscript{2} were detected shortly after the initial O\textsubscript{2} depletion (hereafter referred to as O\textsubscript{2} peaks) in cultures of

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure1.png}
\caption{Denitrification pathway}
\end{figure}

Reduction of nitrate (NO\textsubscript{3}\textsuperscript{--}) to nitrite (NO\textsubscript{2}\textsuperscript{--}), nitric oxide (NO), nitrous oxide (N\textsubscript{2}O), and finally dinitrogen (N\textsubscript{2}). The four reductase enzymes, nitrate reductase (NarGHI), nitrite reductase (NirS), nitric oxide reductase (NorCB), and nitrous oxide reductase (NosZ), are shown above.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure2.png}
\caption{The explored proposed NO cycle in \textit{P. aeruginosa} in the presence of putative dismutation of NO}
\end{figure}

After depletion of O\textsubscript{2}, \textit{P. aeruginosa} utilizes nitrogen oxides as alternative terminal electron acceptors in the denitrification pathway. Nitrogen oxide reductases replace the aerobic terminal oxidases (TOX), performing a stepwise reduction of NO\textsubscript{3}\textsuperscript{--} to NO\textsubscript{2}\textsuperscript{--}, NO, N\textsubscript{2}O, and finally N\textsubscript{2} mediated by the nitrate (NarGHI), nitrite (NirS), nitric oxide (NorCB), and nitrous oxide (NosZ) reductases, respectively. NO can be detoxified, anaerobically, through reduction to N\textsubscript{2}O. In oxic conditions, NO can be removed by the dioxygenase activity of flavohemoglobin (Fhp) generating NO\textsubscript{3}\textsuperscript{--} from oxidation of NO by O\textsubscript{2}. In a NorCB-deficient mutant (\(\Delta\text{norB}\)) NO accumulates, resulting in increased amount of NO available for dismutation to O\textsubscript{2} and N\textsubscript{2}. Blue arrows = oxic processes. Red arrows = anoxic processes. Based on Robinson and Brynildsen (2016).
WT and ΔnorB supplemented with NO₂− (Figure 3). The O₂ peaks in the WT cultures were significantly amplified (p = 0.0017) in LB media supplemented with 1 mM NO₂−. The WT P. aeruginosa has a complete denitrification pathway and thus produces NO from NO₂− supplementation followed by removal by the subsequent nitric oxide reductase step—lacking in ΔnorB. Accordingly, the O₂ peaks in the ΔnorB cultures were significantly enhanced with supplementation of the LB media with as little as 0.1 mM NO₂− (p = 0.0018) and with 1 mM NO₂− (p < 0.0001). In addition, the O₂ peaks in the ΔnorB cultures were significantly higher than in the WT at 0.1 mM NO₂− and 1 mM NO₂− (p = 0.0002) with peaks reaching a mean of 23.1 ± 1.4 μM O₂ (mean ± SEM) (Figure 4). To exclude a possible leaking of incoming O₂ from the surrounding, vials loaded with LB-media equilibrated in an anaerobic bench showed no sign of incoming O₂ when measured at atmospheric surrounding (Figure S1). NO may interfere with some optical sensors of O₂ (Klaus et al., 2017). This possible interference was ruled out in our set-up by measuring vials loaded with LB-media equilibrated in an anaerobic bench and added 100 μM of the NO donor DPTA NONOate. No increase of the stable low signal for O₂ was seen even though 100 μM of DPTA NONOate may release 200 μM NO with a half-life of 3 h at pH 7.4, 37°C (Hrabie et al., 1993; Keefer et al., 1996) (Figure S2). To verify that the increased signal for O₂ induced by NO₂− is mediated by NO, we demonstrated a dose-dependent reduction of the O₂ peaks by varying amounts of CARBOXY-PTIO, which is an NO scavenger, to cultures of ΔnorB supplemented with 1 mM NO₂−. The dose-dependent decrease of the measured O₂ further emphasizes the involvement of NO in the transient oxygen increase by P. aeruginosa at the onset of anoxia. Further evidence for the involvement of NO in the O₂ peaks may be obtained by examining P. aeruginosa with engineered expression of the bacterial nitric oxide synthase.

Lack of O₂ peaks in anoxically initiated cultures
Cultures initiated normoxically, and supplemented with 1 mM NO₂−, displayed the characteristic O₂ peaks but in anoxically initiated cultures of both WT and ΔnorB, also supplemented with 1 mM NO₂−, no O₂ peaks were detected (Figure 4).

Lack of repetitive O₂ peaks by additional NO₂−
We have previously demonstrated that anoxic cultures of P. aeruginosa rapidly deplete NO₂− (Line et al., 2014), presumably resulting in a transient accumulation of NO. Accordingly, subsequent injection of NO₂− could be expected to trigger a further burst of NO to supply a putative nitric oxide dismutase (Nod) activity to generate a secondary O₂ peak (Ettwig et al., 2010). Despite this, a significant secondary O₂ peak could not be stimulated by addition of extra NO₂−, and the effect of addition of extra NO₂− was insignificant (Figure 5). These data suggest that the generation of the O₂ peaks is upregulated during the transition from aerobic to anaerobic lifestyle and by the presence of NO. When the anaerobic lifestyle has been established the O₂ peaks could not be stimulated by NO.
Role of NO in oxygen release

The apparent lack of Nod activity directly linking NO and the observed O2 dynamics led us to further validate the role of NO in stimulating O2 peaks by studying O2 dynamics in *P. aeruginosa* strains with mutations in enzymes implicated in the NO cycle (Figures 2 and 6). As expected, both addition of 1 mM NO2/C0 and addition of the NO donor DPTA NONOate resulted in increased O2 peaks in both the WT and the ΔnorB (p < 0.002). Supporting the idea that O2 peak formation is mediated by denitrification to NO (in both WT and ΔnorB supplemented with NO2/C0) is the absence of detectable O2 peaks seen in ΔnirS-N, which is unable to generate NO from NO2/C0 both with and without 1 mM NO2/C0. The requirement of NO2/C0 reduction could be bypassed by addition of the NO donor DPTA NONOate, which increased the O2 peak significantly (p < 0.0001) by the ΔnirS-N and thus further confirms the direct involvement of NO in the generation of the transient O2 peaks (Figure 6).

The peaks of the Δfhp mutant were not affected by additional NO2/C0 or NO, indicating lack of impact of fhp on the generation of O2 peaks in our experiments (Figure 6).

Endogenous NO release precedes O2 peaks

Concentrations of NO and O2 in a ΔnorB culture with 1 mM NO2/C0 were recorded simultaneously (Figure 7). These measurements demonstrated that the initiation of the O2 peaks was preceded by an increase in the concentration of NO (Figure 7). This sequence validated that a raise in the concentration of NO may lead to release of O2 in cultures of *P. aeruginosa*. In addition, because this experiment employed electrochemical sensors, the specificity of our measurement of NO-induced O2 peaks by optical O2 sensors was confirmed.

Removal of released O2

We asked whether the released O2 was subsequently removed by the aerobic TOX or by the aerobic NO detoxifying Fhp. In an experiment, 2 mM potassium cyanide (KCN) was added to a ΔnorB culture close to the observed O2 peak to inhibit the TOX with KCN (Jurtshuk et al., 1975). A small increase in O2 was observed just after injection of both 0 mM and 2 mM KCN, as the solutions were prepared in normoxic conditions. In the control injection (0 mM), O2 was rapidly removed, whereas a gradual increase in O2 was observed following injection of 2 mM KCN (Figure S4). The O2 concentration then decreased modestly ~0.5 h after KCN injection.

DISCUSSION

The rapid depletion of the initial normoxic O2 (~200 μM) in the cultures can trigger the switch from aerobic to anaerobic respiration by denitrification of NO2/C0 via Nir to generate NO (Schobert and Jahn, 2010). In
NorB, which lacks Nor activity, NO is presumed to accumulate, leading to the hypothesis that NO may mediate O$_2$ release by *P. aeruginosa*. In an effort to identify the source of the observed O$_2$ peaks we considered several possibilities.

O$_2$ release could not be linked to known homologues for nitric oxide dismutation
Unlike the study of Robinson et al. (2017), where culture vials were open to allow diffusion of atmospheric O$_2$, our experiments were performed in sealed, airtight vials excluding the possibility of an external O$_2$ source. Initially, the novel possibility of dismutation of NO to O$_2$ in *P. aeruginosa* was considered. Such a pathway is plausible in energetic terms ($2$ NO + O$_2$ + N$_2$. $\Delta G^{\circ} = -173.1$ kJ/mol O$_2$) with a similar dismutation of chlorite (ClO$_2^-$) by a chlorite dismutase observed in chlorate-reducing bacteria (Mehboob et al., 2009; van Ginkel et al., 1996). In addition, evidence for this pathway and putative nitric oxide dismutase (Nod) enzymes has been uncovered in the anaerobic alkane oxidizers *Candidatus* Methylomirabilis oxyfera, Hdn1, and isolates from contaminated aquifers and wastewater treatment centers, suggesting that NO dismutation could be widespread in bacteria (Ettwig et al., 2010; Zhu et al., 2017). However, no homologous enzyme in *P. aeruginosa* was found via BLAST searches of putative Nod enzyme sequence alignments to the sequenced *P. aeruginosa* genome (pseudomonas.com). We did, however, find high-level sequence similarity in other homologous enzymes such as denitrification enzyme genes. This resemblance is in line with the hypothesis of the Nod enzyme being a heme/copper terminal oxidase-like membrane protein (Ettwig et al., 2012).

NO mediates release of O$_2$ in *P. aeruginosa*
Observations from the present study indicate that formation of O$_2$ peaks in liquid cultures of *P. aeruginosa* are (1) dependent on initial presence of O$_2$ via normoxic initiation of cultures and (2) amplified by indirect donation of NO via reduction of NO$_2^-$ during denitrification. A lack of Nor activity has been suggested to result in a tighter regulation of NO production in *P. aeruginosa* via decreased nitrite reduction and increased scavenging by iron-containing enzymes as a survival strategy (Borrero-de Acuña et al., 2016; Yoon et al., 2007). To further support the role of NO in the formation of O$_2$ peaks, direct donation was also demonstrated with DPTA NONOate. With both NO$_2^-$ and DPTA NONOate, the greatest induction of O$_2$ peaks was observed in strains lacking Nor activity. The Δfrp mutant was apparently not able to impact the observed O$_2$ peaks most likely because oxic conditions are required for the conversion of NO and O$_2$ → NO$_3^-$ (Gardner and Gardner, 2002) and in this mutant Nor was still actively reducing NO to N$_2$O. Accordingly, we hypothesize that the peaks generated by NO result either from the release of stored O$_2$ or from de novo synthesis. Interestingly, the peaks of O$_2$ may reach 20 μM and are derived from $2.5 \times 10^7$ bacteria/mL. Assuming the volume of one single *P. aeruginosa* to vary from $10^{-15}$ L to $1.45 \times 10^{-15}$ L, we have calculated the internal concentration of O$_2$ to approximate 1 M. It is, however, difficult to explain storage of O$_2$ at concentrations as high as 1 M by biological or relevant physical mechanisms. Therefore, we...
propose that the observed NO-mediated oxygen peaks are resulting from de novo production. Such a mechanism is highly possible as demonstrated by the nitric oxide dismutase activity in M. oxyfera (Ettwig et al., 2010), but further investigations are needed to identify the enzymatic set-up for NO-mediated generation of O₂ by P. aeruginosa. Likewise, the missing knowledge of the exact origin of O₂ is a limitation of this study and could be examined by isotopic labeling using ¹⁸O-labeled NO₂/CO₂ (Ettwig et al., 2010).

We speculate that this phenomenon of NO-mediated appearances of O₂ may play a role for P. aeruginosa in adapting to a switch between aerobic and anaerobic metabolism by providing a small O₂ burst at the onset of denitrification. Feeding of the aerobic metabolism by the extra peaks of O₂ is evidenced by the observation that the peak was shown to be removed mainly by the aerobic TOX (Figure S4). In contrast, the subsequent modest reduction in O₂ concentration in the inhibited TOX could be due to Fhp activity. A small O₂ burst at the onset of anoxia may offer improved energy generation for instance to aid transcription of the many denitrification modules (Borrero-de Acuña et al., 2016) and/or Fhp-mediated protection from NO generated by the host response during chronic infections (Arai et al., 1997; Kolpen et al., 2014b; Wheeler et al., 1997). Furthermore, the observed peaks of O₂ may affect the depletion of dissolved O₂ when estimating O₂ consumption rates measured by microrespirometry and may thus lead to overestimating the actual specific respiration rates.

Our demonstration of the involvement of NO in the generation of O₂ peaks in near anoxic conditions suggests the presence of an NO-sensing mechanism that is active at low O₂ tensions in P. aeruginosa. Interestingly, at low O₂ tensions Anr activates the expression of Dnr (Arai et al., 1997). The ability of Dnr to respond to NO by transcriptional activation may provide cues to the regulation of the putative enzymes engaged in the generation of the O₂ peaks.

NO is already implicated in a long list of cellular functions including signaling, motility, toxicity, and regulatory functions (Arora et al., 2015; Bowman et al., 2011; Hossain and Boon, 2017; Radi, 1996). Accordingly, Nor is an important enzyme for modulating virulence of P. aeruginosa (Arai and Iiyama, 2013). Evidence for a hybrid chain of aerobic and anaerobic respiration has been previously proposed, as denitrification genes are upregulated and electrons accepted by denitrification are increased under microoxic conditions (Alvarez-Ortega and Harwood, 2007; Chen et al., 2003), and the addition of NO₂⁻ to aerobic cultures induces increased expression of multiple transcripts involved in denitrification (Filiatrault et al., 2005). Furthermore, two of P. aeruginosa’s five terminal oxidases for aerobic respiration, cbb₃-1 and cbb₃-2, which have high affinity for O₂, are more highly upregulated in anoxic conditions (Hamada et al., 2014; Kawakami et al., 2010) and accordingly may be able to respond to small O₂ releases during transitions from aerobic to anaerobic conditions where an overlap between aerobic and anaerobic metabolism in P. aeruginosa may happen at low O₂ concentrations. This could be beneficial during chronic infection, where dynamic or low supplies of
O$_2$ are observed (Wessel et al., 2014; Worlitzsch et al., 2002) and simultaneous expression of the two pathways would allow flexibility to adapt to such environmental changes. However, further studies are required to determine the precise physiological implications of our observations.

Our proposed nitric oxide dismutase activity may provide important benefits in the setting of nitrate removal by bypassing the denitrification step leading to N$_2$O formation. Thus, the Nod has the potential to allow NO$_3^-$/CO$_2$ removal with emission of O$_2$ and N$_2$, but without emission of N$_2$O, which is one of the most powerful greenhouse gases.

In conclusion, we demonstrate a role for NO in cellular O$_2$ dynamics where accumulation of NO during hypoxic conditions lead to increased concentrations of O$_2$, potentially bridging the conversion between aerobic and anaerobic metabolism.

Limitations of the study
Although this study has demonstrated the direct role of NO in the observed O$_2$ release, we did not identify the origin of this release whether genetic or enzymatic. The source of the released O$_2$ could potentially be identified by isotopic labeling, and the potential involved genetic setup may be revealed by transcriptomic analysis.

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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103404.
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AUTHOR CONTRIBUTIONS
Conceived and outlined the study: LL, TB, POJ; collected data: LL, VS, THJ; analyzed data: ML, LL, VS, THJ, MK, TB, POJ; created mutant strains: MR, TTN, MT, NN; wrote the manuscript: ML, LL, POJ with editorial inputs from all remaining authors.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** | | |
| *Pseudomonas aeruginosa* holloway PAO1 (WT) | Holloway et al. (1979) | NA |
| *Pseudomonas aeruginosa* nir operon deletion mutant (ΔnirS-N) | Toyofuku et al. (2014) | NA |
| *Pseudomonas aeruginosa* norB deletion mutant (ΔnorB) | This paper | NA |
| *Pseudomonas aeruginosa* fhp deletion mutant (Δfhp) | This paper | NA |
| *Pseudomonas aeruginosa* fhp, norB double deletion mutant (ΔfhpΔnorB) | This paper | NA |
| *Escherichia coli* mobiliser strain (S17-1) | Simon et al. (1986) | NA |
| *Escherichia coli* cloning strain (DH5α) | Simon et al. (1986) | NA |
| **Chemicals, peptides, and recombinant proteins** | | |
| LB | | |
| NaNO₂ | Sigma, Denmark | CAS number: 7632-00-0 |
| DPTA NONOate | Cayman Chemical, USA | CAS number: 146,724-95-0 |
| Carboxy-PTIO | Sigma, Denmark | CAS number: 148,819-94-7 |
| **Oligonucleotides** | | |
| norB-F. | This paper | 5'-CGGAATTCCGCCGTTTATACGCCGCAGG-3' |
| norB-R. | This paper | 5'-GCCAAGCTTGTAGGTCGCGACGCC-3' |
| fhp-UpF. | This paper | 5'-GGGGACAAAGTTTTACAAAAAGCAGGCTCATCGTAGGATCGGGCAGG-3' |
| fhp-UpR. | This paper | 5'-GGCCGGAGCCAAGAACTCGGCACGTTGGCATTGGAC-3' |
| fhp-DnF. | This paper | 5'-GAGTTCTCGGTCCCGGC-3' |
| fhp-DnR. | This paper | 5'-GGGGACCACTTTGTACAAGAAAGCCTGGGTAAGGAAGAAGCTGGGTAAGGAAG-3' |
| **Recombinant DNA** | | |
| pHSG398. plC type cloning vector, Cm<sup>R</sup> | TaKaRa, Japan | NA |
| pG19II. Derivative of pK19 mob sacB, Gm<sup>R</sup> | Maseda et al. (2004) | NA |
| pG19norB. norB deletion cassette in pG19II | This paper | NA |
| pDONRPEX18Gm. Cloning vector, Gm<sup>R</sup> | Hmel et al. (2015) | NA |
| pENTRfhp. fhp deletion cassette in pDONRPEX18Gm | This paper | NA |
| pRK600. Mobilization vector, Cm<sup>R</sup> | Kessler et al. (1992) | NA |
| * Cm<sup>R</sup> = Chloramphenicol resistant; Gm<sup>R</sup> = Gentamicin resistant | | |
| **Software and algorithms** | | |
| GraphPad prism 8.4.3 | GraphPad Software | https://www.graphpad.com |
| Pyro oxygen logger | PyroScience | https://www.pyroscience.com |
| Sensortrace suite | Unisense | https://www.unisense.com |
| **Other** | | |
| Micro-respiration vials | PyroScience, Germany | OXVIAL4 |
| Adapter ring | PyroScience, Germany | ADVIAL4 |
| Optical fiber | PyroScience, Germany | SPFIB-BARE |

(Continued on next page)
RESOURCES AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Peter Østrup Jensen (peter.oestrup.jensen@regionh.dk).

Materials availability
Mutant strains generated for use in this study will be made available on request but we may require a completed Materials Transfer Agreement if there is potential for commercial application.

Data and code availability
The article includes all datasets generated or analyzed during this study.
- Data reported in this paper will be shared by the lead contact upon reasonable request.
- This study did not generate any code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacterial strains and deletion mutant construction
The strains and plasmids utilized in this study are listed in the key resources table.

The ΔnirS-N mutant defective for nitrite reduction to nitric oxide by Nir was constructed as previously described by allelic exchange (Ma et al., 1999; Toyofuku et al., 2014).

The pG19II derived pG19norB plasmid carrying a deletion cassette of norB of the norCB operon encoding nitric oxide reductase was constructed using the method described previously (Maseda et al., 2004). In brief, the norB region was PCR amplified using primers norB-F/norB-R (Key resources table), and the PCR fragment was subsequently restriction digested with EcoRI and HindIII and then ligated into the multi-cloning site of cloning vector pHSG398. The norB fragment was deleted by PstI digestion followed by self-ligation. The deletion fragment was subcloned into pG19II, restriction digested by EcoRI and HindIII, to generate pG19norB which was introduced into the mobiliser E. coli strain S17-1 and conjugated into P. aeruginosa PAO1 (Maseda et al., 2004) yielding the ΔnorB mutant defective in nitric oxide reduction by nitric oxide reductase. Deletion was confirmed with PCR.

The fhp deletion vector was constructed essentially using the method published by Hmelo and colleagues (Hmelo et al., 2015). In brief, upstream and downstream regions flanking fhp were amplified using fhp-UpF/fhp-UpR and fhp-DnF/fhp-DnR primer pairs containing external attB attachment sites. The in-frame deletion cassette was constructed from the two fragments using splicing by overlap extension PCR and cloned into the donor vector pDONRPEX18Gm using Gateway BP Clonase (Thermo Fischer Scientific, Denmark). Introduction of the reaction into E. coli DH5α created pENTRfhp.

The integrity of the cassette was confirmed by sequencing of the vector. Deletion of fhp in P. aeruginosa PAO1 and ΔnorB was performed by introducing pENTRfhp using trip parental mating and plasmid pRK600 as the conjugation helper. Integration of the vector into the P. aeruginosa chromosome by a single cross-over event was confirmed by selection for transconjugants on plates containing gentamicin. Excision of the vector backbone by a second cross-over event was selected for by sacB-mediated counter selection on plates containing sucrose. The second cross-over event was confirmed by restored sensitivity toward gentamicin. Finally, clones containing the fhp deletion allele were selected using colony PCR yielding the Δfhp deletion mutant and the ΔfhpΔnorB double deletion mutant (See Table 1).
METHOD DETAILS

Growth conditions

Strains were plated from frozen cultures and single colonies used to initiate normoxic overnight cultures in Luria Bertani (LB) media. For normoxically initiated experiments, cultures were adjusted to OD\textsubscript{600} = 0.1 and regrown for \sim 2 h in LB to OD\textsubscript{600} = 0.4 to ensure all cells were actively growing. For anoxically initiated experiments, the regrowth was performed in anoxic LB. As anoxic growth is slow and did not reach OD\textsubscript{600} = 0.4, the regrowth was performed for 2 h - still representing an active growth phase and reaching OD\textsubscript{600} = 0.2.

Anoxic media was prepared in an anoxic bench (Concept 400, Thermo Scientific, Denmark) with O\textsubscript{2} < 0.02%, as routinely monitored with a luminescent dissolved O\textsubscript{2} sensor (HQ40d multi, HACH Company, USA). Stable solutions of LB media were established by addition of NaNO\textsubscript{2} (Sigma, Denmark). Anoxic LB media was produced by sealing with parafilm (Bemis, USA) followed by equilibration in the anoxic bench for a minimum of 3 days to eliminate O\textsubscript{2}. Unstable solutions containing DPTA NONOate (Cayman Chemical, USA) were prepared as stock solutions in 0.01 M NaOH (stable at 0°C for 24 h) and immediately prior to experiments, the stock solutions were freshly diluted in anoxic media in the anoxic bench and vigorously shaken to remove the remaining O\textsubscript{2}.

In NO\textsubscript{2}\textsuperscript{−} supplemented media we chose 100 μM NO\textsubscript{2}\textsuperscript{−} and 1 mM NO\textsubscript{2}\textsuperscript{−}, corresponding to non-toxic physiological concentrations able to allow rapid O\textsubscript{2} depletion (Major et al., 2010; Yoon et al., 2006). For the serial injection of NO\textsubscript{2}\textsuperscript{−}, 100 μM was chosen as this is still representative of a physiological concentration, able to induce significant O\textsubscript{2} peaks while minimizing toxic effects of NO accumulation from NO\textsubscript{2}\textsuperscript{−} reduction. Similarly, 100 μM of the NO donor DPTA NONOate was used to minimize toxicity while providing a robust NO donation, corresponding to release of 200 μM NO with a half-life of 3 h at pH 7.4, 37°C (Hrabie et al., 1993; Keefer et al., 1996).

Microrespirometric O\textsubscript{2} measurements

Measurements of O\textsubscript{2} dynamics were performed in micro-respiration vials (OXVIAL4, PyroScience, Germany), i.e. 4 mL glass vials fitted with a contactless optical O\textsubscript{2} sensor spot for detection of dissolved O\textsubscript{2} (0.02–100%) with a response time of <15 s. Vials were sealed by airtight lids fitted with rubber septa. Excitation and emission of the sensor was achieved with an adapter ring (ADVIAL4, PyroScience, Germany) connected via an optical fiber (SPFIB-BARE, PyroScience, Germany) to a fiber-optic O\textsubscript{2} meter with 4 O\textsubscript{2} channels and 1 temperature sensor channel (FireStingO\textsubscript{2}, PyroScience, Germany). The four O\textsubscript{2} respiration vials were fitted with 2 mm glass-coated magnetic stirrer bars placed on a magnetic stirring plate stirring at 700 rpm. The entire system was placed in a room maintained at 37°C, as monitored with a submersible temperature sensor (TSUB21, PyroScience, Germany) with an accuracy of ±0.5°C. Sensors were calibrated according to factory settings and O\textsubscript{2} signals, compensated by temperature detection were logged with Pyro Oxygen Logger software (PyroScience, Germany). Each micro-respiration vial was filled with the relevant regrown P. aeruginosa cultures diluted to a final OD\textsubscript{600} = 0.04, corresponding to a cell density capable of rapid O\textsubscript{2} depletion. Care was taken to avoid enclosure of air bubbles when closing and tightening the lid of the vials. For anoxic experiments, micro-respiration vials were filled with anoxic medium and sealed inside the anoxic bench. A representative trace of the O\textsubscript{2} concentration change over time is shown in Figure 3.

For NO\textsubscript{2}\textsuperscript{−} supplementation, P. aeruginosa cultures were simply diluted into LB containing 1 mM NO\textsubscript{2}\textsuperscript{−} in the respiration vials. Due to toxicity concerns and an inhibitory effect on initial O\textsubscript{2} depletion (Mason et al., 2006), DPTA NONOate was injected to the vial by syringe and needle through the septum to a final concentration of 100 μM after initial O\textsubscript{2} depletion. Subsequent NO\textsubscript{2}\textsuperscript{−} stimulation and LB controls were injected in the same way. Injections were prepared in anoxic media in the anoxic bench to avoid O\textsubscript{2} injection into the system.

Simultaneous O\textsubscript{2} and NO measurements

Micro-respiration microsensors for detection of O\textsubscript{2} and NO concentrations were applied simultaneously using a micro-respiration system with a custom-made glass double chamber (4mL volume) (UniSense A/S, Aarhus, Denmark). The double chamber was equipped with micro stir-bars (0.2 mm, glass coated) and filled with the donor P. aeruginosa culture diluted to a final OD\textsubscript{600} = 0.04. The lids were closed with an overflow to avoid entrapment of air bubbles. The O\textsubscript{2} microsensor was two-point calibrated in 100%
air-saturated LB medium (purged with air using an aquarium pump) and in anoxic LB medium (purged with 
nitrogen gas). The NO microsensor was linearly calibrated via chemical NO synthesis according to manu-
facturer’s recommendation (UniSense A/S, Aarhus, Denmark) using NaNO₂ as an NO source, which 
included the following NO concentrations: 1, 2, 4, 8, and 16 μM.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Data from biological replicates were normalized by logarithmical transformation. Normalized data were 
compared by two-way ANOVA with Bonferroni multiple comparisons correction with Prism (v. 8.4.3, Graph-
Pad Software). p < 0.05 was considered statistically significant.