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Two-pronged survival strategy for the major cystic fibrosis pathogen, *Pseudomonas aeruginosa*, lacking the capacity to degrade nitric oxide during anaerobic respiration

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Protection from NO gas, a toxic byproduct of anaerobic respiration in *Pseudomonas aeruginosa*, is mediated by nitric oxide (NO) reductase (NOR), the *norCB* gene product. Nevertheless, a *norCB* mutant that accumulated ~13.6 μM NO paradoxically survived anaerobic growth. Transcription of genes encoding nitrate and nitrite reductases, the enzymes responsible for NO production, was reduced >50- and 2.5-fold in the *norCB* mutant. This was due, in part, to a predicted compromise of the $[4Fe–4S]^{2+}$ cluster in the anaerobic regulator ANR by physiological NO levels, resulting in an inability to bind to its cognate promoter DNA sequences. Remarkably, two O_2-dependent dioxygenases, homogentisate-1,2-dioxygenase (HmgA) and 4-hydroxyphenylpyruvate dioxygenase (Hpd), were derepressed in the *norCB* mutant. Electron paramagnetic resonance studies showed that HmgA and Hpd bound NO avidly, and helped protect the *norCB* mutant in anaerobic biofilms. These data suggest that protection of a *P. aeruginosa norCB* mutant against anaerobic NO toxicity occurs by both control of NO supply and reassignment of metabolic enzymes to the task of NO sequestration.

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Introduction

The pathogen *Pseudomonas aeruginosa* (*PA*) (Holloway, 1969) is capable of aerobic or anaerobic growth. The latter is either via anaerobic respiration, using an inorganic oxynitrogen terminal electron acceptor (Gennis and Stewart, 1996), or slow growth by arginine substrate level phosphorylation (Mercenier *et al*, 1980). Anaerobic respiration requires nitrate (NO_3^-), nitrite (NO_2^-) or nitrous oxide (N_2O) as terminal electron acceptors. NO_3^- reduction occurs via two routes, through an assimilatory pathway, where NO_3^- is reduced to NH_3 and used as a nitrogen source, or by a dissimilatory pathway, where NO_3^- is reduced to N_2 by respiration (Sias *et al*, 1980). Dissimilatory NO_3^- reduction occurs only under anaerobic conditions and involves a sequential eight-electron reduction of NO_3^- to N_2, with intermediates that include NO_2^-, nitric oxide (NO) and N_2O (Figure 1A). These reactions are catalyzed by metalloenzymes embedded in the inner membrane and periplasm. Loci involved in the four-step reduction of NO_3^- to N_2 are termed *nar* (nitrate reductase) (Sias *et al*, 1980), *nir* (nitrite reductase) (Silvestrini *et al*, 1989), *nor* (nitric oxide reductase) (Arai *et al*, 1995), and, finally, *nos* (nitrous oxide reductase) genes (Arai *et al*, 2003), respectively.

Transcriptional control of genes involved in anaerobic respiration and arginine substrate level phosphorylation in *PA* is dependent upon ANR (anaerobic nitrate regulator), an FNR (fumarate/nitrate regulator/CRP)-like transactivator (Galimand *et al*, 1991; Lu *et al*, 1999). ANR is also required for transcription of the downstream regulator DNR (Arai *et al*, 1997). ANR also participates in controlling *PA* hydrogen cyanide production under reduced oxygen tension (Laville *et al*, 1998; Pessi and Haas, 2000), conditions that exist in the thick mucus lining the airways of cystic fibrosis (CF) patients (Worlitzsch *et al*, 2002; Yoon *et al*, 2002).

In denitrifying bacteria, the denitrification pathway is tightly controlled to minimize the adverse effects of NO, a toxic byproduct. During anaerobiosis, NO levels are maintained between 1 and 65 nM, depending on the bacterial species and the experimental conditions implemented (Goretski *et al*, 1990). Although NO toxicity mechanism(s) are unclear, accumulating evidence indicates that NO-derived nitrosative species can damage DNA (Woodmansee and Imlay, 2003) and compromise protein function by modifying moieties, including Fe–S clusters (Soum and Drapier, 2003), tyrosine residues (Schopfer *et al*, 2003), heme (Mayburd and

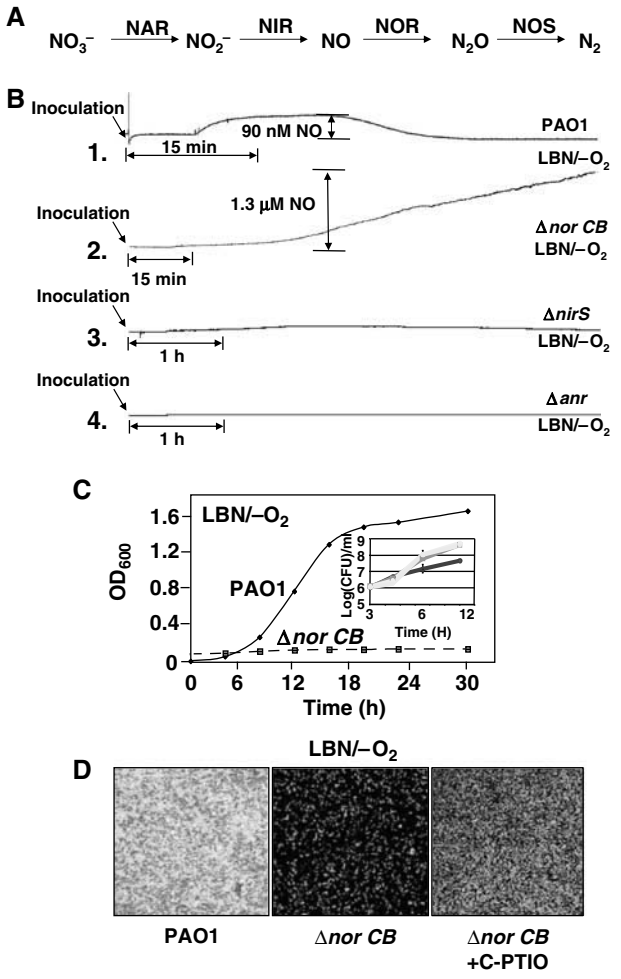

Figure 1 A *PA norCB* mutant maintains anaerobic viability despite high *in vivo* NO levels. (**A**) Anaerobic respiratory (denitrification) pathway. Enzymes involved in each reduction step are termed nitrate reductase (NAR), nitrite reductase (NIR), nitric oxide reductase (NOR) and nitrous oxide reductase (NOS), respectively. (**B**) NO tracings of wild type, *norCB*, *nirS* and *anr* mutants during anaerobic culture. Aerobic, stationary phase precultures were diluted 30-fold in 3 ml of LBN and placed in the NO electrode chamber. Before inoculation, the NO signal baseline was stabilized for at least 30 min. Note the different time scale between PAO1 and the mutant strains. (**C**) Anaerobic growth curve of wild-type and *norCB* mutant grown in LBN. Aerobic starter cultures were diluted 100- and 10-fold for wild-type and *norCB* mutant bacteria, respectively. Because of this, the initial turbidity was higher in the culture of the *norCB* mutant to demonstrate that anaerobic growth of the *norCB* mutant is virtually ceased. A 10-fold more initial bacterial load in *norCB* mutant, but OD_{600} remained constant. The inset indicates anaerobic wild-type and *norCB* mutant bacteria colony forming units in presence versus absence of 10 mM C-PTIO when grown in medium containing 10 mM KNO_3 at 3, 6 and 12 h. Because the C-PTIO is stoichiometrically exhausted after 12 h, the final viable cell count was enumerated at this time point. Gray line (PAO1), white line (*norcB* mutant + C-PTIO), black line (*norCB* mutant). (**D**) Confocal images of anaerobic biofilms of wild-type PAO1 and the *norCB* mutant. Bacteria were grown anaerobically for 24 h in LBN and stained for viability assessment using the BacLite® live/dead stain. Green and red organisms represent those that are either live or dead, respectively (for color figure see online version).

Kassner, 2002) and sulfhydryl groups (Spallarossa *et al*, 2003). The potency of high intracellular NO was demonstrated by revealing that a mutant lacking the global quorum sensing regulator, RhlR, committed a metabolic suicide by overproduction of anaerobic NO (Yoon *et al*, 2002). More recently, acidified NO_2^- contributed to killing the mucoid form of *PA*, a variant that severely complicates the prognosis for CF patients, through NO evolution and/or through nitrosation of sulfur or metal centers downstream from HNO_2 (Yoon *et al*, 2006).

The molecular basis underlying NO resistance in bacteria is poorly understood. In human disease, some intracellular pathogens replicate within host macrophages that produce high NO levels (Nathan and Shiloh, 2000). The most extensively studied proteins involved in NO detoxification are flavohemoglobin (Hmp or NOD (Hausladen *et al*, 2001) and flavorubredoxin (FlavoRb) in *Escherichia coli*. Hmp possesses an oxygen-dependent NO dioxygenase (NOD) activity (Gardner *et al*, 1998; Poole and Hughes, 2000), while FlavoRb possesses anaerobically induced NO reductase (NOR) activity (Gardner *et al*, 2002). Recent data indicate that Hmp is activated in response to NO even under anaerobic conditions, and NO-mediated modification of FNR, an *E. coli* ANR homolog, was suggested as a link between NO stress and anaerobic *hmp* transcription (Cruz-Ramos *et al*, 2002). NO exposure also triggers activation of the *soxRS* regulon, members of which include the superoxide dismutase (SOD) and antibiotic efflux pumps in both *E. coli* and *Salmonella typhimurium* (Vasil'eva *et al*, 2001; Coban and Durupinar, 2003). Because NO can react with superoxide (O_2^-) to produce highly destructive peroxynitrite ($ONOO^-$), higher levels of SOD are required for protection of NO-treated bacteria (Nunoshiba *et al*, 1993). Similar to the binding of NO to FNR (Cruz-Ramos *et al*, 2002), oxidation of the $[2Fe–2S]^{2+}$ cluster present in SoxR by NO leads to SoxS activation that subsequently results in transcription of genes under its control (Koo *et al*, 2003). In the CF airway, although low pH favors $ONOO^-$ protonation and ONOOH-associated toxicity (Yoon *et al*, 2002; Ricciardolo *et al*, 2004), anaerobic conditions may deprive NADPH oxidase of substrate (Worlitzsch *et al*, 2002).

Although some bacteria are capable of anaerobic growth using even NO as a terminal electron acceptor (Pichinoty *et al*, 1978), the major function of NOR is to detoxify NO generated anaerobically by NIR. In *P. stutzeri*, a *norCB* mutant perished when forced to respire via anaerobic NO_3^- reduction (Braun and Zumft, 1991). Previously, we showed that a *norCB* mutant of *PA* was also impaired in anaerobic NO_3^- reduction, yet the bacteria mysteriously survived under such conditions (Yoon *et al*, 2002). We postulated that these organisms adapted to anaerobic growth by either dramatically slowing their rate of growth or developing countermeasures to combat potentially toxic endogenous NO levels.

In this study, we addressed the basis for survival of the *norCB* mutant of *PA* and present evidence that it involves an elegant two-pronged defense mechanism. NO-linked control of ANR-mediated transcription is used to both stem the supply and purge the cytoplasm of NO by derepression of two dioxygenase encoding genes, whose gene products have inherent NO-scavenging capacity.

Results

A PA norCB mutant maintains anaerobic viability despite producing high endogenous NO levels

To determine the role of *PA* NOR in anaerobic growth, we first measured NO levels produced endogenously by strain PAO1

and a *norCB* mutant. In wild-type bacteria, NO was detected ~7 min post-inoculation (where oxygen was depleted), reaching a maximum of ~90 nM, and returning to baseline levels within 20 min (Figure 1B, line 1). In contrast, NO levels generated by the *norCB* mutant increased linearly to 1.3 μM over a 2-h incubation (Figure 1B, line 2). As expected, NO was not detected in a *nirS* mutant, that is deficient in NIR activity, and, as such, is incapable of generating anaerobic NO (Figure 1B, line 3). This supports the notion that the NO generated by the *norCB* mutant is mediated solely by NIR activity. In an *anr* mutant, where the enzyme involved in NO production (NIR) is not synthesized, NO was also not detected (Figure 1B, line 4). The amount of NO detected was independent of the initial NO_3^- concentration, suggesting that additional products downstream of *nar* and *nir* may be produced but are dependent on inoculum size, confirming that NO production is of biological origin (data not shown). During anaerobiosis, the growth rate of the *norCB* mutant was dramatically impaired, whereas wild-type bacteria demonstrated a typical growth curve (Figure 1C). The addition of the NO scavenger (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (C-PTIO) allowed the *norCB* mutant to grow at a rate equivalent to that of wild-type bacteria (Figure 1C, inset). However, C-PTIO was stoichiometrically exhausted after 12 h of NO scavenging in the mutant strain (data not shown). No differences in aerobic growth rate or cell yield between PAO1 and the *norCB* mutant were observed (data not shown).

Others and we have demonstrated via a confocal laser scanning analysis of *PA* biofilms that a live/dead stain is useful in determining viability of biofilm bacteria (Yoon *et al*, 2002; Webb *et al*, 2003). Figure 1D shows that *norCB* mutant biofilm bacteria were mostly alive, although the cell density in these biofilms was significantly lower when compared to wild-type biofilms that harbored a mixture of live and dead cells. The addition of C-PTIO restored near wild-type biofilm growth. These results suggest that there is a dramatic and sustained increase in NO levels in the *norCB* mutant that persist for more than 2 h without a loss of viability of this strain. We next dissected the molecular basis underlying this phenomenon.

A PA norCB mutant has reduced NAR/NIR activity and nar/nir gene transcription relative to wild-type bacteria

To elucidate the survival mechanism(s) of the *norCB* mutant under anaerobic conditions, we postulated that one strategy involves a downregulation of *nar* and *nir* transcription and concomitant NAR and NIR activities. Collectively, this would reduce the amount of NO that could kill the *norCB* mutant. First, single-copy *narK1-lacZ* and *nirS-lacZ* promoter fusions, representing the first genes of the *PA* operons involved in NAR and NIR biosynthesis, were constructed and placed in the neutral *attB* locus on the *PA* wild-type and *norCB* mutant genomes. Figure 2A shows that *narK1* transcription was barely detectable, while that of *nirS* was reduced ~2.5-fold in the *norCB* mutant when compared to wild-type expression. The reduction in *narK1* and *nirS* transcription was specific, as levels of the constitutively expressed genes *katA* (encoding the major catalase, KatA (Hassett *et al*, 1999; Ma *et al*, 1999)), and *crc* (encoding the catabolite repressor control protein (MacGregor *et al*, 1991)) were not significantly altered (data not shown). Biochemical complementation with C-PTIO

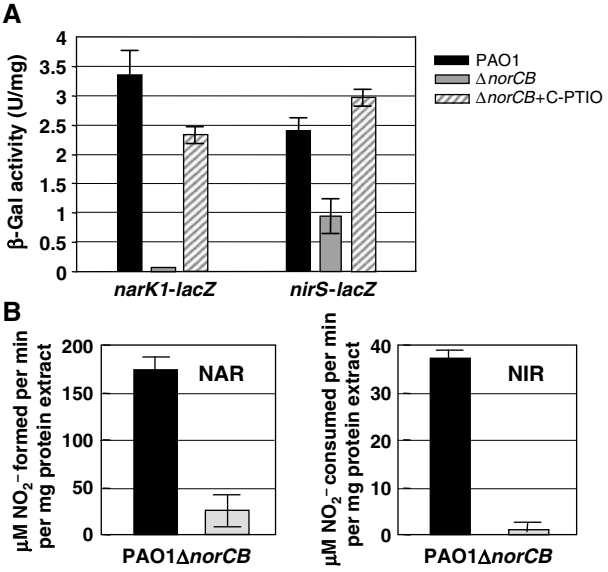

Figure 2 The *norCB* mutant has dramatically reduced NAR/NIR activities and *nar/nir* gene transcription relative to wild-type bacteria. (**A**) Cell extracts of wild-type and *norCB* mutant bacteria were prepared after anaerobic growth in LBN for 24 h. Wild-type PAO1 and *norCB* mutant bacteria harboring single-copy *narK1-lacZ* and *nirS-lacZ* fusions were assayed for β-galactosidase reporter activity in triplicate and mean ± s.e.m. is presented. C-PTIO at 10 mM was added for biochemical complementation purposes for the *lacZ*. (**B**) Nitrate (NAR) and nitrite (NIR) reductase activity of cell extracts of wild-type and *norCB* mutant bacteria after anaerobic growth in LBN for 24 h. Each assay was performed in triplicate and the mean ± s.e.m. is shown.

nearly restored both *narK1-lacZ* and *nirS-lacZ* to wild-type levels. Similarly, Figure 2B demonstrates that NAR and NIR activity were reduced ~85 and 97%, respectively, in the *norCB* mutant relative to wild-type levels. Not surprisingly, the NO_2^- levels remaining in the *norCB* mutant were only ~16 μM, whereas wild-type supernatants contained 12.3 mM (data not shown).

Metabolic NO inactivates ANR, altering its metal center, resulting in a loss of DNA binding activity in the norCB mutant

Since there was little or no *narK1* and *nirS* transcription in the anaerobic *norCB* mutant (Figure 2B), we postulated that ANR-mediated transcription had been compromised. To determine if endogenous NO inactivated ANR in *norCB* bacteria, anaerobic ANR was first purified (Figure 3A). The physical characteristics of ANR were similar to *E. coli* FNR (Khoroshilova *et al*, 1997), with the monomeric M_r being ~27 kDa (Figure 3A), while dimeric ANR was 54 kDa. ANR is brown, consistent with the presence of a $[4Fe–4S]^{2+}$ cluster, as found in *E. coli* FNR (Lazazzera *et al*, 1996). This was confirmed by the UV/visible spectrum of Figure 3B, which shows the characteristic optical bands of a $[4Fe–4S]^{2+}$ cluster with a shoulder at 320 nm and a broad peak with an absorption maximum of ~420 nm. Upon exposure to air, the absorbance decreased in the visible region (Figure 3B1) (Khoroshilova *et al*, 1997). Because the absorbance at 420 nm did not drop to zero, but rather shifted to a slightly longer wavelength, it is likely that the putative $[4Fe–4S]^{2+}$ cluster is not destroyed, but is rather converted to a $[2Fe–2S]^{2+}$ cluster, as observed for FNR. However, we caution our

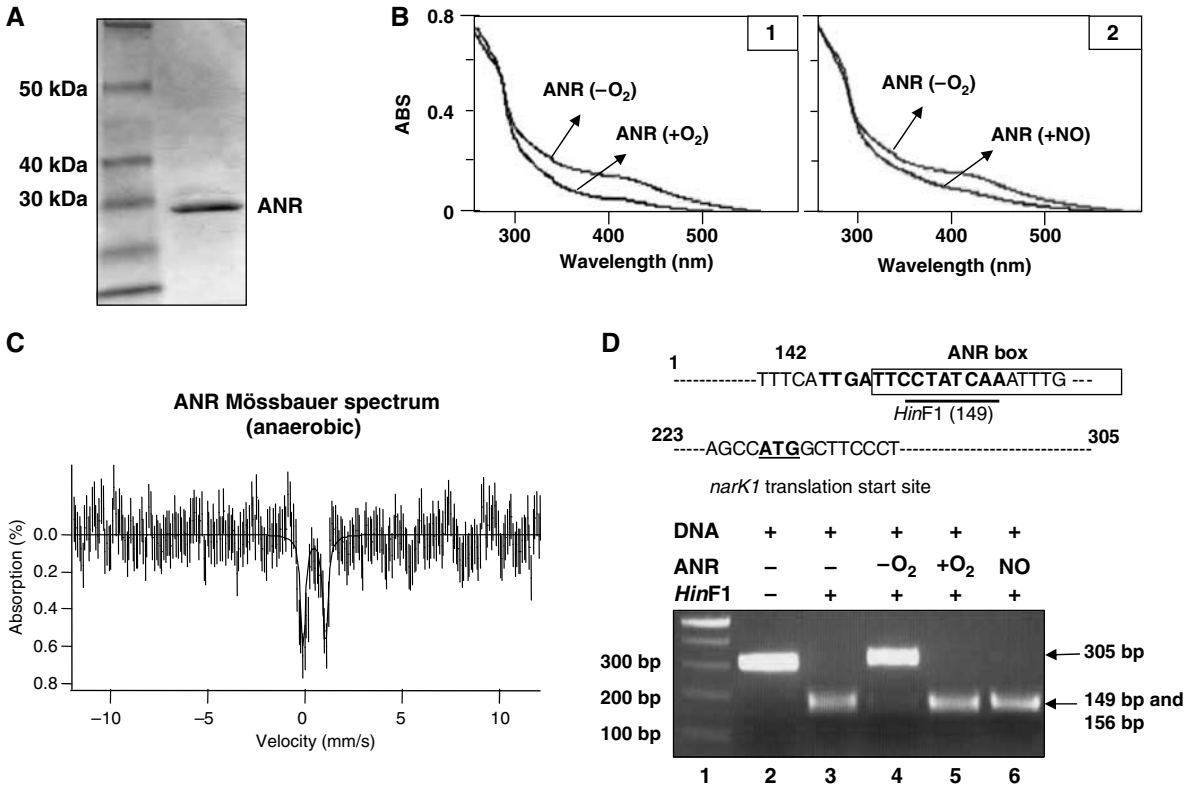

Figure 3 Purification of *PA* ANR and inactivation by NO. (**A**) SDS–PAGE of purified ANR. Lane 1, molecular weight standard (kDa); lane 2, 1 μg of purified ANR. (**B**) Absorption spectra of anaerobic ANR (1.2 mg/ml), air-treated and NO-treated ANR. The latter treatment is a 1% gaseous NO (balance) argon exposure for 1 h. The ratio of 1% NO (19 μM NO in solution) to 1.2 mg/ml ANR protein is 1:1 on a molar molar basis. To obtain spectra of anaerobic and NO-treated ANR, samples (200 μl) were placed in sealed cuvettes in an anaerobic chambe and scanned. (**C**) The 4.2 K Mössbauer spectra of anaerobically prepared ANR. The solid line is a doublet for $[4Fe–4S]^{2+}$ simulated with $E_Q = 1.2$ mm/s and $\delta = 0.43$ mm/s. (**D**) *Hin*F1 restriction protection assay. The *narK1* promoter sequence, in which an ANR box overlaps a *Hin*F1 recognition site, was digested with *Hin*F1 in the absence (lane 3) or presence of ANR (lanes 4, 5 and 6). Before digestion, the promoter sequence was incubated with anaerobic (lane 4), air-treated (lane 5) or NO-treated (lane 6) ANR for 30 min. DNA fragments after *Hin*F1 digestion were separated on a 1.5% agarose gel and photographed. Lane 1, DNA ladder (New England Biolabs Inc.); lane 2, DNA only.

interpretation of these data, for further experimental evidence is necessary to prove this postulate. A similar pattern of protein modification was also mediated by the addition of NO at levels generated endogenously by *norCB* mutant bacteria (Figure 3B2). To definitively identify the type of iron–sulfur cluster, ^{57}Fe-ANR was prepared for Mössbauer spectroscopy. Fitting of a preliminary spectrum revealed the presence of a quadrupole doublet with an isomer shift $\delta \approx 0.43$ mm/s and a quadrupole splitting of $E_Q \approx 1.2$ mm/s, similar to that observed for the $[4Fe–4S]^{2+}$ cluster of FNR (Figure 3C; Khoroshilova *et al*, 1997). Addition of O_2 or NO to ANR broadened the quadrupole doublet, consistent with a change in the cluster, but the formation of a $[2Fe–2S]^{2+}$ cluster could not be confirmed due to the poor signal to noise ratio of the spectrum (data not shown).

We next assessed the effect of NO on the ability of ANR to bind to its cognate recognition promoter sequence using a restriction site protection assay (D'Autreaux *et al*, 2002). Briefly, a 305-bp DNA fragment (Figure 3D, lane 2) harboring the *narK1* promoter was incubated with ANR under anaerobic or aerobic conditions, or with NO-treated ANR under anaerobic conditions prior to *Hin*F1 digestion. Complete *Hin*F1 digestion resulted in two small fragments of 149 and 156-bp (Figure 3D, lane 3). Full-length DNA was expected when the proper binding of ANR to its cognate recognition sequences occurred. Consistent with this expectation, anaerobic ANR successfully protected the *narK1* promoter from *Hin*F1 digestion (Figure 3D, lane 4), while air- or NO-treated ANR failed to protect the DNA (Figure 3D, lanes 5 and 6). These results indicate that ANR loses its ability to bind DNA when exposed to physiological concentrations of anaerobic NO.

Two genes encoding oxygen-dependent enzymes are derepressed in the anaerobic norCB mutant

We next tested whether other compensatory mechanisms were used by the *norCB* mutant that enable it to survive anaerobic growth. We examined whole-cell protein synthesis profiles in anaerobic wild-type versus *norCB* mutant bacteria similar to Wu *et al* (2005). A total of 300 spots were reproducibly separated by two-dimensional (2-D) gel electrophoresis, and 16 proteins that showed altered expression were analyzed by Matrix-Associated Laser Desorption Ionozation-Time-of-Flight (MALDI-TOF) mass spectrometry (Supplementary Figure S1). Of these, 14 were identified and are listed in Supplementary Table S1. Several proteins were expected to be absent in the *norCB* mutant. These included the NirQ regulator (spot 11), NirS (spot 16) and OprE (spot 5) (Supplementary Figure S1). NirQ is required for expression of *nir* and *nor* genes and is controlled by ANR (Arai *et al*, 1994),

and OprE expression is induced anaerobically (Yamano *et al*, 1998). The paucity of NirS further supported the significantly reduced NIR activity in the *norCB* mutant (Figure 2B). Interestingly, synthesis of two O_2-dependent enzymes, homogentisate-1,2-dioxygenase (HmgA) and 4-hydroxyphenylpyruvate dioxygenase (Hpd) was highly induced in anaerobic *norCB* bacteria, and not detected in wild-type extracts (Figure 4A). Induced synthesis of these proteins in the *norCB* mutant was also verified transcriptionally by examining *hmgA* and *hpd* reporter activity. Figure 4B shows that *hmgA* and *hpd* transcription in anaerobic *norCB* bacteria was ~4.2- and ~3.8-fold higher than in anaerobic extracts of wild-type bacteria, respectively. The addition of C-PTIO to the *norCB* mutant significantly reducted both *hmgA-lacZ* and *hpd-lacZ* activity (clear bars).

Crc was recently found to be upregulated 1.9-fold during anaerobic growth of *PA* and to negatively control aerobic expression of HmgA and Hpd in *P. putida* (Morales *et al*, 2004). In addition, ANR also controls transcription of *dnr*, encoding a nitrogen oxide-responsive regulator that, when overexpressed in an *anr* mutant background, restores anaerobic growth and *nar*, *nir* and *nor* transcription (Arai *et al*, 1997). Thus, we elected to test the hypothesis that the increased transcription of *hmgA* and *hpd* might be Crc- and/or DNR-mediated. To test this postulate, we constructed *norCB crc* and *norCB dnr* double mutants and measured *hmgA* and *hpd* transcription using *lacZ* reporter fusions. Our hypothesis was refuted, however, when we discovered that double *norCB crc* and *norCB dnr* mutants possessed identical *hmgA-lacZ* and *hpd-lacZ* as the *norCB* mutant (data not shown).

We next constructed isogenic mutants in each of the other 12 genes encoding proteins that were identified in our 2-D gel/MALDI-TOF analysis, and tested them for their ability to grow via anaerobic NO_3^- respiration (Supplementary Table S1, graphic to right). As expected, a *nirQ* mutant grew poorly when compared to growth of wild-type bacteria. Without NIR (a *nirS* mutant), *PA* cannot derive energy from NO_2^- respiration, although it is still capable of gaining energy from NO_3^- respiration. Thus, this strain was able to grow, albeit poorly. However, four mutants with defects in flagellin type-B synthesis, a probable aldehyde dehydrogenase (PA5312), the outer membrane protein OprE (PA0291) and an acetyl-CoA acetyltransferase (PA1999) showed a reduction in O.D.$_{600}$ of ~0.5.

HmgA and Hpd scavenge NO, thereby contributing to protection of anaerobic norCB bacteria in biofilms

Because HmgA and Hpd are Fe(II) containing enzymes, and NO binds with high affinity to Fe(II) (Arciero *et al*, 1983; Ford *et al*, 2002; Ford and Lorkovic, 2002), we postulated that these proteins may contribute to protection of the *norCB* mutant by their inherent ability to scavenge NO. To test this hypothesis, recombinant HmgA and Hpd were purified (Figure 5A1, 2) and their enzymatic activities were confirmed (data not shown). The NO binding properties of both enzymes were next examined using liquid helium temperature electron paramagnetic resonance (EPR) spectroscopy. Figure 5A1, 2 clearly shows that NO bound avidly to HmgA and Hpd to produce a characteristic spectrum with intense resonances near $g = 4$ (and 2) (Arciero and Lipscomb, 1986). The binding of homogentisate to HmgA caused only a small change in line shape, but the NO affinity is high with or without substrate. In the case of Hpd, 4-hydroxyphenylpyruvate binding caused a large increase in NO affinity, even at near stoichiometric NO levels, as is commonly observed for Fe(II) containing dioxygenases (Arciero *et al*, 1985). While we do not anticipate high levels of the Hpd substrate to be present under anaerobic conditions, these enzymes typically bind a wide range of aromatic compounds as inhibitors, and these complexes also result in a significant increase in NO affinity (Arciero *et al*, 1985).

We next used a genetic approach to test the hypothesis that HmgA and Hpd could help protect the *PA norCB* mutant growing as anaerobic biofilms by constructing an isogenic *norCB hmgA hpd* triple mutant. Interestingly, NO levels in the *norCB hmgA hpd* mutant steadily increased to nearly 16 μM and did not return to baseline, while the *norCB* mutant produced 13.6 μM NO (Figure 5B). When *hmgA* and *hpd* were provided *in cis*, NO levels were reduced to 10.3 μM. An absence of the two dioxygenases in an *hmgA hpd* mutant had

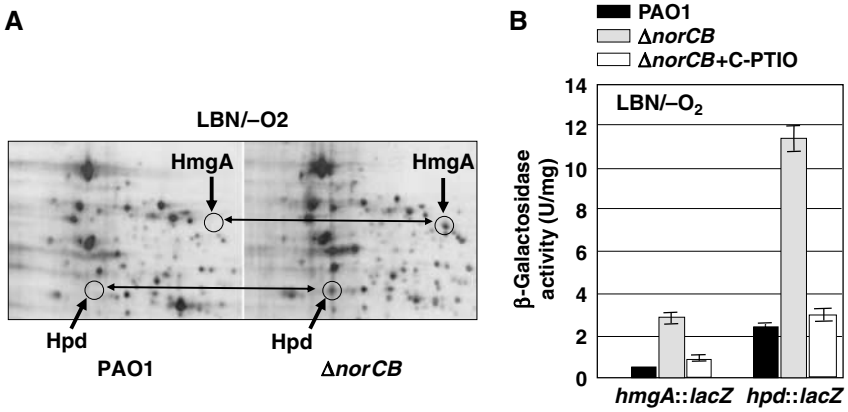

Figure 4 Upregulation of two oxygen-dependent enzymes in the anaerobic *norCB* mutant. (**A**) A portion of the *norCB* 2-D gel that contains spots for HmgA and Hpd proteins was compared with the same area of a 2-D gel from wild-type bacteria. A 60 μg weight of whole-cell extracts was separated in the 2-D gel system. The *norCB* mutant suspension was diluted 10-fold in the main anaerobic culture to obtain comparable amounts of cells with wild-type PAO1. Bacteria were grown in LBN under anaerobic conditions for 24 h. The identity of HmgA and Hpd was confirmed by MALDI-TOF mass spectrometric analysis. For the full 2-D gel images and the complete list of identified proteins, refer to the Supplementary data. (**B**) Measurements of transcript levels of *hmgA* and *hpd* in wild-type PAO1 (black bar), *norCB* mutant (gray bar) and norCB mutant bacteria + 10 mM C-PTIO (white bars) under anaerobic conditions. Cultures were assayed for β-galactosidase activity in triplicate as described in the Materials and Methods and mean ± s.e.m. is shown.

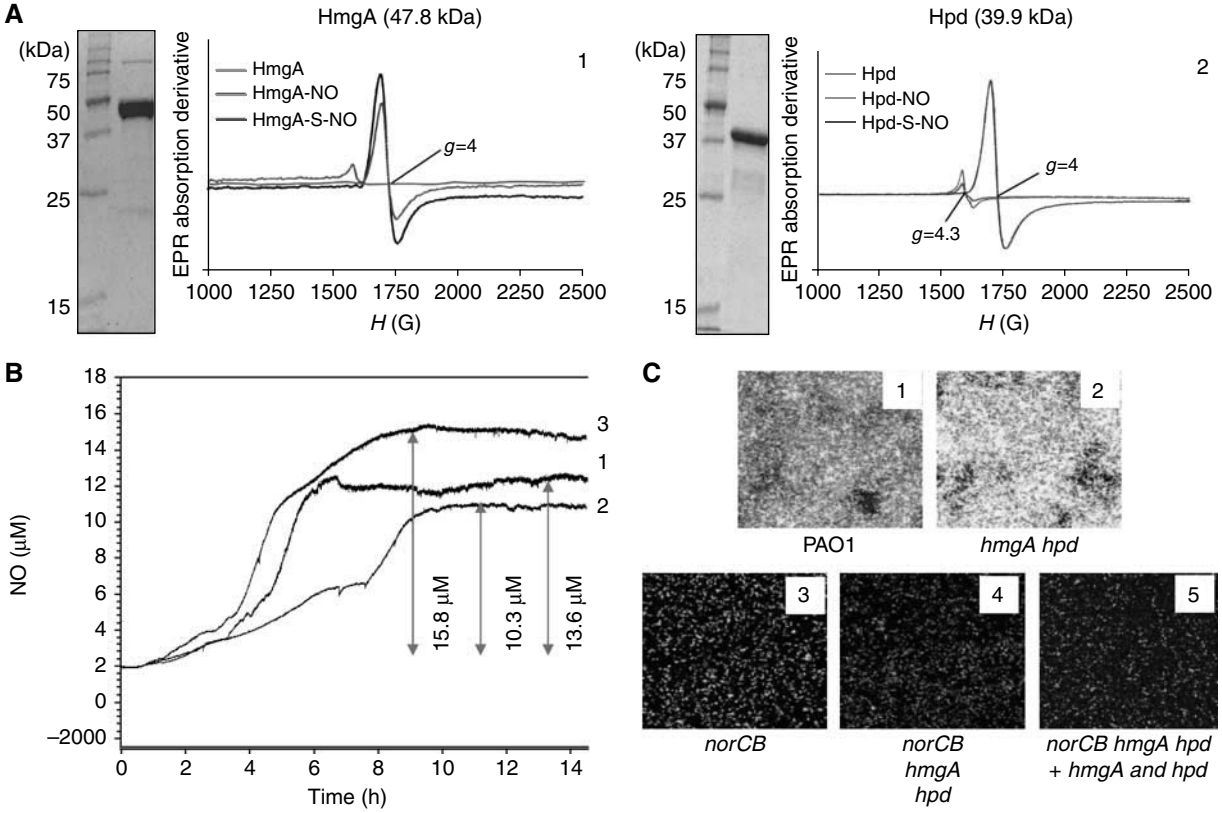

Figure 5 Binding of NO by HmgA and Hpd helps protect *norCB* mutant bacteria in anaerobic biofilms. (**A**) EPR spectra of 13 μM HmgA and Hpd in the absence or presence of NO. To establish that the Fe(II)–NO complex forms specifically at the catalytic center of these enzymes, spectra with the addition of stoichiometric levels of substrate (homogentisate for HmgA (HmgA-S-NO) and 4-hydroxyphenylpyruvate for Hpd (Hpd-S-NO)) are also shown. EPR conditions are as follows: temperature, 2 K; microwave frequency, 9.64 GHz; microwave power, 50 μW; modulation frequency 100 kHz; modulation amplitude, 10 G. Purity of HmgA or Hpd (lane 2) is shown by SDS–PAGE with molecular weight standards in kDa (lane 1). (**B**) NO tracings of *norCB* (line 1) and *norCB hmgA hpd* triple mutants (line 3) and the triple mutant + hmgA and hpd (line 2) during anaerobic culture. Experimental conditions were identical to those in Figure 1B. (**C**) Confocal images of anaerobic biofilms of wild-type PAO1 (panel 1), *hmgA hpd* (panel 2), *norCB* (panel 3), *norCB hmgA hpd* (panel 4), *norCB hmgA hpd* + *hmgA* and *hpd* in attB site (panel 5).

no adverse effects on anaerobic biofilm structure or viability, where NO was efficiently removed by NOR. However, anaerobic *norCB* (panel 3) and *norCB hmgA hpd* (panel 4) mutants formed very poor biofilms resulting from poor anaerobic growth of these strains (compare to wild-type bacteria, panel 1). Note, however, based upon the live:dead ratios, that the amount of dead bacteria was significantly higher in the *norCB hmgA hpd* mutant (>85%), relative to *norCB* single mutant (<1%). Finally, genetic *in cis* complementation of both *hpd* and *hmgA* in the *attB* site restored viability of the triple mutant to the *norCB* mutant phenotype (Figure 5C).

hmgA and hpd transcription is derepressed in norCB and anr mutants

We next tested whether increased anaerobic *hmgA* and *hpd* transcription in the *norCB* mutant resulted from ANR inactivation. To test this postulate, single-copy *hmgA-lacZ* and *hpd-lacZ* promoter fusions were introduced into the *anr* mutant chromosome. In wild-type bacteria, *hmgA* and *hpd* transcription was predictably decreased under anaerobic relative to aerobic conditions (Figure 6A and B). Consistently, in separate microarray analyses, we found that *hmgA* and *hpd* gene expression was reduced by factors of 14.9- and 7.3-fold in anaerobic versus aerobic wild-type bacteria, respectively (data not shown). Under aerobic conditions, *hpd* transcription was >2-fold higher than comparable levels of *hmgA* in

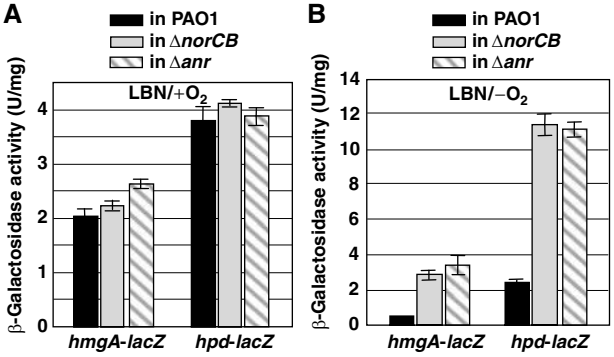

Figure 6 *norCB* and *anr* mutants show similar anaerobic phenotypes with regard to *hmgA* and *hpd* regulation. (**A**, **B**) Measurements of transcript levels of *hmgA* and *hpd* in wild-type PAO1 (black bar), *norCB* mutant (gray bar) and *anr* mutant (hatched bar) under both aerobic (A) and anaerobic (B) conditions. Cultures were assayed for β-galactosidase activity in triplicate as described in Materials and Methods.

either PAO1, *norCB* and *anr* mutant strains, and no significant transcriptional differences were observed in these strains (Figure 6A). Under anaerobic conditions, however, *hmgA* and *hpd* expression was increased in the *anr* mutant to the same levels observed in the *norCB* mutant (Figure 6B), suggesting that anaerobic *hmgA* and *hpd* repression is

ANR dependent. Taken together, these data suggest that the anaerobic behavior of the *norCB* mutant is nearly identical to that of the *anr* mutant *vis-à-vis hmgA/hpd* gene regulation. Finally, upregulation of *hmgA* and *hpd* in the anaerobic *norCB* mutant is likely because of an NO-mediated inactivation of ANR.

Discussion

The important CF pathogen *PA* forms highly problematic and antibiotic/phagocyte refractory biofilms while enmeshed in thick anaerobic airway mucus (Singh *et al*, 2000; Hassett *et al*, 2004). Furthermore, when the organism converts to the formidable mucoid form during the chronic stages of the disease, the pulmonary performance of CF patients diminishes dramatically (Govan and Harris, 1986). Although conventional antibiotics keep *PA* titers at roughly 10^8 CFU/g sputum, such titers still represent a huge problem, because the antimicrobial power of phagocytes in this complex niche is significantly diminished.

NO is an important antimicrobial component of our innate host defense system and a cell signaling molecule. However, in the context of treatment of recalcitrant *PA* biofilms enmeshed in the anaerobic airway mucus of CF patients, NO likely has little relevance from an antimicrobial perspective. Recently, however, we have unraveled two important clues to help eradicate this organism that relate to the ability of *PA* to cope with intracellular or extracellular NO. First, we discovered that paralysis of *PA rhl* quorum sensing during anaerobic biofilm growth, cause such organisms to commit a metabolic suicide by overproduction of metabolic NO (Yoon *et al*, 2002). This suggests that novel drugs that can paralyze *PA* quorum sensing could help eradicate *PA* from the CF airways. It is for this reason that many laboratories are currently examining quorum sensing inhibitors as potential therapeutic agents for treatment of CF and other infections (Passador *et al*, 1996; Smith *et al*, 2003; Suga, 2003; Kim *et al*, 2004). Second, the results of this study also builds on a major potential therapy for treating what we coined the 'Achilles' heel' of mucoid *PA* that specifically involves an inability to cope with nitrogen oxides downstream from extracellular $NaNO_2$ at the slightly acidic pH (6.4–6.5) of the CF airways (Yoon *et al*, 2006).

This study was initiated because of an intriguing observation that initially appeared to be a glaring biological paradox; a *PA norCB* mutant, that is incapable of detoxifying endogenously generated NO during anaerobic respiration, did not perish from its inherent toxicity. Our goal was to define the mechanism(s) and the machinery underlying the ability of this microorganism to resist metabolic NO. Our first experiments honed in on the major enzymes involved in *PA* anaerobic respiration. These include NAR and NIR, the only anaerobic enzymes that collectively mediate metabolic NO production, and the master regulator of *nar* and *nir* gene transcription, the anaerobic transcriptional regulator, ANR. Since we found that both *nar* and *nir* transcription and NAR and NIR enzymatic activity in the *norCB* mutant was markedly reduced relative to that of wild-type bacteria, we elected to examine whether the function of ANR was compromised. The *E. coli* ANR homolog, FNR, is sensitive to O_2^- and NO-mediated inactivation (Cruz-Ramos *et al*, 2002). The disruptive poisoning of the *E. coli* FNR $[4Fe–4S]^{2+}$ cluster by O_2 and NO causes a conversion of the $[4Fe–4S]^{2+}$ cluster and a change in the oligomeric state of FNR. Thus, exposure of FNR to NO derepressed transcription of *hmp*, encoding flavohemoglobin (also called NOD (nitric oxide dioxygenase)) that is the major NO detoxifier in *E. coli* (Gardner *et al*, 1998; Poole and Hughes, 2000). Reduced DNA binding activity of FNR due to NO-mediated modification of $[4Fe–4S]^{2+}$ was proposed as a molecular mechanism for NO sensing. In addition to FNR as a NO sensing regulator, CydR protein of *Azotobacter vinelandii* that is more homologous to ANR (88% identical) was also reported to show decreased binding to its cognate DNA recognition sequence when poisoned by NO (Poole and Hughes, 2000; Wu *et al*, 2000).

In the case of ANR, our spectrophotometric survey and restriction endonuclease protection assays clearly indicate that NO has deleterious effects on the ANR $[4Fe–4S]^{2+}$ cluster and its concomitant transcriptional potential. A model of how NO regulates this process is depicted in Figure 7. It is of interest that iron-nitrosyl clusters can both be targets of *S*-nitrosothiol toxicity and promote *S*-nitrosothiol metabolism (Vanin *et al*, 2004), although airway acidification should promote *S*-nitrosothiol formation in the CF airway (Ricciardolo *et al*, 2004; Yoon *et al*, 2006). *S*-nitrosothiol levels in the CF airway are actually quite low, perhaps—at least in part—because of prokaryotic catabolism (Grasemann *et al*, 1999). One of the most compelling arguments indicating that the transcriptional capacity of ANR was compromised in the *norCB* mutant is the near identical behavior of anaerobic *anr* and *norCB* mutant bacteria in both planktonic and biofilm cultures.

DNR, another transcription factor in the same CRP/FNR family, is also essential for anaerobic growth in *PA* and is responsive to NO_2^- and NO, but not NO_3^- (Arai *et al*, 1997). Although *in trans* provision of DNR to the *anr* mutant restored the ability to denitrify, transcription of *dnr* is under the control of ANR, suggesting that ANR is the master regulator under anaerobic condition in *PA* (Arai *et al*, 1997). Construction of a *norCB dnr* mutant revealed that *hmgA/hpd* transcription was identical to that of an anaerobic *norCB* mutant (data not shown). Interestingly, it was also shown that the transcriptional activity of *anr* was >10-fold higher than that of *dnr* in anaerobic bacteria, suggesting that ANR is more abundantly synthesized than DNR under anaerobic conditions and plays a more important role in oxygen sensing and the initiation of the denitrification pathway (Arai *et al*, 1997). Still, Arai *et al* (1997) have suggested that the denitrification genes in *PA* are not directly controlled by ANR, but rather indirectly by DNR. However, the mechanistic basis behind this mode of transcriptional control is unknown. Structural studies have been initiated that hope to determine precisely how the DNR-based regulatory system functions.

Although anaerobic growth was significantly impaired because of repression of genes required for denitrification in the *norCB* mutant, fundamental biological anaerobic processes of this strain still remained active. This observation forced us to probe potential additional survival mechanisms of the *norCB* mutant under anaerobic conditions. Using MALDI-TOF analyses, the synthesis of eight of 16 identified proteins was increased in the *norCB* mutant compared to the wild-type strain. This clearly implies that the bacteria are at least partially capable of responding to high levels of NO and/or downstream metabolites. Two proteins, HmgA and Hpd,

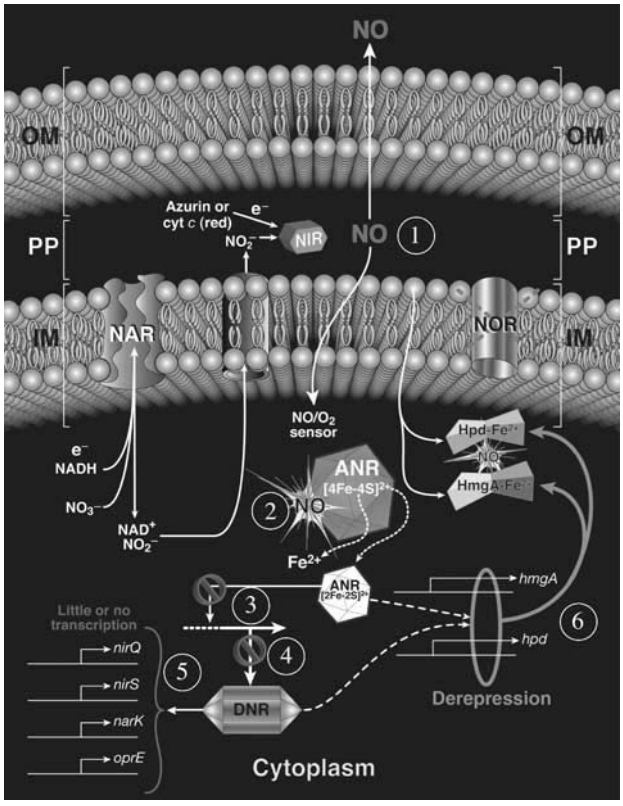

Figure 7 Summary of the protective mechanisms used by a PA *norCB* mutant during anaerobic respiration. (1) NO is produced anaerobically in the periplasmic space by NIR and can either exit the cell or enter the cytoplasm; (2) Once in the cytoplasm, NO can inactivate dimeric ANR, causing release of iron from the [4Fe–4S]$^{2+}$, causing conversion to a putative [2Fe–2S]$^{2+}$ cluster, thus rendering it incapable of transcriptional activation of genes under its control (3), this is indicated by the 'no' sign (e.g., ⃠). In contrast, genes that are anaerobically repressed, including *hmgA* and *hpd*, become derepressed, and function as NO scavengers; (4) because ANR is required for transcription of *dnr*, DNR is not produced; (5) thus, *narK1*, *nirS*, *nirQ* and *oprE* transcripts are either absent or extremely low (denoted by red X); (6) ANR, and perhaps DNR, denoted as a '?', however, loses it activity as a repressor. Thus, genes that would be repressed anaerobically, such as *hmgA* and *hpd*, are now derepressed (denoted by green circle). Because of the inherent NO-binding properties of HmgA and Hpd (refer to Figure 5A and B), elevated levels of these enzymes served to help protect the anaerobic *norCB* mutant against NO-mediated toxicity. OM, outer membrane; PP, periplasmic space; IM, inner membrane; NarK1/2, putative NO_2^- extrusion pump; CytC (red), reduced cytochrome *c*; Azurin, periplasmic protein donating electrons to NIR; e^-, electron flow; NADH, electron donor for NAR (for color figure see online version).

are involved in the tyrosine degradation pathway, which occurs under aerobic conditions. Oxidative decarboxylation followed by hydroxylation and ultimately cleavage of the aromatic ring catalyzed by dioxygen splitting are mediated by Hpd and HmgA, respectively, in two consecutive steps of the pathway (Milcamps and de Bruijn, 1999; Fritze *et al*, 2004). The dioxygenase class of enzymes, of which HmgA and Hpd are members, typically contains iron as a cofactor within their active site (Que and Ho, 1996). Dioxygenases that utilize Fe^{2+} bind NO with high affinity, and this affinity increases even further when they bind substrate (Arciero *et al*, 1983). Two well-characterized examples, catechol-2,3-dioxygenase from the related organism *P. putida* and protocatechuate-4,5-dioxygenase from *P. testosteroni*, have been shown to bind NO with K_d values well below 1 μM (Arciero *et al*, 1985). Definitive proof that HmgA and Hpd are capable of scavenging NO emerged from two separate experiments. First, we demonstrated by EPR spectroscopy that NO bound to both purified HmgA and Hpd (Figure 5A). Most importantly, we showed that HmgA and Hpd contributed to protection of the anaerobic *norCB* mutant in demonstrating that a triple *norCB hmgA hpd* mutant was killed during the anaerobic biofilm mode of growth because of sustained NO production (Figure 5B and C). The *PA* genome harbors 22 genes that could potentially produce at least 16 different dioxygenases (www.pseudomonas.com) Although at this juncture, it is not clear whether other dioxygenases or proteins capable of binding NO are also induced in response to NO stress and confer protective roles against NO, the results presented in Figure 5C clearly indicate that there are alternative functions of two aerobic enzymes under anaerobic conditions in the presence of high NO levels. Of note, this increased NO binding of HmgA/Hpd by anaerobic *PA* could account, at least in part, for the observation that antipseudomonal therapy increases exhaled concentrations of NO in CF patients (Jaffe *et al*, 2003), in the airways of whom *PA* growth is largely anaerobic (Worlitzsch *et al*, 2002; Yoon *et al*, 2002).

The dramatic upregulation of HmgA and Hpd in the anaerobic *norCB* mutant was initially perplexing, because it is counterintuitive to synthesize oxygen-dependent enzymes in an anaerobic environment. As expected, in wild-type strain PAO1, transcription of *hmgA* and *hpd* was highly repressed under anaerobic conditions (Figure 6A and B). Identical increases in *hmgA* and *hpd* transcription was also observed in both *norCB* and *anr* mutant strains. These results strongly suggest that repression of *hmgA* and *hpd* transcription during anaerobic growth is mediated by intact, dimeric ANR, and derepression of these genes in the *norCB* mutant is due to an NO-mediated inactivation of ANR. This evidence clearly establishes that the *norCB* mutant behaves identically to the *anr* mutant strain under anaerobic conditions.

In summary, this study describes a novel two-tiered, 'circuit breaker' mechanism involving the paradoxical survival of a mutant lacking the protective enzyme, NOR, during anaerobic respiration by *PA* (Figure 7). We found that *PA* possesses an inherent homeostatic mechanism for maintaining NO levels at sublethal concentrations. First, we found that increased NO levels leads to a reduction in the expression of the enzymes that produce NO from NO_3^- and NO_2^- (NAR and NIR). Second, we found that the production of enzymes that can sequester NO (HmgA and Hpd) via an ANR-dependent derepression, were significantly increased under these conditions. Furthermore, we found that this unique protective regulatory duality occurs via the direct NO-mediated inactivation of the master anaerobic regulator, ANR, which contains an NO-sensitive [4Fe–4S]$^{2+}$ cluster.

Materials and methods

Bacterial growth conditions

All bacteria were isogenic derivatives of *PA* strain PAO1. Bacteria were grown in either L-broth (10 g tryptone, 5 g NaCl, 5 g yeast extract per liter) or LB/1% KNO_3 (LBN). Overnight cultures were grown aerobically in LB to stationary phase. Wild-type bacteria were diluted 100-fold and the *norCB* mutant was diluted 10-fold for anaerobic cultures, unless otherwise indicated. Anaerobic growth was achieved in a Coy anaerobic chamber (Coy labs, Grass Lake, MI).

Construction of isogenic mutants and lacZ transcriptional fusions

Isogenic *PA* mutants were constructed by two different allelic exchange procedures as described previously (Schweizer and Hoang, 1995). Other *PA* mutants were obtained from the University of Washington mutant library collection, each of which was confirmed by DNA sequencing. To construct single-copy *lacZ* transcriptional fusions, DNA fragments containing the promoter region of *narK1* (representing the first gene of the *nar* operon, PA3877) and *nirS* (representing the first gene of *nir* operon, PA0519) were flanked by *Pst*I and *Bsp*HI restriction sites. Three fragments of (i) an *Nco*I–*Sal*I fragment of pZ1918 (Schweizer, 1993) containing the *lacZ* reporter gene with its own ribosomal binding site, (ii) *Pst*I–*Bsp*H1 promoter sequences and (iii) *Pst*I–*Sal*I digest of mini-CTX1 (Hoang *et al*, 2000) were ligated, and putative clones were verified by DNA sequencing. *E. coli* SM10 harboring each transcriptional *lacZ* fusion was used as a donor strain in biparental matings with strain PAO1, and isogenic *norCB, norCB crc, norCB dnr* or *anr* mutants. Each promoter-*lacZ* construct was integrated at the nonessential *attB* locus within the *PA* chromosome, yielding single-copy transcriptional fusions (Hoang *et al*, 2000).

NO electrode measurements

NO levels were measured polarimetrically using a NO electrode system (World Precision Instruments Inc., Sarasota, FL). The chamber was prepared with a stably positioned electrode within the cavity of the bacterial suspension, as previously described (Gardner *et al*, 2003). All NO signals were calibrated from the stoichiometric conversion of acidified NO_2^- to NO as specified by the vendor. In an experiment to trace NO generated by anaerobically growing *PA*, strains were inoculated into the closed chamber through a port of ~0.6 mm. A 100 µl volume of aerobic overnight culture was inoculated into 2.5 ml of LBN at 37°C. The NO signal was recorded and saved with APOLLO 4000™ software (World Precision Instruments Inc., Sarasota, FL). C-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, Molecular Probes, Eugene, OR) or deoxy-hemoglobin, stoichiometric NO scavengers, were added to confirm that the signal is specific to NO, when needed.

Overexpression and purification of PA ANR, HmgA and Hpd

The *hmgA* and *hpd* genes were amplified by PCR and positionally cloned into pET23a (Novagen) and *anr* into pET19b (Novagen), and transformed into *E. coli* BL21 (DE3). The resulting pET19b-*anr* plasmid was transformed into *E. coli* Tuner™ (DE3, Novagen). For overexpression of HmgA and Hpd, *E. coli* was grown aerobically in LB, while for ANR overexpression, organisms were grown anaerobically in LBN/0.2% glucose. Expression was induced in mid-logarithmic phase cultures with 0.4 mM IPTG. Recombinant His-tagged ANR, HmgA or Hpd was bound to a nickel-NTA column (Qiagen, Valencia, CA) and eluted with 250 mM imidazole. ANR was purified under anaerobic conditions following the procedure used for purification of *E. coli* FNR (Sutton and Kiley, 2003). HmgA and Hpd activity assays were performed as previously described (Rodríguez *et al*, 2000; Kavana and Morna, 2003). ANR was prepared anaerobically and aliquots exposed either to ambient air or 1% NO gas balanced with argon for 1 h under anaerobic conditions.

Reporter assays

β-Galactosidase activity was measured as follows: *PA* strains were first grown to mid-logarithmic phase and the OD_{600} of each culture was measured. For *norCB* and *anr* mutants, the suspensions were diluted 10-fold from aerobic stationary phase cultures and incubated anaerobically at 37°C. A 100 µl volume of each culture was mixed with 0.9 ml of Z buffer (100 mM sodium phosphate buffer, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 25 mM β-mercaptoethanol). A 20 µl volume each of 2% SDS and chloroform were added to 1 ml aliquots of cell suspension, and the mixture was vortexed to facilitate cell lysis. After a 5-min incubation at 28°C, 0.2 ml of a 4 mg/ml solution of *o*-nitrophenyl-β-D-galactopyranoside was added, and the incubation was continued until development of a yellow color. Reactions were stopped by adding 0.5 ml of 1 M Na_2CO_3 and the reaction time was recorded. Cell debris was removed and the OD_{420} was measured immediately. Units of specific activity were expressed as $OD_{420}/(OD_{600} \times \text{min} \times \text{ml of culture})$.

Microscopic examination of PA biofilms

For examination of biofilm architecture and cell viability, an eight-chambered coverslip system (Lab-Tek Inc., Campbell, CA) was used. LBN (0.4 ml) was inoculated with 4 µl of an aerobically grown overnight LB starter culture of wild-type and various isogenic mutants. All stationary phase strains grown aerobically had approximately the same value of CFU/ml. After 24 h at 37°C, anaerobic biofilms were washed three times with 0.9% saline and stained with 0.4 ml of a LIVE/DEAD *Bac*Light bacterial viability stain for 15 min (Molecular Probes Inc., Eugene, OR). Images were acquired on a Zeiss LSM 510 laser scanning confocal unit attached to an Axiovert microscope using a 63×1.4 NA oil-immersion objective (Carl Zeiss MicroImaging Inc., Thornwood, NY). For two-color images, samples were scanned sequentially at 488 and 546 nm. Syto 9 (green fluorescence) was detected through a 505–530 nm bandpass filter and propidium iodine (red fluorescence) was detected through a 560 nm longpass filter and presented in two channels of a 512×512 pixel, 8-bit image.

NAR and NIR activity assays

NAR and NIR activities were measured using cell extracts as described previously, with minor modifications (Yoon *et al*, 2002). Briefly, 0.4 mM methyl viologen reduced by the addition of 2 mM sodium thiosulfite was used as the electron donor in the NAR activity assay, while 5 mM NADH coupled with 20 mM phenazine methosulfate (PMS) was used for NIR assays. The amount of NO_2^- in the reaction mixture was measured at specified intervals with the Griess reagent (Green *et al*, 1982). The increase or decrease in NO_2^- levels was measured using the NAR and NIR assays, respectively.

Optical spectroscopy

A Genesys 5 spectrophotometer (Spectronic, Rochester, NY) was used to record the optical spectrum of purified anaerobic ANR between 250–600 nm. Anaerobiosis was maintained by capping quartz cuvettes containing anaerobically purified and buffered ANR that were previously filled in the anaerobic chamber.

Restriction site protection assays

The 305-bp promoter region of *narK1* (P_{narK1}), in which the ANR box overlaps a *Hin*F1 restriction site, was amplified by PCR and cleaned using a PCR purification kit (Qiagen Inc., Valencia, CA). P_{narK1} DNA (100 ng) was incubated with 2 µg of ANR for 30 min in 25 mM Tris–HCl (pH 7.8) and 50 mM NaCl at 37°C. All restriction digestions were performed by adding 1 U of *Hin*F1 (New England Biolabs Inc., Beverly, MA) for 1 h. EDTA (25 mM) was added to stop each reaction. Agarose gel (1.5% agarose) electrophoresis was used to separate the DNA fragments.

2-D gel electrophoresis and MALDI-TOF analysis

2-D gel and in-gel trypsin digestion were performed as described previously (Yoon *et al*, 2002). MALDI-TOF was used to obtain all peptide mass fingerprinting profiles. Mass-to-charge (*m/z*) ratios of each fragment generated after trypsin digestion were queried from a public database (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm) and a protein with highest statistical certainty was selected for identification.

EPR and Mössbauer spectroscopy

EPR spectra of the Fe-nitrosyl complex of purified *PA* HmgA and Hpd was monitored as described previously (Hassett *et al*, 2000). Mössbauer spectra of ^{57}Fe-labeled ANR were obtained following the procedures described elsewhere (Khoroshilova *et al*, 1997).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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