Three *Pseudomonas putida* FNR Family Proteins with Different Sensitivities to O$_2$

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Susan A. Ibrahim, Jason C. Crack, Matthew D. Rolfe, José Manuel Borroto-de Acuña, Andrew J. Thomson, Nick E. Le Brun, Max Schobert, Melanie R. Stapleton, and Jeffrey Green

From the 1Krebs Institute, Molecular Biology and Biotechnology, University of Sheffield, Sheffield, S10 2TN, United Kingdom, the 2Centre for Molecular and Structural Biochemistry, School of Chemistry, University of East Anglia, Norwich, NR4 7TJ, United Kingdom, and 3Institut für Mikrobiologie, Technische Universität, D-38106 Braunschweig, Germany

**Background:** FNR proteins are O$_2$-responsive bacterial transcription factors.

**Results:** *Pseudomonas putida* possesses three FNR proteins with iron-sulfur clusters that have different sensitivities to O$_2$.

**Conclusion:** The mechanism of the iron-sulfur cluster reaction with O$_2$ is conserved among FNR proteins.

**Significance:** Differential sensitivity of multiple FNR proteins extends the range of O$_2$-responsive gene expression within a single bacterium.

The *Escherichia coli* fumarate-nitrate reduction regulator (FNR) protein is the paradigm for bacterial O$_2$-sensing transcription factors. However, unlike *E. coli*, some bacterial species possess multiple FNR proteins that presumably have evolved to fulfill distinct roles. Here, three FNR proteins (ANR, PP_3233, and PP_3287) from a single bacterial species, *Pseudomonas putida* KT2440, have been analyzed. Under anaerobic conditions, all three proteins had spectral properties resembling those of [4Fe-4S] proteins. The reactivity of the ANR [4Fe-4S] cluster with O$_2$ was similar to that of *E. coli* FNR, and during conversion to the apo-protein, via a [2Fe-2S] intermediate, cluster sulfur was retained. Like ANR, reconstituted PP_3233 and PP_3287 were converted to [2Fe-2S] forms when exposed to O$_2$, but their [4Fe-4S] clusters reacted more slowly. Transcription from an FNR-dependent promoter with a consensus FNR-binding site in *P. putida* and *E. coli* strains expressing only one FNR protein was consistent with the in vitro responses to O$_2$. Taken together, the experimental results suggest that the local environments of the iron-sulfur clusters in the different *P. putida* FNR proteins influence their reactivity with O$_2$, such that ANR resembles *E. coli* FNR and is highly responsive to low concentrations of O$_2$, whereas PP_3233 and PP_3287 have evolved to be less sensitive to O$_2$.

Fumarate-nitrate reduction regulator (FNR)$^2$ proteins are a major subgroup of the cyclic-AMP receptor protein family of bacterial transcription regulators (1). The major function of FNR proteins is the reprogramming of gene expression to coordinate the switch from aerobic to anaerobic metabolism when facultative anaerobes like *Escherichia coli* are starved of O$_2$ (2–7). The paradigm for O$_2$-sensing transcription factors is the *E. coli* FNR protein. The N-terminal region of FNR contains four essential cysteine residues that coordinate an O$_2$-sensitive [4Fe-4S] cluster (8, 9). In the absence of O$_2$, the [4Fe-4S] cluster is stable, and FNR exists as a homodimer that is capable of high affinity, site-specific DNA binding to an FNR box (TTGATNNNNATCAA) (9, 10). When bound to target DNA, FNR activates the expression of genes encoding proteins required for anaerobic metabolism and represses those utilized under aerobic conditions (2, 4), such that when O$_2$ is available, anaerobic metabolism is shutdown in favor of the more energetically efficient aerobic respiratory metabolism.

Molecular oxygen reacts with the FNR [4Fe-4S] cluster in a series of steps that ultimately yields the apo form of the protein (Equations 1–3) (11–13).

**Step 1:**
\[
[4Fe-4S]^{2+} + O_2 \rightarrow [3Fe-4S]^{3+} + Fe^{2+} + O_2^{-} \quad (Eq. 1)
\]

**Step 2:**
\[
[3Fe-4S]^{3+} \rightarrow [2Fe-2S]^{2+} + 2S^{2-} + Fe^{3+} \quad (Eq. 2)
\]

**Step 3:**
\[
[2Fe-2S]^{2+} \rightarrow (apo) + 2S^{2-} + 2Fe^{3+} \quad (Eq. 3)
\]

Recent work has shown that step 2 (Equation 2) is more complex than previously envisaged because it involves the conversion of the [3Fe-4S]$^{3+}$ cluster to a persulfide-coordinated [2Fe-2S]$^{2+}$ form. The [4Fe-4S] to [2Fe-2S] conversion can therefore be written as in Equation 4 (14).

\[
[4Fe-4S(CysS)_4]^{2-} + O_2 \rightarrow [2Fe-2S(CysS)_2(CysS)_2]^{2-} + Fe^{3+} + Fe^{2+} + \text{reduced O}_2 \text{ species} \quad (Eq. 4)
\]

The retention of cluster sulfide (as CysSS) permits facile repair of the FNR [4Fe-4S] cluster in the presence of Fe$^{2+}$ and a reducing agent (14). Molecular oxygen-dependent conversion of the [4Fe-4S] cluster to the persulfide-ligated [2Fe-2S] causes con-
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Formations (19). The with persistence of this obligate aerobe under anaerobic condi-
tions was the monomeric apo form, which is capable of acquiring a [4Fe-4S] cluster via the
action of the lsc (iron-sulfur cluster) biosynthetic machinery, such that FNR continually monitors the cytoplasm for the availability of O$_2$ (16–18).

Unlike many bacteria, *Pseudomonas putida* and *Burkholderia* spp. possess multiple FNR family proteins that retain the characteristic cluster of cysteine residues in the N-terminal sensory domain. Transcriptomic analysis of the opportunistic pathogen *Burkholderia cenocepacia* revealed the presence of a 50-gene low oxygen-activated (*lxa*) locus that was associated with persistence of this obligate aerobe under anaerobic conditions (19). The *lxa* locus includes the FNR protein BCAM0287, which was induced 17-fold under low O$_2$ (microaerobic) conditions. In addition, two other FNR protein encoding genes were induced during growth under a 6% O$_2$ atmosphere, BCAM0049 (induced 77-fold, compared with aerobic conditions) and BCAM1483 (induced 3.5-fold) (19). Although an FNR box-like motif was associated with many genes induced under microaerobic conditions, the functions of the multiple FNR regulators in *B. cenocepacia* are poorly defined. Similarly, the properties and functions of the three FNR proteins of *P. putida* KT2440 are poorly understood. Here for the first time, three FNR proteins (PP$_{3233}$, PP$_{3287}$, and PP$_{4265}$, the last of which is also known as ANR) from a single bacterial species, *P. putida* KT2440, have been isolated, and their responses to O$_2$ in vivo and in vitro have been assessed.

**Experimental Procedures**

Overproduction and Purification of Proteins—The ANR open reading frame was amplified from *P. putida* KT2440 genomic DNA using the primers MS87 (5’-TTTTTCTAGACATGTCGAGGCCATCAAAT-3’) and MS88 (5’-TTTTTCTCGAGTCAGGCTCCATCACA-3’) containing engineered XbaI and XhoI sites, respectively, and ligated into pGEX-KG to give construct pGS2268 (see Table 1). The resulting GST-ANR fusion protein was overproduced following IPTG induction in aerobic cultures of *P. putida* BL21 harboring pGS2414, pGS2403, or pGS2413, respectively, for 24 h at 15 °C. The fusion proteins were purified from cell-free extracts on nickel-charged Hi-Trap chelating columns (GE Healthcare) equilibrated with 20 mM sodium phosphate, 0.5 M NaCl, pH 7.4, and were eluted by application of a linear imidazole gradient (0–1 M) following by desalting into 25 mM HEPES, 100 mM NaCl, 100 mM NaNO$_3$, pH 7.5. Protein concentration was determined by the Bio-Rad protein reagent (20).

The ANR, PP$_{3233}$, and PP$_{3287}$ proteins were reconstituted overnight under anaerobic conditions at 25 °C in 25 mM HEPES, 100 mM NaCl, 100 mM NaNO$_3$, pH 7.5, to which 0.5 mM L-cysteine, 12.5 mM DTT, an 8-fold molar excess of (NH$_4$)$_2$Fe(SO$_4$)$_2$ and 0.2 μM NiFs cysteine desulfurase were added. Reconstituted proteins were purified on a heparin column (GE Healthcare) and eluted in 25 mM Tris-HCl containing 500 mM NaCl and 2 mM EDTA, and the standards used to calibrate the column were blue dextran, hemoglobin, ovalbumin, cytochrome c, and aprotinin.

UV-visible and CD Spectroscopy—Sealed anaerobic cuvettes containing reconstituted FNR proteins were injected with increasing amounts of air-saturated buffer as indicated and were incubated at 25 °C for 10 min following by spectroscopic analysis. Absorbance measurements were made with a Cary UV-visible spectrophotometer. Changes in absorbance at 405 nm were used to monitor the conversion of the clusters. The extinction coefficient for the *E. coli* FNR iron-sulfur cluster ($\epsilon_{405\text{ nm}}=16,200\text{ M}^{-1}\text{ cm}^{-1}$) was used to calculate the amount of [4Fe-4S]$^{2+}$ cluster in the reconstituted proteins. The spectra shown are typical of at least three experiments. CD measurements were made with a Jasco J-810 spectropolarimeter. Aliquots of ANR (680 μl) were diluted to 29.8 μM iron-sulfur cluster under anaerobic conditions for initial measurements before treating with oxygenated buffer (~220 μM O$_2$) to give ~2-fold molar excess O$_2$ and then incubated for 15 min at room temperature prior to further measurements.

Kinetic Measurements—Reactions were initiated by the injection of air-saturated buffer (final concentration, ~100 μM O$_2$) into sealed anaerobic cuvettes containing reconstituted ANR, PP$_{3233}$, or PP$_{3287}$ proteins (final concentration, ~6–9 μM [4Fe-4S]) at 25 °C. The dead time of mixing was ~5 s. Changes in absorbance at 420 nm were used to monitor the conversion of the clusters. The A$_{420\text{ nm}}$ decay data were fitted to a single or double
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... exponential function (as appropriate) using the program Origin (version 8; OriginLab). Where a double exponential function was fitted, the higher rate constant was assumed to correspond to the initial reaction with O₂. Reported rate constants are mean values with standard errors from three repeats.

Liquid Chromatography-Mass Spectrometry of ANR—For LC-MS, an aliquot of ANR (20 μl, 80 μM [4Fe-4S]) was combined with an equal volume of oxygenated buffer (~220 μM O₂) or anaerobic buffer and allowed to react for 15 min. Samples were diluted to 2.9 μM final concentration, with an aqueous mixture of 1% (v/v) acetonitrile, 0.3% (v/v) formic acid, sealed, removed from the anaerobic cabinet, and loaded (5 μl) onto a ProSwift RP-1S column (4.6 x 50 mm) (Thermo Scientific) on a Ultimate 3000 UHPLC system (Dionex, Leiden, The Netherlands). Protein analysis was eluted (0.2 ml/min) using a linear gradient (15 min) from 1% to 100% (v/v) acetonitrile, 0.1% (v/v) formic acid. The eluent was continuously infused into a Bruker microQTOF-QIII mass spectrometer, running Hystar (Bruker Daltonics, Coventry, UK), using positive mode electrospray ionization. Compass Data Analysis with MaxEntrophy v1.3 (Bruker Daltonics, Coventry) was used for processing of spectra under LC peak. The mass spectrometer was calibrated with ESI-L tuning mix (Agilent Technologies).

Construction of Plasmids and Bacterial Strains—To investigate the responses of the three P. putida FNR proteins in vivo, it was necessary to create P. putida KT2440 strains that only expressed one of the three FNR proteins encoded by the genome. Two different strategies were used to create unmarked deletion mutants. The P. putida gene PP_4265 encoding ANR was deleted using sacb counter selection and FLP recombinase excision as described by Hoang et al. (22). The primer pairs oAS23 (5'-GGAATTCAGCCAGATCGGCGACCTGTA-3') and oAS26 (5'-CTTCGTCCAGTCAAGTGGGCTCAGCACCCAGAGTGCCAT-3') containing engineered EcoRI and BamHI, and XbaI restrictions sites (as indicated by underlining) were used to amplify 511- and 533-bp DNA fragments flanking the PP_4265 gene. The fragments were cloned into the suicide vector pEX18Ap flanking the gentamicin resistance cassette from plasmid pPS858 and used to generate the unmarked P. putida PP_4265 mutant strain (22).

For the generation of unmarked gene deletion mutants of the genes encoding PP_3233 and PP_3287, the I-SceI endonuclease was used to amplify upstream and downstream regions of PP_3233 and join both fragments by sewing PCR: Upstream-Fwd (5'-GCTTCGTCCAGGCTCAC-3'), Upstream-Rev (5'-CTTCGTCCAGGCTCAC-3'), Downstream-Fwd (5'-CTTCGTCCAGGCTCAC-3'), Downstream-Rev (5'-CTTCGTCCAGGCTCAC-3'), and join both fragments by sewing PCR: Upstream-Fwd (5'-GCTTCGTCCAGGCTCAC-3'), Upstream-Rev (5'-CTTCGTCCAGGCTCAC-3'), Downstream-Fwd (5'-CTTCGTCCAGGCTCAC-3'), Downstream-Rev (5'-CTTCGTCCAGGCTCAC-3'), and Downstream-Rev (5'-CTTCGTCCAGGCTCAC-3'), engineered HindIII site underlined). For PP_3287 the following primers: PP_3287 Upstream-Fwd (5'-GAAATTCGCGACATCGGCTAGACCTGTC-3'), engineered EcoRI site underlined), PP_3287 Downstream-Fwd (5'-AGGCTCTCTCGATTGACGGTCACACGTTC-3'), engineered BamHI site underlined), PP_3287 Downstream-Rev (5'-GGATCCCCACGTTGCATGATCTTGGTCGAGCCTTCGCAG-3'), engineered BglII site underlined), PP_3287 Downstream-Rev (5'-GGATCCCCACGTTGCATGATCTTGGTCGAGCCTTCGCAG-3'), engineered BglII site underlined), and PP_3287 Downstream-Rev (5'-GGATCCCCACGTTGCATGATCTTGGTCGAGCCTTCGCAG-3'), engineered BglII site underlined). The PCR products were ligated into the suicide vector pEMG and used to generate double knock-out mutants P. putida PP_3233 PP_4265 (JRG6721) and P. putida PP_3287 PP_4265 (JRG6722), as well as P. putida PP_3233 PP_3287 (JRG6723) following the protocol described by Martinez-Garcia and de Lorenzo (23).

The PP_3233 and PP_3287 genes including their promoter regions were amplified by PCR from P. putida KT2440 genomic DNA using the primer pairs PP_3233 (5'-TTTTGGAATTCCTCGATCAACAGTGAA-3' and 5'-TTTTGGAATTCCTCGATCAACAGTGAA-3') and PP_3287 (5'-TTTTGGAATTCCTCGATCAACAGTGAA-3' and 5'-TTTTGGAATTCCTCGATCAACAGTGAA-3'). The PCR products were ligated into the suicide vector pPS858 containing engineered EcoRI and Xhol sites (underlined) for ligation into pBBR1MCS-5 to give pPS2508 and pGS2509, respectively (see Table 1).

For the heterologous reporter system, expression plasmids for use in E. coli JRG6348 as well as an equivalent E. coli fnr expression plasmid to act as a control were created (see Table 1). The open reading frames corresponding to FNR, ANR, PP_3233, and PP_3287 were amplified by PCR to incorporate a Xhol restriction site downstream of the open reading frames: MS125 (5'-ATCCCCAAAAGGCAATTAT-3') and MS126 (5'-TTTTGACGACGACAGTCG-3') for ANR; MS122 (5'-TTTTGACGACGACAGTCG-3') for MS88 (5'-TTTTGACGACGACAGTCG-3') and MS92 (5'-TTTTGACGACGACAGTCG-3') for MS124 (5'-TCAGCTCGCTCGAATAT-3') and MS90 (5'-TTTTGACGACGACAGTCG-3') for MS288. After digestion with XhoI, the products were ligated into pBADHisB (Invitrogen) following Ncol and Xhol digestion and filling in the Ncol site so that the ATG start codon was provided by the vector and the encoded proteins lacked His tags. The authenticity of all constructs was confirmed by DNA sequencing.

In Vivo Transcription Assays—P. putida KT2440 mutants, JRG6721, JRG6722, and JRG6723 with deletions of two of the three FNR encoding genes, i.e. capable of expressing either PP_3233, PP_3287, or anr only, were transformed with the FNR-dependent reporter plasmid pGS810 (pFF-41.5; see Table 1). Where indicated, JRG6721 and JRG6722 were transformed with pGS810 and either pGS2508 or pGS2509 (expressing PP_3233 or PP_3287 under the control of their respective native promoters: see Table 1). Cultures were grown in L-broth supplemented with appropriate antibiotics—tetracycline (35 μg ml⁻¹) and gentamicin (20 μg ml⁻¹)—in 50 ml of shaking (200 rpm) conical flasks containing 10, 20, 30, 40, or 50 ml of medium at 30 °C for 3 h. To test the effects of nitric oxide on ANR, PP_3233, and PP_3287 activities, 1-hydroxy-2-oxo-3-((200 rpm) conical flasks containing 10, 20, 30, 40, or 50 ml of medium at 30 °C for 3 h. To test the effects of nitric oxide on ANR, PP_3233, and PP_3287 activities, 1-hydroxy-2-oxo-3-(N-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7, 20 μM; 40 μM nitric oxide) was added to anaerobic cultures grown in mineral medium 154 (1.4 g KH₂PO₄, 5.7 g Na₂HPO₄, 0.6 g NaCl, 1.7 g K₂SO₄, 0.55 mg MnSO₄·4H₂O, 50 mg MgSO₄·7H₂O, 3 mg/liter FeCl₃) supplemented with 0.4% (w/v)
yeast extract and 30 mM L-arginine and appropriate antibiotics.

The effects of oxidative stress were tested in aerobic L-broth cultures supplemented with paraquat (0.2 mM). All *P. putida* cultures were incubated at 30 °C for 3 h.

**H9252**-Galactosidase activities were measured as described by Miller (24).

RNA Isolation and qRT-PCR— Cultures of *E. coli* JRG6348 transformed with the pBAD-HisB-derivatives pGS2350, pGS2351, pGS2352, or pGS2353 (encoding FNR, PP_4265 (ANR), PP_3233, and PP_3287) all expressed under the control of the pBAD promoter to eliminate any differential transcriptional control over the production of the regulators (see Table 1) were grown under anaerobic conditions (sealed tubes filled to the neck) in M9 minimal medium supplemented with L-broth (5%, v/v), glycerol (0.4%, v/v), trimethylamine N-oxide (20 mM), sodium fumarate (20 mM), and ampicillin (100 μg ml⁻¹) at 37 °C until the *A₆₀₀* reached 0.2 (2). Aliquots were removed, and mRNA was stabilized by the addition of 0.4 volume of ice-cold ethanol-phenol (95%:5%) at pH 4.5. The cultures were then exposed to air by shaking, and incubation was continued for 20 min before taking further samples for total RNA preparation using the RNeasy RNA purification kit (Qiagen) according to the manufacturer’s instructions. RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Relative lacZ RNA quantities were determined for triplicate cultures as previously described (25).
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To determine the abundances of the *anr*, PP$_{3233}$ and PP$_{3287}$ transcripts in *P. putida* strains, qRT-PCR was used with RNA samples isolated as described above. The genes for normalization were *gyrA* and *gyrB*. The primers used were: *anr*, 5’-TCTTTGCTGAACCTGGGAAG-3’ and 5’-AGGCAAAA-CTGTCAACCCTG-3’; *gyrA*, 5’-GTCAGGTTCCAGCCTGTA-3’ and 5’-TCCCGGTTGATGACGTAGC-3’; *gyrB*, 5’-GCAAGCTTATCATCATA-3’ and 5’-GGGTCTCAACACAG-3’; PP$_{3233}$, 5’-AGGAATGAGCCTGACC-3’ and 5’-GAAAAATTCTTGATGACGTAGC-3’; and PP$_{3287}$, 5’-GAATTTCGCAACCTGACCATG-3’ and 5’-TGCGGATGTCTCGTGAAG-3’.

Results and Discussion

*P. putida*—KT2440 possesses three FNR proteins: PP$_{3233}$, PP$_{3287}$ and PP$_{4265}$ (hereafter ANR). Compared with the *E. coli* FNR protein, ANR is 53% identical (76% similar over 226 amino acid residues), PP$_{3233}$ is 46% identical (67% similar over 225 amino acid residues), and PP$_{3287}$ is 41% identical (58% similar over 224 amino acid residues). The four cysteine residues that coordinate the [4Fe-4S] cluster that is essential for the function of *E. coli* FNR are conserved, and thus all three *P. putida* FNR proteins were predicted to contain cysteinylated [4Fe-4S] clusters; however, the amino acid residues in the vicinity of the clusters differ (Figs. 1 and 2). Previous studies have shown that replacement of amino acid residues adjacent to cluster coordinating cysteine residues can have profound effects on the reactivity of the *E. coli* FNR iron-sulfur cluster with O$_2$ (12, 26). These observations suggested that the three *P. putida* FNR proteins might have evolved different sensitivities to O$_2$.

The Reaction of the *P. putida* ANR Iron-Sulfur Cluster with O$_2$ Reminisces That of *E. coli* FNR—*ANR* was released from a GST-ANR fusion by “on-column” treatment with the protease thrombin. Application of the resulting apo-ANR protein to a calibrated gel filtration column indicated that unlike apo-FNR, which is monomeric (10), apo-ANR was dimeric, despite retaining Asp-154 (FNR numbering) that is proposed to cause a charge clash preventing dimerization of apo-FNR (Fig. 1A and Ref. 15). This suggests that additional residues in the dimer interface also contribute to determine the oligomeric state of ANR and FNR. After anaerobic iron-sulfur cluster reconstitution, the iron content of ANR was 4.1 ± 0.3 iron atoms per subunit (n = 3), based on protein estimation by total amino acid analysis. The anaerobic UV-visible spectrum of ANR was characteristic of a [4Fe-4S] protein (ε$_{405}$nm = ~18,000 M$^{-1}$ cm$^{-1}$), and upon addition of O$_2$ the spectrum changed to resemble that of a [2Fe-2S] protein, with broad absorbance bands at 320, 420, and 550 nm (Fig. 3A). Upon prolonged (16 h) exposure to air, the [2Fe-2S] form was degraded to the apo-ANR protein. Titration of reconstituted ANR with aerobic buffer revealed a progressive decrease in absorbance in the 400–420-nm region associated with conversion of the [4Fe-4S] form to the [2Fe-2S] form (Fig. 3A). The CD spectrum of reconstituted [4Fe-4S] ANR exhibited positive bands at 296, 325, 375, and 420 nm, and upon addition of O$_2$ (~2-fold molar excess), these bands were replaced by a broad spectrum with two positive bands at 325 and 450 nm and one negative band at 375 nm, similar to the [2Fe-2S] form of FNR (Fig. 3B) (21). Treatment of the [4Fe-4S] form of ANR with 2 molar equivalents of O$_2$ for 15 min followed by analysis of the resulting [2Fe-2S] form by LC-MS revealed the presence of up to five sulfur adducts, with one and two additional sulfurs as the major species (Fig. 3C). Thus, it was concluded that the reaction of the ANR [4Fe-4S] cluster with O$_2$ proceeds via the same mechanism as that described for FNR, including the retention of cluster sulfide (14). The retention of cluster sulfide as S$^0$ has implications for the repair of [4Fe-4S] clusters (14). Anaerobic incubation of [2Fe-2S] ANR with 4-fold molar excess of ferrous ions in the presence of the reducing agent DTT regenerated the [4Fe-4S] form, as judged by the UV-visible spectrum of the protein (Fig. 3D). Thus, the mechanism of [4Fe-4S] repair proposed for *E. coli* FNR is likely to be a common feature of this family of regulators and probably other iron-sulfur proteins (14).

Reactions of the [4Fe-4S] Clusters of PP$_{3233}$ and PP$_{3287}$ with O$_2$ Result in Conversion to [2Fe-2S] Forms—Several attempts were made to overproduce the *P. putida* PP$_{3233}$ and PP$_{3287}$ proteins, but they were consistently found as insoluble aggregates when expressed at high levels, except when fused to the C terminus of the chaperone Trigger factor (Tig). Therefore, PP$_{3233}$ and PP$_{3287}$ were isolated as Tig fusions, and a Tig fusion of ANR was also generated to permit direct comparisons.

Anaerobic reconstitution of the iron-sulfur clusters of the three Tig fusion proteins resulted in UV-visible spectra characteristic of [4Fe-4S] proteins, with a broad absorbance at 400–420 nm (Fig. 4, A–C). Titration with aerobic buffer resulted in spectral changes that were consistent with conversion from...
FIGURE 3. Oxidation and repair of the *P. putida* ANR protein. A, UV-visible spectrum of reconstituted ANR containing ~15 μM [4Fe-4S] cluster under anaerobic conditions (thick line). The changes in the ANR spectrum upon successive additions of aerobic buffer (25 mM Tris-HCl containing 500 mM NaCl, pH 7.5) (thin lines) are presented along with the final spectrum ([2Fe-2S] form) shown in gray. B, CD spectra of [4Fe-4S] ANR (29.8 μM) before (solid line) and after (dashed line) exposure to O₂ (~2-fold molar excess). The arrow indicates the movement of spectral features in response to O₂. The buffer was 9 mM Tris, 17 mM HEPES, 1.7 mM CaCl₂, 236 mM NaCl, 66 mM NaNO₃, pH 7.5. C, detection of persulfide forms of apo-ANR after exposure of [4Fe-4S] ANR to O₂. Mixtures of ANR reconstituted under anaerobic conditions (initially 80 μM [4Fe-4S]₂²⁺ cluster) were analyzed by LC-MS after incubation with anaerobic buffer for 15 min (gray line) and after treatment with 2 molar equivalents of O₂ for 15 min (black line). The peak at 28,343 Da corresponds to the peak ANR monomer (mass, 28,347 Da) with two disulfide bonds. The peaks labeled S₀–5S₀ correspond to successive S₀ additions (Δ32 Da). D, restoration of the ANR [4Fe-4S] cluster by treatment of purified [2Fe-2S] ANR (40 μM cluster) with ferrous ions (4-fold molar excess) and DTT (3 mM). The gray line shows the initial spectrum of [2Fe-2S] ANR, the dashed and solid black lines show the spectra obtained 50 and 160 min after the addition of ferrous ions and DTT.

FIGURE 4. Absorbance spectra of Tig-tagged *P. putida* FNR proteins after treatment with increasing amounts of O₂. Absorbance spectra obtained by titration of anaerobic solutions of proteins with air-saturated (220 μM O₂ at 25 °C) buffer (25 mM Tris-HCl containing 500 mM NaCl, pH 7.5). The initial and final spectra are shown as bold lines. After each O₂ addition, the sample was incubated for 10 min at 25 °C before obtaining the spectrum shown. The arrows indicate the direction of spectral change during the titration. A, ANR. B, PP_3233. C, PP_3287. The insets show the spectral changes in the visible region with an expanded ordinate (absorbance) scale. The spectra shown are typical of at least three measurements.
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FIGURE 5. Kinetics of O₂-mediated [4Fe-4S] cluster conversion. Samples of reconstituted Tig fusions of ANR (A, inset untagged ANR), PP_3233 (B), and PP_3287 containing −8 μM [4Fe-4S] cluster (C) were mixed with a 13-fold molar excess of O₂ as aerobic buffer at 25 °C. The buffer was 25 mM HEPES, 100 mM NaCl, 100 mM NaNO₃, pH 7.5. Loss of the [4Fe-4S] clusters was monitored at 420 nm as a function of time (gray lines). Data were fitted to exponential functions as described in the text (black lines). The upper data set (not fitted) in each panel shows the response when anaerobic buffer was used in place of aerobic buffer. The rate constants reported in the text from these experiments are mean values with standard errors from three repeats.

[4Fe-4S] to [2Fe-2S] forms (Fig. 4, A–C). The response of the Tig-ANR fusion (Fig. 4A) was similar to that of the untagged ANR protein (Fig. 3A), suggesting that the Tig tag did not impair cluster acquisition or O₂-mediated cluster conversion. Thus, it was concluded that all the P. putida FNR proteins acquired [4Fe-4S] clusters that underwent conversion to [2Fe-2S] clusters in the presence of O₂.

PP_3233 and PP_3287 React More Slowly than ANR with O₂ in Vitro—Under pseudo-first order reaction conditions (O₂/[4Fe-4S] ratio of ~13), the A₄₂₀ nm decays for ANR (both ANR and the Tig-ANR fusion), Tig-PP_3233, and Tig-PP_3287 were measured (Fig. 5). For ANR and Tig-ANR, the data were best fitted to a double-exponential function with observed rate constants (kobs) for the first reaction of 0.034 ± 0.007 s⁻¹ for ANR and 0.028 ± 0.0015 s⁻¹ for the Tig-ANR fusion (Fig. 5A). This again indicates that fusion to Tig did not significantly affect the reactivity of the ANR iron-sulfur cluster, and thus it was assumed that a Tig tag would not affect the reactivity of the PP_3233 and PP_3287 clusters. For the Tig-PP_3233 and Tig-PP_3287 fusion proteins, the data were best fitted to a single-exponential function yielding kobs values of 0.0038 ± 0.0002 s⁻¹ for Tig-PP_3233 and 0.0055 ± 0.0001 s⁻¹ for Tig-PP_3287 (Fig. 5, B and C). Division of the observed rate constants by the O₂ concentration provides an estimate of the apparent second order rate constants for the fusion proteins: ANR, 280 M⁻¹ s⁻¹ (cf. 309 M⁻¹ s⁻¹ for the untagged ANR); PP_3233, 38 M⁻¹ s⁻¹; and PP_3287, 55 M⁻¹ s⁻¹. These values indicate that the [4Fe-4S] cluster of ANR displays similar sensitivity to O₂ as previously reported for E. coli FNR (278 M⁻¹ s⁻¹), but the iron-sulfur clusters of PP_3233 and PP_3287 were significantly less reactive with O₂ in vitro, more closely resembling the previously characterized variant FNR-S24F (80 M⁻¹ s⁻¹), which is also less responsive to O₂ in vivo (11, 12).

The Responses of PP_3233 and PP_3287 to Increased Culture Aeration Are Weaker than That of ANR—To determine whether ANR, PP_3233, and PP_3287 act as O₂ sensors in vivo, three double mutant strains of P. putida were created in which two of the three genes encoding FNR proteins were deleted (Table 1). Cultures of these strains were grown in 50-ml conical flasks at 30 °C with shaking (200 rpm). For aerobic cultures, the flasks contained 10 ml of medium; for O₂-limited cultures, the flasks contained 40 ml of medium. Strains that lacked anr exhibited impaired growth under O₂-limited conditions, and strains that lacked either PP_3233 or PP_3287 were impaired under aerobic conditions (Fig. 6). This is consistent with relative O₂ sensitivities of the ANR, PP_3233, and PP_3287 iron-sulfur clusters.

For E. coli FNR, it has been shown that Glu-209, Ser-212, and Arg-213 in the DNA recognition helix make the major interactions with the FNR box: TTAGATCTAGATCAA (FF site). The amino acid sequences of the DNA recognition helices of the P. putida FNR proteins are very similar to those of E. coli FNR (PP_3287 has Cys in place of Ser), suggesting that ANR, PP_3233, and PP_3287 may act as O₂ sensors. Preliminary electromobility shift assays indicated that all three P. putida FNR proteins could bind to the FF site under anaerobic conditions. Therefore, the P. putida mutants were transformed with plasmid pGS810, which carries the FNR-dependent FF-41.5 (pFF-41.5) promoter fused to lacZ (Table 1). Cultures were grown under O₂-limited conditions (50 ml of medium in a 50-ml shaking conical flask) and aerobic conditions (10 ml of medium in a 50-ml shaking conical flask). Measurement of β-galactosidase activity for P. putida JRG6723 (expresses only anr) showed that ANR activity decreased ~5-fold in response to enhanced aeration (Fig. 7, A and B). Measurement of the anr transcript by qRT-PCR and ANR protein by Western blotting with anti-serum raised against E. coli FNR for cultures grown in 50-ml shake flasks
TABLE 1
Bacterial strains and plasmids used in this work

| Relevant characteristics | Source |
|--------------------------|--------|
| **Bacterial strain**     |        |
| JRG6348                  | Dr. David Lee (University of Birmingham, Birmingham, UK) |
| JRG6725                  | Laboratory collection |
| JRG6721                  | This work |
| JRG6722                  | This work |
| JRG6723                  | This work |
| JRG1728                  |        |
| **Plasmid**              |        |
| PAS12                    | This study |
| pEX18Ap                  | Ref. 22 |
| pFLP2                    | Ref. 35 |
| pGS2422                  | Ref. 36 |
| pGS652                   | Ref. 37 |
| pGS810 (pF2-41.5)        | Ref. 38 |
| pGS2268                  | This work |
| pGS2350                  | This work |
| pGS2351                  | This work |
| pGS2352                  | This work |
| pGS2353                  | This work |
| pGS2403                  | This work |
| pGS2413                  | This work |
| pGS2414                  | This work |
| pGS2508                  | This work |
| pGS2509                  | This work |
| pPS858                   | Ref. 22 |
| pSW1                     | Ref. 40 |

Responses of Three P. putida FNR Proteins to O₂

Table 1 contains data on bacterial strains and plasmids used in the study. The table lists the bacterial strains, plasmids, and relevant characteristics along with their sources. The table provides a comprehensive overview of the experimental setup, including the use of E. coli and P. putida strains, along with their respective plasmids and characteristics such as antibiotic resistances (Amp, Cm, Gm, Kan, Tet) and control plasmids (pAS12, pEX18Ap, pFLP2).

The study focuses on the expression and regulation of FNR-dependent promoters in P. putida under oxygen-limited conditions. The data indicates that the level of expression of PP_3233 was ~10-fold lower, and PP_3287 was ~5-fold lower than anr. Low levels of PP_3233 and PP_3287 mRNA were consistent with the hypothesis that expression of the three P. putida FNR proteins is likely to be temporally and/or spatially distinct. Therefore, to increase expression of PP_3233 and PP_3287, these genes and their respective promoter regions were ligated into the broad host range vector pBBR1-MCS-5 (Table 1). The resulting expression plasmids were used to transform P. putida JRG6722 and JRG6721 carrying the pGS810 reporter plasmid creating strains that only expressed PP_3233 or PP_3287. Cultures expressing PP_3233 from pGS2508 (ANR^-, PP_3233^+, PP_3287^-) or PP_3287 from pGS2509 (ANR^-, PP_3233^-, PP_3287^+) were grown under O₂-limited and aerobic conditions, and qRT-PCR showed that the level of PP_3233 and PP_3287 expression was increased by ~10- and ~5-fold compared with the expression of chromosomal anr. Unfortunately, the corresponding increase in PP_3233 and PP_3287 proteins could not be determined because the E. coli FNR anti-serum did not cross-react with these proteins. Nevertheless, for both overexpression strains, FNR-dependent β-galactosidase activity decreased significantly with increased aeration (Fig. 7A).
Responses of Three P. putida FNR Proteins to O₂

FIGURE 6. Growth of P. putida under aerobic and O₂-limited conditions. P. putida mutants that lack two of the three fnr genes present in the wild-type strain were grown under aerobic conditions (A, 10 ml of medium in a 50-ml conical flask with shaking at 200 rpm, 30 °C) or O₂-limiting conditions (B, 40 ml of medium in a 50-ml conical flask with shaking at 200 rpm, 30 °C). Growth was monitored by measuring the optical density of the cultures (A) or qRT-PCR after exposure of anaerobic cultures to O₂ for 20 min (B). The data points show the means and standard deviations (n = 6). *p < 0.05, significance difference between: wild-type (A) and all mutant strains and strains possessing the anr gene (i.e. wild-type and JRG6723) and those lacking anr (JRG6721 and JRG6722) (p ≤ 0.05) (B).

It was concluded that transcription activation by PP_3233 and PP_3287 was inhibited by O₂.

To confirm the in vivo O₂ responsiveness of the P. putida FNR proteins, a heterologous reporter system consisting of an E. coli fnr, lac mutant (JRG6348) with a chromosomal copy of the FNR-dependent FF-41.5 promoter fused to lacZ was transformed with plasmids expressing FNR, ANR, PP_3233, or PP_3287 under the control of the pBAD promoter (Table 1). Measurement of the decrease in lacZ transcript abundance by qRT-PCR after exposure of anaerobic cultures to O₂ for 20 min showed that the activities of E. coli FNR and all three P. putida regulators decreased, with FNR- and ANR-dependent transcription showing the greatest responses (Fig. 7C). The weaker responses of PP_3233 and PP_3287 suggested that these proteins were less sensitive to O₂ compared with FNR and ANR, consistent with the in vitro data presented above.

Signal Specificity—Transcription factors that utilize iron-sulfur clusters as sensory modules have been shown to respond to O₂ (e.g. FNR), redox state (e.g. SoxR), nitric oxide (e.g. NsrR), and iron-sulfur cluster/iron homeostasis (e.g. IscR) (27, 28). Some of these transcription factors respond to more than one of these signals. Hence, the E. coli FNR and SoxR proteins respond to nitric oxide in addition to their primary signals, O₂ and redox cycling, respectively. In vitro kinetic measurements with the [4Fe-4S] form of FNR indicated that it is much more sensitive to nitric oxide than it is to O₂. However, in vivo, FNR is only nitrosylated when the major nitric oxide sensors (e.g. NsrR and NorR) and detoxification systems (e.g. NorVW, NrfA, and Hmp) are overwhelmed. Thus, FNR serves primarily as an O₂ sensor with a secondary nitric oxide sensing role (25). By contrast, the iron-sulfur clusters of regulators that are primarily nitric oxide sensors (e.g. NsrR and Wbl proteins) or redox sensors (e.g. SoxR) are generally stable for several hours in the presence of O₂ (29–31). The data described above show that the three P. putida FNR proteins respond to O₂ in vitro and in vivo, suggesting that they are primarily O₂ sensors. To determine whether they also share the nitric oxide- or redox-responsive characteristics of E. coli FNR and SoxR, respectively, anaerobic cultures of P. putida expressing only one of the three FNR proteins and carrying the FNR-dependent pFF-41.5 fused to lacZ were supplemented with the nitric oxide donor NOC-7; in addition, aerobic cultures were exposed to the redox cycling agent paraquat. The responses of PP_3233 and PP_3287 were similar, nitric oxide had little or no effect under anaerobic conditions, and paraquat had no effect under aerobic conditions (Fig. 8). However, for ANR, nitric oxide significantly inactivated anaerobic reporter gene expression, whereas paraquat again had no effect under aerobic conditions (Fig. 8). Thus, the response of ANR was similar to that reported previously for E. coli FNR, further confirming the similarities between these two proteins, but PP_3233 and PP_3287 were less responsive with both O₂ and nitric oxide compared with ANR (25). Nevertheless, in all cases the greatest responses were provoked by culture aeration, and therefore, it was concluded that O₂ is the major modulator of the activity of all three P. putida FNR proteins.

Conclusions—The research described here suggests that the three FNR proteins of P. putida have evolved to fulfill distinct but overlapping roles. All three regulators, ANR, PP_3233, and PP_3287, acquired [4Fe-4S] clusters under anaerobic conditions and were converted to [2Fe-2S] forms upon exposure to O₂ in vitro. ANR has the least number of nonconservative amino acid substitutions in the vicinity of the cluster-ligating cysteine residues compared with E. coli FNR and hence was expected to exhibit similar cluster reactivity to FNR (Fig. 1). The double-exponential nature of the ANR [4Fe-4S] cluster reaction with O₂, the detection of sulfur adducts after conversion of [4Fe-4S]²⁺ ANR to the [2Fe-2S] form, and the capacity to repair the [4Fe-4S] cluster by simply providing ferrous ions under reducing conditions are consistent with the reaction scheme for E. coli FNR and O₂ proposed by Zhang et al. (14) in which the [2Fe-2S]²⁺ cluster is ligated by one (Equations 5 and 6) or two (Equations 5 and 7) cysteine persulfides (CysS²⁻).

\[
[4Fe-4S(CysS)_2]^--O_2\rightarrow[3Fe-4S(CysS)_2]^-- + CysS^- + O_2^- + Fe^{2+} \quad (\text{Eq. 5})
\]

\[
[3Fe-4S(CysS)_2]^-- + CysS^- + O_2 + 2H^+ \rightarrow[2Fe-2S(CysS)_2CysS]^-- + Fe^{3+} + S^- + H_2O_2 \quad (\text{Eq. 6})
\]
Responses of Three \( P. \) putida FNR Proteins to \( O_2 \)

\[ \text{[3Fe-4S(CysS)]}^2^- + \text{CysS}^- + \text{O}_2 + 4\text{H}^+ \rightarrow \text{[2Fe-2S(CysS)S]}^2^- + \text{Fe}^{3+} + 2\text{H}_2\text{O} \]  

(Eq. 7)

Furthermore, ANR resembled FNR in exhibiting a secondary response when cultures were exposed to micromolar levels of nitric oxide (Fig. 8). Thus, the observations reported here are consistent with \( P. \) putida ANR acting as an \( E. \) coli-type \( O_2 \) sensor regulator, in accordance with its ability to regulate the expression of multiple terminal oxidases of the \( P. \) putida respiratory chain and the ability of the closely related \( P. \) aeruginosa \( \text{anr} \) gene (encoded protein 88% identical, 94% similar over 244 amino acids) to complement the anaerobic growth phenotype of an \( E. \) coli \( \text{fur} \) mutant (32, 33).

The [4Fe-4S] clusters of \( \text{PP}_3233 \) and \( \text{PP}_3287 \) also underwent conversion to [2Fe-2S] clusters upon exposure to \( O_2 \), but these reactions were slower than that of ANR, and the responses of these proteins when cultures were exposed to nitric oxide were weaker than that observed for ANR (Fig. 8). The kinetic data for the reaction of the \( \text{PP}_3233 \) and \( \text{PP}_3287 \) [4Fe-4S] clusters with \( O_2 \) fitted well to a single-exponential function rather than a double-exponential function, implying that, unlike FNR and ANR, the initial cluster oxidation step to generate the [3Fe-4S]\(^{1+} \) intermediate (Equation 5) was much slower than the subsequent decay of the [3Fe-4S]\(^{1+} \) to the [2Fe-2S]\(^{2+} \) form (Equations 6 and 7). Thus, it is suggested that the mechanism for [4Fe-4S] to [2Fe-2S] cluster conversion in \( \text{PP}_3233 \) and \( \text{PP}_3287 \) was similar to that described for FNR (11) and ANR, but the \( \text{PP}_3233 \) and \( \text{PP}_3287 \) [4Fe-4S] clusters appear to be more stable when bacteria are exposed to air or nitric oxide. The relative rates of ANR, \( \text{PP}_3233 \), and \( \text{PP}_3287 \) cluster reactions with \( O_2 \) results in differential responses to \( O_2 \) availability.

The \textit{in vivo} properties of ANR, \( \text{PP}_3233 \), and \( \text{PP}_3287 \) were consistent with the observed reactivities of the [4Fe-4S] clusters with \( O_2 \). Previous work with \( E. \) coli FNR showed that replacement of Ser-24, which is located immediately adjacent to the cluster ligand Cys-23, by Arg resulted in significant aerobic FNR activity, indicative of stabilization of the FNR-S24R iron-sulfur cluster (12). Interestingly, \( \text{PP}_3287 \) has Arg in the position equivalent to Ser-24 in FNR (Figs. 1 and 2), and thus this amino acid substitution could at least partially account for the lower reactivity of \( \text{PP}_3287 \) with \( O_2 \). On the other hand, \( \text{PP}_3233 \) resembles \( E. \) coli FNR by retaining a Ser residue at the equivalent of position 24 (Figs. 1 and 2); however, amino acid substitutions in other locations are known to influence the reactivity of the \( E. \) coli FNR iron-sulfur cluster with \( O_2 \) (26, 34). Like S24R, another amino acid substitution that promoted aer-

\begin{align*}
\text{ANR} & \rightarrow \text{ANR}^+ \\
\text{PP}_3233 & \rightarrow \text{PP}_3233^+ \\
\text{PP}_3287 & \rightarrow \text{PP}_3287^+
\end{align*}

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![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

**FIGURE 8. Responses of P. putida FNR proteins to nitric oxide and oxidative stress in vivo.** P. putida strains capable of expressing only arr from the chromosome (ANR) or only PP_3233 from a multicopy plasmid (PP_3233 $^{+}$) or only PP_3287 from a plasmid (PP_3287 $^{-}$) were transformed with the FF-41.5-lacZ reporter plasmid pGS810. Cultures were grown at 30°C for 3 h under anaerobic (50 ml of anaerobic minimal medium 154 supplemented with 0.4% (w/v) yeast extract and 30 mML L-arginine in a sealed 50-ml conical flask) or aerobic (10 ml of L-broth in a 50-ml conical flask, 200 rpm shaking) conditions in the presence and absence of NOC-7 (20 L) as indicated. Samples were taken for measurement of β-galactosidase activity as a proxy for FNR protein activity: ANR (A), PP_3233 $^{+}$ (B), and PP_3287 $^{-}$ (C). The error bars are the standard deviation from the mean values ($n = 3$). p values were determined by Student’s t test. **, $p < 0.01$; *, $p < 0.1$; NS, $p > 0.5$.

by analogy, replacement of the acidic Asp residue might alter the redox properties of the PP_3233 iron-sulfur cluster, such that it is less O$_2$ reactive.

Although the observations reported here resolve several aspects of the properties of the three FNR proteins possessed by P. putida KT2440, many questions remain, including: (i) What are the conditions encountered by P. putida that induce PP_3233 and PP_3287 target gene expression? (ii) Do the three P. putida FNR proteins control distinct but overlapping regulons, perhaps by making productive interactions with additional transcription factors or alternative sigma factors? (iii) What is the imperative for employing multiple FNR proteins to extend the range of O$_2$-responsive gene expression? Further detailed biochemical and physiological studies are now required to address these questions and in so doing discern the mechanism of the observed differential sensitivities to O$_2$ of these closely related proteins and the broader implications for the control of gene expression in P. putida.

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