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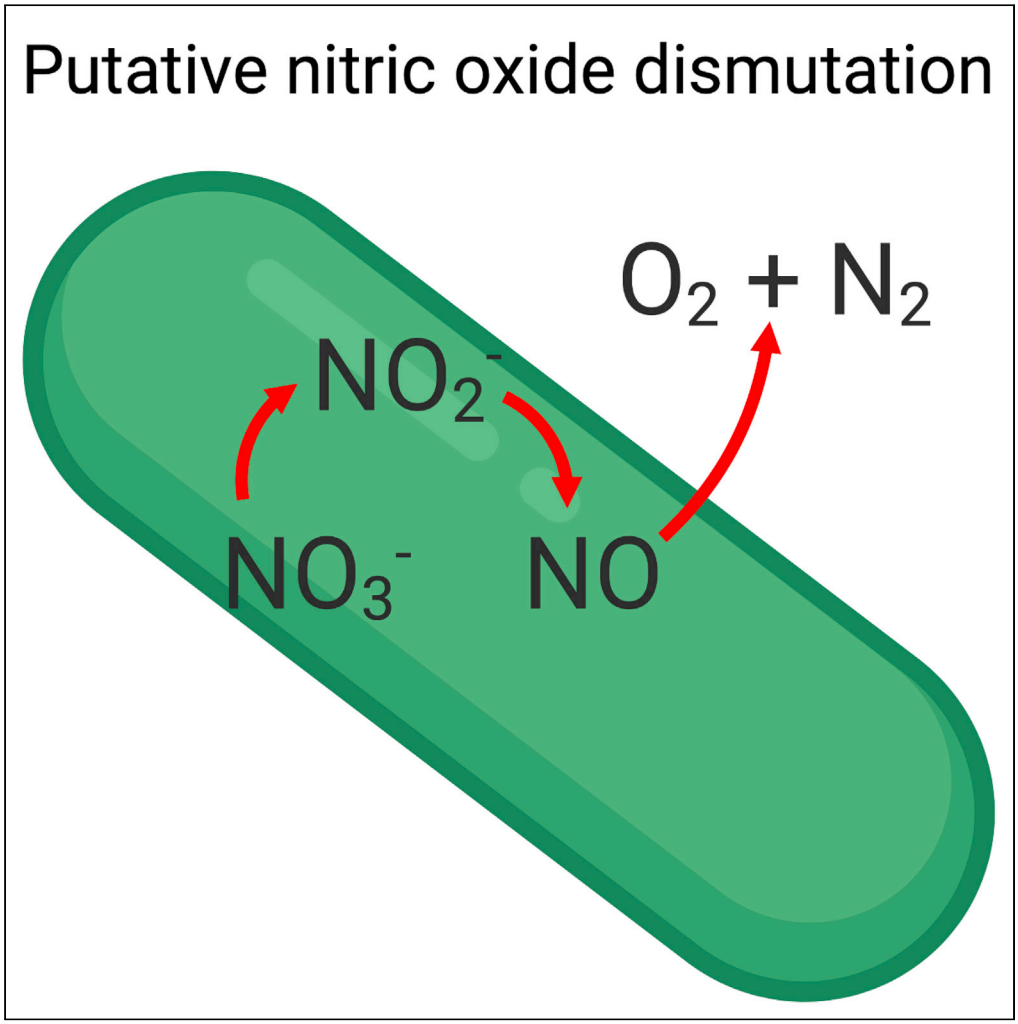

Mads Lichtenberg, Laura Line, Verena Schrameyer, ..., Michael Kühl, Thomas Bjarnsholt, Peter Østrup Jensen

peter.oestrup.jensen@regionh.dk

Highlights

Pseudomonas aeruginosa was found to release O_2 at the onset of anoxia

Peaks of O_2 were amplified in a nitric oxide reductase (NOR) mutant

The O_2 release was mediated by nitric oxide (NO)

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

Mads Lichtenberg,[1] Laura Line,[1] Verena Schrameyer,[2] Tim Holm Jakobsen,[1] Morten Levin Rybtke,[1] Masanori Toyofuku,[3] Nobuhiko Nomura,[3] Mette Kolpen,[4] Tim Tolker-Nielsen,[1] Michael Kühl,[2] Thomas Bjarnsholt,[1,4] and Peter Østrup Jensen[1,4,5,6,*]

SUMMARY

Denitrification supports anoxic growth of *Pseudomonas aeruginosa* in infections. Moreover, denitrification may provide oxygen (O_2) resulting from dismutation of the denitrification intermediate nitric oxide (NO) as seen in *Methylomirabilis oxyfera*. To examine the prevalence of NO dismutation we studied O_2 release by *P. aeruginosa* in airtight vials. *P. aeruginosa* rapidly depleted O_2 but NO supplementation generated peaks of O_2 at the onset of anoxia, and we demonstrate a direct role of NO in the O_2 release. However, we were not able to detect genetic evidence for putative NO dismutases.

The supply of endogenous O_2 at the onset of anoxia could play an adaptive role when *P. aeruginosa* enters anaerobiosis. Furthermore, O_2 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 (O_2) is missing (Zumft, 1997). The denitrification pathway is comprised of stepwise reductions of nitrate (NO_3^-) to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), and finally dinitrogen (N_2) 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 N_2O, *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; Høiby 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 O_2 for the generation of the toxic reactive oxygen species superoxide (O_2^-) 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 (Kragh 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 NO_3^- to N_2 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 O_2 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 NO_3^- (<1 mM NO_3^-) are able to support growth of *P. aeruginosa* comparable with the slow rates observed during chronic

[1]Costerton Biofilm Center, Department of Immunology and Microbiology, Faculty of Health Sciences, University of Copenhagen, 2200 Copenhagen, Denmark

[2]Marine Biological Section, Department of Biology, University of Copenhagen, 3000 Helsingør, Denmark

[3]Microbiology Research Center for Sustainability (MiCS), Faculty of Life and Environmental Sciences, University of Tsukuba, 305-8577 Tsukuba, Japan

[4]Department of Clinical Microbiology, Rigshospitalet, 2100 Copenhagen, Denmark

[5]Center for Rheumatology and Spine Diseases, Institute for Inflammation Research, Rigshospitalet, 2100 Copenhagen, Denmark

[6]Lead contact

*Correspondence: peter.oestrup.jensen@regionh.dk

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Figure 1. Denitrification pathway

Reduction of nitrate (NO_3^-) to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O), and finally dinitrogen (N_2). The four reductase enzymes, nitrate reductase (NarGHI), nitrite reductase (NirS), nitric oxide reductase (NorCB), and nitrous oxide reductase (NosZ), are shown above.

CF lung infection (Kragh et al., 2014; Line et al., 2014). Likewise, physiological concentrations of NO_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; Cutruzzolà 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). *P. aeruginosa* expresses two known enzymatic NO detoxification mechanisms, with NO being reduced to N_2O by Nor in the denitrification pathway under anoxic conditions (Kumita et al., 2004) and by the nitric oxide dioxygenase activity of flavohemoglobin (Fhp) generating NO_3^- from O_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 *Methylomirabilis oxyfera*, where dismutation of NO, nitric oxide dismutase (Nod), results in the generation of O_2 and N_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 *P. aeruginosa* by employing microrespirometry of appropriate precursors and knockout mutants according to a proposed NO cycle in *P. aeruginosa* in the presence of putative dismutation of NO (Figure 2).

RESULTS

Oxygen release in nitric oxide reductase mutant *P. aeruginosa*

Initial observation of O_2 concentration in airtight liquid cultures initiated in normoxic conditions (~200 μM O_2) of *P. aeruginosa* WT and Δ*norB* displayed rapid depletion of O_2 as expected due to the energetic preference of aerobic respiration in *P. aeruginosa* (Chen and Strous, 2013). Unexpectedly, however, small peaks of O_2 were detected shortly after the initial O_2 depletion (hereafter referred to as O_2 peaks) in cultures of

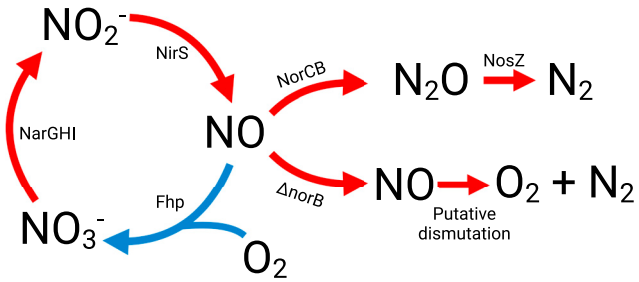

Figure 2. The explored proposed NO cycle in *P. aeruginosa* in the presence of putative dismutation of NO

After depletion of O_2, *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_3^- to NO_2^-, NO, N_2O, and finally N_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_2O. In oxic conditions, NO can be removed by the dioxygenase activity of flavohemoglobin (Fhp) generating NO_3^- from oxidation of NO by O_2. In a NorCB-deficient mutant (*ΔnorB*) NO accumulates, resulting in increased amount of NO available for dismutation to O_2 and N_2. Blue arrows = oxic processes. Red arrows = anoxic processes. Based on Robinson and Brynildsen (2016).

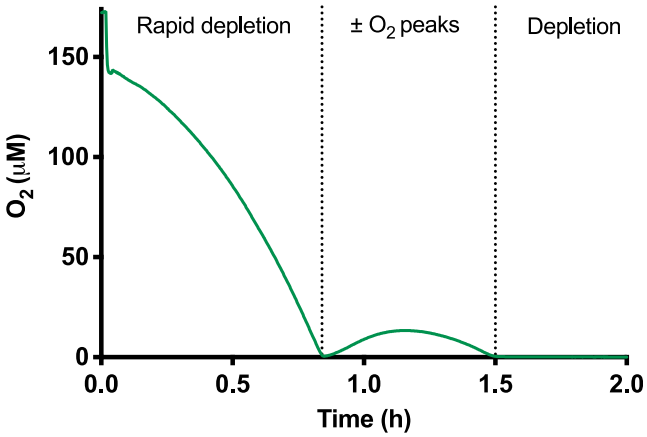

Figure 3. Example of microrespirometry O_2 trace from normoxically initiated *ΔnorB* culture supplemented with 1 mM NO_2^-

Rapid depletion phase demonstrated sharp decrease of O_2 in the normoxic, cell-free media upon cell addition. O_2 was then depleted by the growing culture. ± O_2 peaks phase indicates that in some cultures after the initial O_2 was depleted, O_2 subsequently increased in a transient peak. Depletion phase indicates the following plateau where O_2 had been completely depleted.

WT and Δ*norB* supplemented with NO_2^- (Figure 3). The O_2 peaks in the WT cultures were significantly amplified ($p = 0.0017$) in LB media supplemented with 1 mM NO_2^-. The WT *P. aeruginosa* has a complete denitrification pathway and thus produces NO from NO_2^- supplementation followed by removal by the subsequent nitric oxide reductase step—lacking in Δ*norB*. Accordingly, the O_2 peaks in the Δ*norB* cultures were significantly enhanced with supplementation of the LB media with as little as 0.1 mM NO_2^- ($p = 0.0018$) and with 1 mM NO_2^- ($p < 0.0001$). In addition, the O_2 peaks in the Δ*norB* cultures were significantly higher than in the WT at 0.1 mM NO_2^- and 1 mM NO_2^- ($p = 0.0002$) with peaks reaching a mean of 23.1 ± 1.4 μM O_2 (mean ± SEM) (Figure 4). To exclude a possible leaking of incoming O_2 from the surrounding, vials loaded with LB-media equilibrated in an anaerobic bench showed no sign of incoming O_2 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 O_2 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 O_2 induced by NO_2^- is mediated by NO, we demonstrated a dose-dependent reduction of the O_2 peaks by varying amounts of CARBOXY-PTIO, which is an NO scavenger, to cultures of Δ*norB* supplemented with 1 mM NO_2^-. The dose-dependent decrease of the measured O_2 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 O_2 peaks may be obtained by examining *P. aeruginosa* with engineered expression of the bacterial nitric oxide synthase.

Lack of O_2 peaks in anoxically initiated cultures

Cultures initiated normoxically, and supplemented with 1 mM NO_2^-, displayed the characteristic O_2 peaks but in anoxically initiated cultures of both WT and Δ*norB*, also supplemented with 1 mM NO_2^-, no O_2 peaks were detected (Figure 4).

Lack of repetitive O_2 peaks by additional NO_2^-

We have previously demonstrated that anoxic cultures of *P. aeruginosa* rapidly deplete NO_2^- (Line et al., 2014), presumably resulting in a transient accumulation of NO. Accordingly, subsequent injection of NO_2^- could be expected to trigger a further burst of NO to supply a putative nitric oxide dismutase (Nod) activity to generate a secondary O_2 peak (Ettwig et al., 2010). Despite this, a significant secondary O_2 peak could not be stimulated by addition of extra NO_2^-, and the effect of addition of extra NO_2^- was insignificant (Figure 5). These data suggest that the generation of the O_2 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 O_2 peaks could not be stimulated by NO.

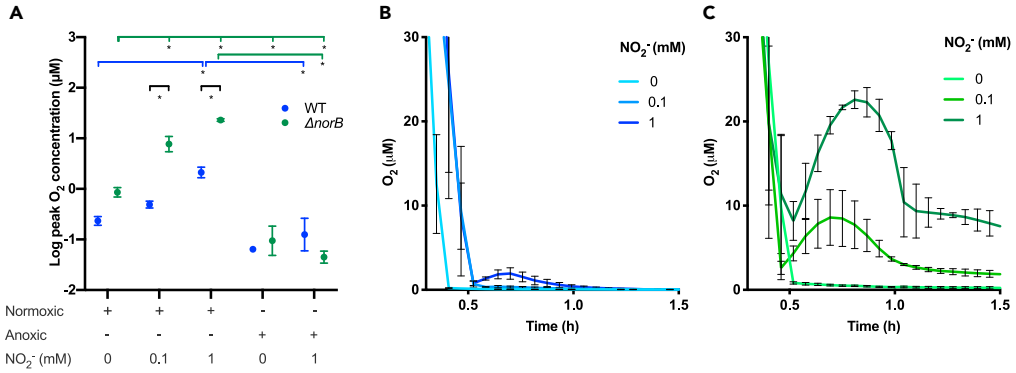

Figure 4. O_2 release is amplified in *ΔnorB* mutants and is dependent on initial presence of O_2

Amplitude of O_2 peaks in normoxically and anoxically initiated cultures supplemented with increasing concentrations of NO_2^-. Blue = wild-type (WT), green = *ΔnorB* mutant. Brackets depict significant differences. (A) Log transformed maximum O_2 peak concentrations under the different conditions (means ± SEM; three biological replicates). In the WT there was a significant O_2 peak increase only upon addition of 1 mM NO_2– ($p < 0.002$). In the *ΔnorB* mutant, a significant effect on O_2 peak amplitude was seen both upon addition of 0.1 ($p < 0.002$) and 1 mM NO_2^- ($p < 0.0001$). Significant O_2 peak formation was dependent on initial presence of O_2 in both WT ($p < 0.002$) and the *ΔnorB* mutant ($p < 0.0001$). Finally, the *ΔnorB* mutant showed higher O_2 peaks than the WT at both 0.1 mM ($p < 0.0002$) and 1 mM NO_2^- supplement ($p < 0.0002$). O_2 concentration traces over time in normoxically initiated cultures with increasing concentrations of NO_2^- (means ± SEM; three biological replicates) of (B) WT *P. aeruginosa* and (C) *ΔnorB P. aeruginosa* mutant (means ± SEM; three biological replicates). (*: $p < 0.05$. Data were analyzed by two-way ANOVA).

Role of NO in oxygen release

The apparent lack of Nod activity directly linking NO and the observed O_2 dynamics led us to further validate the role of NO in stimulating O_2 peaks by studying O_2 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 NO_2^- and addition of the NO donor DPTA NONOate resulted in increased O_2 peaks in both the WT and the Δ*norB* ($p < 0.002$). Supporting the idea that O_2 peak formation is mediated by denitrification to NO (in both WT and Δ*norB* supplemented with NO_2^-) is the absence of detectable O_2 peaks seen in Δ*nirS-N*, which is unable to generate NO from NO_2^- both with and without 1 mM NO_2^-. The requirement of NO_2^- reduction could be bypassed by addition of the NO donor DPTA NONOate, which increased the O_2 peak significantly ($p < 0.0001$) by the Δ*nirS-N* and thus further confirms the direct involvement of NO in the generation of the transient O_2 peaks (Figure 6).

The peaks of the *Δfhp* mutant were not affected by additional NO_2^- or NO, indicating lack of impact of fhp on the generation of O_2 peaks in our experiments (Figure 6).

Endogenous NO release precedes O_2 peaks

Concentrations of NO and O_2 in a Δ*norB* culture with 1 mM NO_2^- were recorded simultaneously (Figure 7). These measurements demonstrated that the initiation of the O_2 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 O_2 in cultures of *P. aeruginosa*. In addition, because this experiment employed electrochemical sensors, the specificity of our measurement of NO-induced O_2 peaks by optical O_2 sensors was confirmed.

Removal of released O_2

We asked whether the released O_2 was subsequently removed by the aerobic TOX or by the aerobic NO detoxifying Fhp. In an experiment, 2mM potassium cyanide (KCN) was added to a Δ*norB* culture close to the observed O_2 peak to inhibit the TOX with KCN (Jurtshuk et al., 1975). A small increase in O_2 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), O_2 was rapidly removed, whereas a gradual increase in O_2 was observed following injection of 2 mM KCN (Figure S4). The O_2 concentration then decreased modestly ~0.5 h after KCN injection.

DISCUSSION

The rapid depletion of the initial normoxic O_2 (~200 µM) in the cultures can trigger the switch from aerobic to anaerobic respiration by denitrification of NO_2^- via Nir to generate NO (Schobert and Jahn, 2010). In

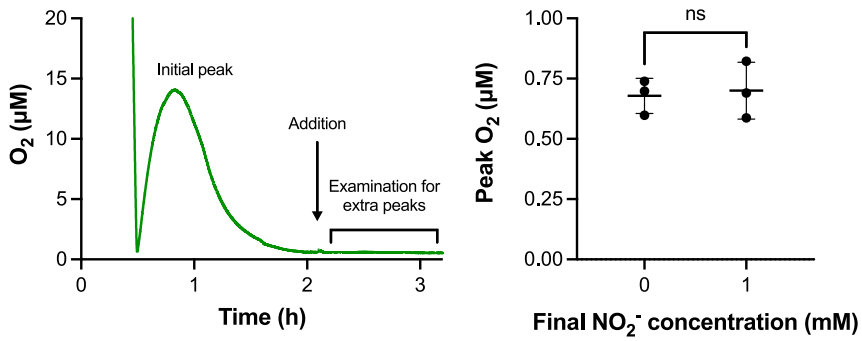

Figure 5. Lack of repeated O_2 peak stimulation suggest exogenous O_2 origin rather than *de novo* synthesis

(Left) Amplitude of O_2 peaks in normoxically initiated *ΔnorB* cultures with 1 mM NO_2^- supplement were added additional LB with NO_2^- (final concentration: 1 mM) or without NO_2^- when the concentration of O_2 declined to the levels before initiation of the first peak. The following hour was examined for extra O_2 peaks according to the highest measured value of O_2 concentration. This is stimulated in series with 100 μM NO_2^- or anoxic LB.).

(Right) Individual points with the maximum O_2 concentration after each supplementation (mean ± SEM; three biological replicates). There was no significant difference between maximum O_2 concentrations of the NO_2^- supplement versus the control ($p > 0.05$; two-way ANOVA).

Δ*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 homoloques 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 ($2\,NO \rightarrow O_2 + N_2$, $\Delta G^{\circ\prime} = -173.1$ 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 *Δfhp* 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 $O_2 \rightarrow NO_3^-$ (Gardner and Gardner, 2002) and in this mutant Nor was still actively reducing NO to N_2O. 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 2.5×10^7 bacteria/mL. Assuming the volume of one single *P. aeruginosa* to vary from 10^{-15} L to 1.45×10^{-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

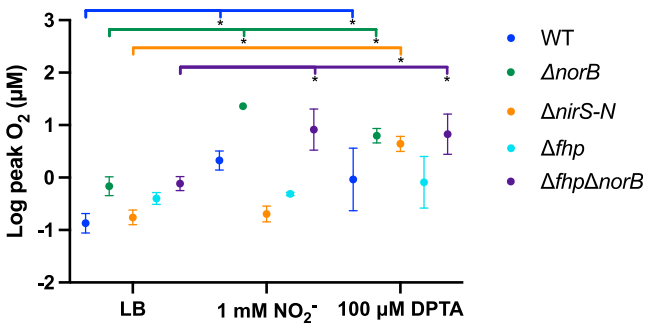

Figure 6. NO mediates O_2 release

Log transformed amplitude of O_2 peaks in normoxically initiated cultures of WT and mutants in *ΔnorB*, *ΔnirS-N*, *Δfhp*, and *ΔfhpΔnorB* treated with ±1 mM NO_2^- or ±100 µM DPTA NONOate (mean ± SEM; three biological replicates). Brackets indicate significant effects of NO precursor or NO donor on the O_2 peak formation. In both WT, *ΔnorB*, and *ΔfhpΔnorB* mutants there was a significant effect of both indirect and direct NO donor addition. In the *ΔnirS-N* mutant, only direct NO donation led to significantly increased O_2 peaks. O_2 peak amplitude was not different between *ΔnorB* and *ΔfhpΔnorB* mutants in the NO_2^- supplement, and no differences were observed between *ΔnorB*, *ΔnirS-N*, and *ΔfhpΔnorB* in the direct NO donor treatment. The *Δfhp* mutant did not show increased O_2 peaks with addition of any of the NO donors (*: $p < 0.05$; two-way ANOVA).

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 O_2 by *P. aeruginosa*. Likewise, the missing knowledge of the exact origin of O_2 is a limitation of this study and could be examined by isotopic labeling using ^{18}O-labeled NO_2^- (Ettwig et al., 2010).

We speculate that this phenomenon of NO-mediated appearances of O_2 may play a role for *P. aeruginosa* in adapting to a switch between aerobic and anaerobic metabolism by providing a small O_2 burst at the onset of denitrification. Feeding of the aerobic metabolism by the extra peaks of O_2 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 O_2 concentration in the inhibited TOX could be due to Fhp activity. A small O_2 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 O_2 may affect the depletion of dissolved O_2 when estimating O_2 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 O_2 peaks in near anoxic conditions suggests the presence of an NO-sensing mechanism that is active at low O_2 tensions in *P. aeruginosa*. Interestingly, at low O_2 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 O_2 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 NO_3^- 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, *cbb_3-1* and *cbb_3-2*, which have high affinity for O_2, are more highly upregulated in anoxic conditions (Hamada et al., 2014; Kawakami et al., 2010) and accordingly may be able to respond to small O_2 releases during transitions form oxic to anoxic conditions where an overlap between aerobic and anaerobic metabolism in *P. aeruginosa* may happen at low O_2 concentrations. This could be beneficial during chronic infection, where dynamic or low supplies of

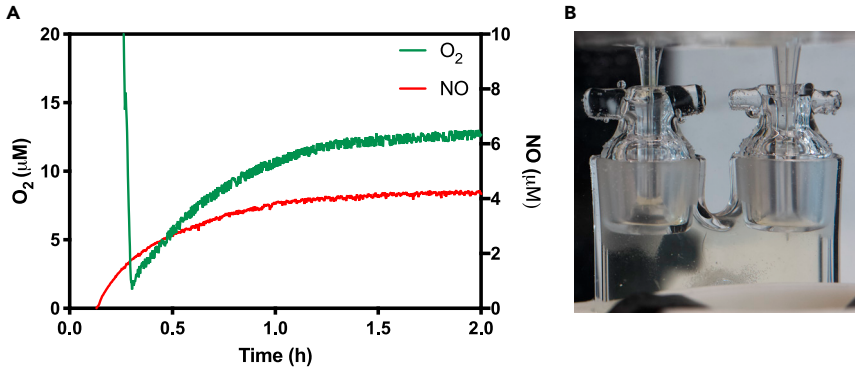

Figure 7. Endogenous NO release precedes O_2 peaks

Simultaneous measurement of O_2 and NO in a normoxically initiated culture of Δ*norB P. aeruginosa* mutant with 1 mM NO_2^- in LB (n = 1).

(A) Dynamics of O_2 and an NO in the culture.

(B) Experimental setup with a O_2 microsensor and an NO microsensor measuring in the same chamber immersed in a water bath at 37°C. The magnetic stirrers are not visible.

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_2O formation. Thus, the Nod has the potential to allow NO_3^- removal with emission of O_2 and N_2, but without emission of N_2O, 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.

STAR★METHODS

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- QUANTIFICATION AND STATISTICAL ANALYSIS

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, PØJ; collected data: LL, VS, THJ; analyzed data: ML, LL, VS, THJ, MK, TB, PØJ; created mutant strains: MR, TTN, MT, NN; wrote the manuscript: ML, LL, PØJ 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 (Δ*nirS-N)*	Toyofuku et al. (2014)	NA
Pseudomonas aeruginosa norB deletion mutant (Δ*norB)*	This paper	NA
Pseudomonas aeruginosa fhp deletion mutant (Δ*fhp)*	This paper	NA
Pseudomonas aeruginosa fhp, norB double deletion mutant (Δ*fhp*Δ*norB)*	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		
$NaNO_2$	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′-CGGAATTCGCCGGTGTTATACGCCGCAGG-3′
norB-R.	This paper	5′-GCCAAGCTTGTCGAGGTCTTCGGCGACGC-3′
fhp-UpF.	This paper	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTCAT CGTAGGGATCGGGCAGG-3′
fhp-UpR.	This paper	5′-GGCCGGACCGAAGAACTCGGCACG TTGGGCATTGGAC-3′
fhp-DnF.	This paper	5′-GAGTTCTTCGGTCCGGCC-3′
fhp-DnR.	This paper	5′-GGGGACCACTTTGTACAAGAAAGCTGGG TAAGGAAGAACGGGCGGAAG-3′
Recombinant DNA		
pHSG398. pUC type cloning vector Cm^R	TaKaRa, Japan	NA
pG19II. Derivative of pK19 *mob sacB*; Gm^R	Maseda et al. (2004)	NA
pG19*norB. norB* deletion cassette in pG19II	This paper	NA
pDONRPEX18Gm. Cloning vector, Gm^R	Hmelo et al. (2015)	NA
pENTR*fhp. fhp* deletion cassette in pDONRPEX18Gm	This paper	NA
pRK600. Mobilization vector, Cm^R	Kessler et al. (1992)	NA
** Cm^R= Chloramphenicol resistant; Gm^R = 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

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Fiber optic O_2 meter	PyroScience, Germany	FireStingO_2
Temperature sensor	PyroScience, Germany	TSUB21
Glass double chamber (O_2/NO measurements)	Unisense A/S, Denmark	https://www.unisense.com/MicroRespiration_System

RESOURCE 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 Δ*nirS-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 pG19*norB* 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 Δ*norB* 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 pENTR*fhp*. The integrity of the cassette was confirmed by sequencing of the vector. Deletion of *fhp* in *P. aeruginosa* PAO1 and Δ*norB* was performed by introducing pENTR*fhp* using triparental 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 Δ*fhp* deletion mutant and the Δ*fhp*Δ*norB* 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_{600} = 0.1 and regrown for ~2 h in LB to OD_{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_{600} = 0.4, the regrowth was performed for 2 h - still representing an active growth phase and reaching OD_{600} = 0.2.

Anoxic media was prepared in an anoxic bench (Concept 400, Thermo Scientific, Denmark) with O_2 < 0.02%, as routinely monitored with a luminescent dissolved O_2 sensor (HQ40d multi, HACH Company, USA). Stable solutions of LB media ± NO_2^- were established by addition of $NaNO_2$ (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_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_2.

In NO_2^- supplemented media we chose 100 μM NO_2^- and 1 mM NO_2^-, corresponding to non-toxic physiological concentrations able to allow rapid O_2 depletion (Major et al., 2010; Yoon et al., 2006). For the serial injection of NO_2^-, 100 μM was chosen as this is still representative of a physiological concentration, able to induce significant O_2 peaks while minimizing toxic effects of NO accumulation from NO_2^- 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_2 measurements

Measurements of O_2 dynamics were performed in micro-respiration vials (OXVIAL4, PyroScience, Germany), i.e. 4 mL glass vials fitted with a contactless optical O_2 sensor spot for detection of dissolved O_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_2 meter with 4 O_2 channels and 1 temperature sensor channel (FireStingO_2, PyroScience, Germany). The four O_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_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_{600} = 0.04, corresponding to a cell density capable of rapid O_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_2 concentration change over time is shown in Figure 3.

For NO_2^- supplementation, *P. aeruginosa* cultures were simply diluted into LB containing 1 mM NO_2^- in the respiration vials. Due to toxicity concerns and an inhibitory effect on initial O_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_2 depletion. Subsequent NO_2^- stimulation and LB controls were injected in the same way. Injections were prepared in anoxic media in the anoxic bench to avoid O_2 injection into the system.

Simultaneous O_2 and NO measurements

Micro-respiration microsensors for detection of O_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 Δ*norB P. aeruginosa* culture diluted to a final OD_{600} = 0.04. The lids were closed with an overflow to avoid entrapment of air bubbles. The O_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 manufacturer's recommendation (UniSense A/S, Aarhus, Denmark) using $NaNO_2$ 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, GraphPad Software). $p < 0.05$ was considered statistically significant.