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Covalently linking the *Escherichia coli* global anaerobic regulator FNR in tandem allows it to function as an oxygen stable dimer

Yue Shan [a], Qing Pan [a], Junyan Liu [a], Feijuang Huang [b], Hongzhe Sun [b], Kunihiko Nishino [c], Aixin Yan [a,*]

[a] School of Biological Sciences, The University of Hong Kong, Hong Kong, China
[b] Department of Chemistry, The University of Hong Kong, Hong Kong, China
[c] Institute of Scientific and Industrial Research, Osaka University, Osaka 567-0047, Japan

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ABSTRACT

The anaerobic global regulator FNR from *Escherichia coli* is a $[4Fe–4S]^{2+}$ cluster containing, O_2 labile dimer that plays an important role in adapting the bacterium to its anaerobic lifestyle. Although functional significance of this global regulator has been well established, its structural and biochemical characterizations have been hindered by the intrinsic O_2 lability of FNR protein. To obtain oxygen stable FNR variants for *in vitro* characterizations, in this study, we utilized the approach of covalently linking two *fnr* alleles in tandem to promote their *in situ* dimerization in the absence of the $[4Fe–4S]^{2+}$ cluster under aerobic conditions. By covalently linking two alleles of *fnrD154A* mutant, which has reduced charge repulsions between two FNR monomers, an FNR variant of FNRD154A-Linker-FNRD154A (designated as $(FNRD154A)_2$) was constructed. $(FNRD154A)_2$ displayed significantly enhanced DNA binding affinities and transcriptional regulatory activities to various FNR dependent promoters under aerobic conditions. Further *in vivo* and *in vitro* studies demonstrated that this variant retains oxygen sensing capability and maintains a similar secondary structure as that formed by native monomers of FNRD154A. We conclude that this novel variant of FNR can be widely used in various biochemical and structural studies of FNR in the presence of O_2.

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1. Introduction

The ability of sensing and rapidly responding to oxygen availability is crucial to facultative anaerobes. In the Gram negative bacterium *Escherichia coli*, this process is primarily controlled by the global transcription regulator FNR (fumarate and nitrate reduction) which is activated under anaerobic conditions and upon activation, regulates the expression of more than one hundred genes in *E. coli* genome [1,2]. However, owing to the strict oxygen lability of native FNR protein, studies of its 3D-structure and its regulation of target genes *in vitro* have been limited.

The oxygen lability of FNR protein is attributed to its oxygen sensing $[4Fe–4S]^{2+}$ cluster, which is assembled into FNR during its *de novo* biosynthesis under anaerobic conditions. Assembly of the $[4Fe–4S]^{2+}$ cluster results in simultaneous FNR dimerization and activity of specific DNA binding and transcription regulation [3]. Upon exposure to oxygen, the $[4Fe–4S]^{2+}$ cluster is rapidly oxidized to $[2Fe–2S]^{2+}$ which renders dissociation of FNR dimer and loss of specific DNA binding and transcription regulation [4].

To facilitate structural and biochemical characterizations of FNR, efforts to isolate oxygen stable FNR mutants have been attempted [5–8]. Among several amino acid substitution mutants which exhibit higher aerobic transcription activities, the mutant FNRD154A is currently widely used in *in vitro* DNA binding assays owing to its capability to dimerize and bind to promoter DNA with decent affinities in the presence of oxygen [8]. Replacing aspartic acid with alanine in FNRD154A eliminates charge repulsions between the two FNR monomers and thus improves its dimerization in the absence of the $[4Fe–4S]^{2+}$ cluster under aerobic conditions [8]. Although FNRD154A is able to activate gene transcription under aerobic conditions, it does not exist exclusively as dimeric form under this condition and the ratio between dimer and monomer varies under different conditions and concentrations, rendering difficulties to characterize the binding of FNR to its regulated promoters [9]. With the development of genome-wide studies which have disclosed multitude of genes regulated by FNR, it becomes compelling to isolate oxygen stable FNR variant to facilitate the characterization of these newly identified genes in FNR regulon.

In the present study, we sought alternative approaches and designed a construct which links two *fnr* alleles in tandem to promote

Abbreviations: FNR, fumarate and nitrate reduction; EMSA, electrophoresis gel mobility shift assay; β-gal, β-galactosidase activity assay.

* Corresponding author. Fax: +852 2559 9114.
E-mail address: ayan8@hku.hk (A. Yan).

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its dimerization and conformational stability under aerobic conditions. This approach has been proved to be effective in generating active protein complexes that function as dimer or multimers, such as the dimeric transcription factor CRP [10], the trimeric multidrug efflux pump AcrB [11], and the hexameric ATPase ClpX [12]. We show that the covalently linked tandem FNR provides as an oxygen stable FNR variant that not only displays high binding affinities to FNR dependent promoters under aerobic conditions but also retains its promoter specificity and conformational stability, thus can be used for various *in vitro* characterizations of FNR as well as its regulation of target genes.

2. Material and methods

2.1. Strains and plasmid construction

Construction of plasmids containing tandem *fnr* alleles was achieved through four major steps as shown below using *fnrD154A-linker-fnrD154A* as an example: Step 1, pAY0871 (pET11a-*fnr*D154A) was constructed by site-direct mutagenesis PCR using pPK823 (pET11a-*fnr*) [8] as template and a pair of complementary primers centered with the desirable Asp to Ala mutant plus 18 bp upstream and 18 bp downstream sequences, respectively. The PCR product was digested with DpnI (final concentration: 200 μg/mL, Promega, USA) to remove the template plasmid before being transformed into DH5α competent cells. The desired plasmid was extracted from the transformants and was verified by DNA sequencing. Step 2, similar site-direct mutagenesis was utilized to remove the stop codon of *fnr*D154A in pAY0871 and replace it with the XhoI restriction site using a pair of complementary primers containing the desirable substitution. The resulting plasmid was designated as pET11a-*fnrD154A-Stop codon:XhoI* (pAY0705). Step 3, a DNA fragment containing the linker sequence followed by the second copy of *fnrD154A* was generated by PCR using pAY0871 (pET11a-*fnr*D154A) as template. The forward primer includes an XhoI restriction site followed by the sequence of the linker and the first 18 bp of *fnr* gene; and the reverse primer includes the last 18 bp of *fnr* gene containing the stop codon followed by the BamHI restriction site. The linker sequence was derived from a peptide containing 20 amino acid residues (ASGAGGSEGGGSEGGTSGAT), which is adopted from the work of Martin et al. [12]. Step 4, the resulting PCR product and the plasmid were digested with XhoI/BamHI enzymes (1000 μg/mL, Promega, USA) and following purification (GE healthcare, UK), were ligated using T4 DNA ligase (1000 μg/mL, Promega, USA). Desired ligation construct was selected by colony PCR and the resulting tandem construct was designated as pET11a-(*fnrD154A*)$_2$. The plasmid was verified by DNA sequencing. Construction of other tandem alleles of *fnr* variants was following the same procedures and are listed in Supplementary Table 1.

Construction of His$_6$-tagged *fnr* for overexpression: pET28a which contains His$_6$ in-frame and plasmid pAY0871 or pAY0706 were double digested with NdeI/BamHI restriction enzymes followed by ligation into pET28a such that the expression of N-terminal His$_6$ tagged FNRD154A or (FNRD154A)$_2$ is expressed under the T7 promoter. The resulting plasmids are pAY0980 and pAY0933, respectively.

2.2. Transcription activity assay

In vivo transcription activities of FNR and its variants were measured by β-galactosidase activity assay (β-gal) of FNR dependent *ydfZ* (PK8203) [8] or *narG* (RZ8480) [13] promoter-*lacZ* fusion in strains containing deletion of chromosomal *fnr* but transformed with plasmids encoded FNR variants. β-Galactosidase activity assay was conducted following the description of Mettert et al. [14]. Cells were grown to A_{600} as ~0.3 in M9 medium (supplemented with 0.2% glucose, 0.2% casamino acids, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 2 μg/mL Vitamine B_1 and 10 μg/mL Ferric Ammonium Citrate) under aerobic or anaerobic conditions. For β-gal assay of *PnarG-lacZ*, 1.4 mM nitrate was added. Chloramphenicol (20 μg/mL) was added to cell culture to terminate cell growth. 200 μL cultures were added to 800 μL Z-buffer and the reaction was initiated by adding 200 μL of 4 mg/mL ONPG. Color development was measured spectrophotometrically at 420 nm and 550 nm following termination of the reaction by 500 μL of 1 M Na_2CO_3. Activities were expressed in Miller units and results are the mean from three independent isolates.

2.3. Western blot

Expression of tandem FNR variants was determined by western blot. Cells were cultured under the identical conditions as for β-galactosidase activity assay. After termination of cell growth, aliquots of cells equivalent to 150 μL cells with $A_{600} \sim 0.3$ were removed and spun down to collect the cell pellets. Cells were lysed with 10 μL SDS sample buffer and cell lysate was separated on SDS–PAGE followed by transferring onto a nitrocellulose membrane. Membranes were then blocked by 5% milk in TBST followed by blotting with the primary (polyclonal anti-FNR, a gift from Prof. Patricia Kiley at the University of Wisconsin-Madison) and secondary antibody (Goat anti-Rabbit IgG-HRP conjugate, BioRad, USA) for 2 h at room temperature, respectively. FNR protein was visualized by exposure to X-ray films following development using ECL Western blotting analysis system (GE healthcare, UK).

2.4. Anaerobic growth curve

PK4811 ([15]) derivatives transformed with different pET11a plasmids containing different *fnr* variants were inoculated in M9 medium supplemented with 0.4% glycerol, 0.2% casamino acids and 20 mM nitrate (initial cell density: 100 cells/mL). A_{600} was recorded every hour from 13 to 20 h to plot the growth curve. Results are the mean from two independent isolates.

2.5. Overexpression and purification of FNR variants

PK22 ([13]) was used as the host strain to overexpress FNR recombinant proteins. Transformants were grown at 37 °C in LB medium with 0.2% glucose and kanamycin (100 μg/mL). Overnight cultures were diluted 1:100 to LB medium and grown to $A_{600} \sim 0.6$. Protein expression was induced by IPTG (0.1 mM) at 25 ° C for 16 h. 100 mL cells were harvested (4800*g* × 10 min) and were lysed by sonication in 4 mL lysis buffer (50 mM KPO_4, 150 mM NaCl, 10% glycerol, 0.5 mM DTT, 0.15 mM PMSF, 0.25 mg/mL lysozyme). Cell debris was removed by ultracentrifugation (20000 rpm × 30 min) at 4 °C and cell lysis was subject to protein purification.

Purification of His$_6$-tagged proteins was performed using Ni Sepharose 6 Fast Flow (GE healthcare, UK). Ni Sepharose was mixed with binding buffer (50 mM KPO_4, 500 mM NaCl, 10% glycerol, 500 mM NaCl, 40 mM imidazole) to make 50% slurry. 4 mL cell lysis and 2 mL slurry were subsequently incubated at 4 °C for 1 h and then were loaded on to the PD-10 column. The Ni Sepharose was washed with 10 mL binding buffer and the protein was then eluted with 2 mL elution buffer (50 mM KPO_4, 10% glycerol, 500 mM NaCl, 500 mM imidazole). Pooled protein elutions were dialysed against buffer A (pH 6.8, 50 mM KPO_4, 300 mM NaCl) to remove imidazole. The concentration of protein was determined by NanoDrop (Thermo, USA).

2.6. Circular Dichroism (CD) spectra

Circular Dichroism (CD) measurements were conducted to compare protein secondary structure. Purified $(FNRD154A)_2$ and FNRD154A were dialysed against buffer B (pH 6.8, 50 mM KPO_4, 200 mM KCl) to remove imidazole and sodium. The final concentrations of both proteins were adjusted to 5 μM (dimer). CD spectra were recorded using the JASCO-J815 spectroporimeter equipped with a 1.0 mm path length quartz cell at room temperature. Samples were measured at a scanning speed of 100 nm/min from 300 to 200 nm with a 1.0 nm data pitch and 1.0 nm bandwidth. The contents of α-helix and β-sheets were predicted by K2D algorithm (http://www.embl.de/~andrade/k2d.html) using values from 200 to 240 nM.

2.7. DIG (digoxigenin) Labeled Electrophoresis gel mobility shift assay (EMSA)

DIG-labeled EMSA was conducted using DIG Gel Shift Kit (2nd Generation, Roche, US). DNA labeling reaction was conducted following the manufacturer's instruction: DNA fragments corresponding to the promoter regions of *ydfZ* (−135 to −42 relative to the translational start site of the gene), *dmsA* (−225 to −113 relative to the translational start site of the gene), and *ompW* (−149 to −31 relative to translational start site of the gene) were diluted to 10 ng/μL by ddH_2O and incubated with labeling buffer, $CoCl_2$-solution, DIG-ddUTP solution and terminal transferase at 37 °C for 15 min. 0.2 mM EDTA was added to stop labeling reaction. Various amounts of purified FNRD154A or $(FNRD154A)_2$ and DNA fragments were mixed and incubated at 37 °C for 20 min in EMSA binding buffer (pH 7.2, 20 mM Tris, 50 mM NaCl, 10 mM EDTA, 4 mM DTT, 5% glycerol, 0.5 mg/mL BSA). Following the binding reaction, samples were electrophoresed on 6% non-denaturing polyacrylamide gel in 0.5×TBE buffer. After electrophoresis, free DNA probes or DNA–protein complexes were transferred to positively charged nylon membrane by contact blotting in 10×SSC buffer. Membrane was incubated with blocking buffer followed by alkaline phosphatase-conjugated antibody (anti-digoxigenin-AP). Films were developed after CDPS treatment. Intensities of DIG-labeled probe or DIG-labeled probe bound with protein were quantified using the Image J software. The equilibrium of free DNA, protein, and DNA–protein complex was under the pseudo-first-order conditions (protein in excess). Dissociation constant was calculated by non-linear least-squares analysis using GraphPad Prism.

3. Results

3.1. Construction and expression of covalently linked FNR dimer

Covalently linking the composing subunits or protomers of a multimeric protein complex has been proved to be an effective approach to generate biochemically functional variants which activities are comparable or even exceed of native proteins. The rational underlying this strategy is by linking two subunits in tandem in a single polypeptide, they are located in close proximity thus can readily form the multimeric protein complex. To create such tandemly expressed FNR dimer, we utilized a linker sequence of ASGAGGSEGGGSEGGTSGAT (Fig. 1A), which is sufficient to provide flexibility between the two subunit copies and is protease resistant. Western bolt analysis of whole cell lysate from cells harboring pET11a-*fnr-fnr* without IPTG induction showed the presence of a protein of ~56 kDa, which is about twice of the native FNR and is reacted with anti-FNR antibody, indicating that tandem FNR (designed as $(FNR)_2$) can be stably expressed (Fig. 1B). Growth assay demonstrated that this tandem protein can support the anaerobic growth of *E. coli* as that of native FNR protein (Fig. 1C), confirming that $(FNR)_2$ is functional in the cell.

3.2. Tandemly linked FNR constructs display elevated transcriptional activity in the presence of O_2

Following successful expression of the covalently linked tandem FNR, we measured *in vivo* transcription activity of this variant. β-galactosidase assay of FNR dependent P_{ydfZ}-*lacZ* showed that the tandem FNR dimer exhibit ~3-fold higher transcription activity than that of single copy (Fig. 2), suggesting that covalently linking of two FNR molecules can improve its activity in the presence of oxygen presumably because of enhanced protein dimerization. To screen for variant with further enhanced activity, we introduced D154A substitution which has been demonstrated to enhance dimerization between two FNR monomers in the presence of oxygen. Western blot analysis showed that this variant can also be stably expressed in *E. coli* (Fig. 1B). β-gal assay demonstrated that introduction of D154A in the tandem construct indeed further enhanced aerobic activity of *PydfZ-lacZ*, resulting in an activity that is ~2-fold higher than that of $(FNR)_2$ and 3-fold higher than that of single copy FNRD154A. The similar growth assays under the condition of anaerobic respiration demonstrated that $(FNRD154A)_2$ is fully functional as that of native FNR protein and $(FNR)_2$ (Fig. 1C).

To test if tandem construct $(FNRD154A)_2$ also activates other FNR dependent promoters under aerobic conditions, we measured β-gal activity of FNR activated P_{narG}-*lacZ*, P_{dmsA}-*lacZ*, and FNR repressed P_{ndh}-*lacZ* (Fig. 2). It was shown that $(FNRD154A)_2$ displayed greater activation or repression on these promoters than the $(FNR)_2$ or single copy FNRD154A. These results together demonstrated that $(FNRD154A)_2$ not only displays enhanced stability to O_2 but also retained promoter specificity as the native FNR, suggesting it can form a dimer resemble that of native FNR. To examine the resemblance of the dimer formed by $(FNRD154A)_2$ with that of native FNR dimer *in vitro*, we compared CD spectrum of His_6 tagged $(FNRD154A)_2$ and the dimer formed by the two monomers of His_6-FNRD154A, which had been shown to have identical secondary structure with that of native FNR dimer [17]. We found that $(FNRD154A)_2$ displayed similar CD spectrum with the dimer formed by two monomers of FNRD154A (Supplementary Fig. 1), further confirming that $(FNRD154A)_2$ folds into a dimer as that of native FNR dimer.

3.3. $(FNRD154A)_2$ retains oxygen responsive capability in vivo

We next examined the activity of $(FNRD154A)_2$ under anaerobic conditions and asked whether it still binds the $[4Fe–4S]^{2+}$ cluster and thus retains oxygen responsive capability. It was shown that under anaerobic conditions $(FNRD154A)_2$ exhibits comparable activity with that of FNR, FNRD154A, and $(FNR)_2$ dimer (Supplementary Fig. 2), suggesting that $(FNRD154A)_2$ is functionally comparable to native FNR protein and did not alter the promoter binding affinity and transcription capacity of FNR under anaerobic conditions. To test whether the anaerobically functional dimer of $(FNRD154A)_2$ was still dependent on the $[4Fe–4S]^{2+}$ cluster and thus retains the O_2 sensing capability, we introduced C122A, which has been shown to abolish the $[4Fe–4S]^{2+}$ cluster binding and transcription activity upon introduction to native FNR [16]. β-gal assay showed that C122A completely abolished anaerobic activity of $(FNR)_2$ or $(FNRD154A)_2$ on P_{ydfZ}-*lacZ*, suggesting that the anaerobic dimer of $(FNRD154A)_2$ was indeed dependent on the $[4Fe–4S]^{2+}$ cluster, thus $(FNRD154A)_2$ retains the capability of sensing and responding to oxygen. This result also suggests that although covalently linking two FNR monomers enhances dimerization by locating the two monomers in close proximity, it does not suppress the dimerization induced by the assembly of the $[4Fe–4S]^{2+}$ cluster

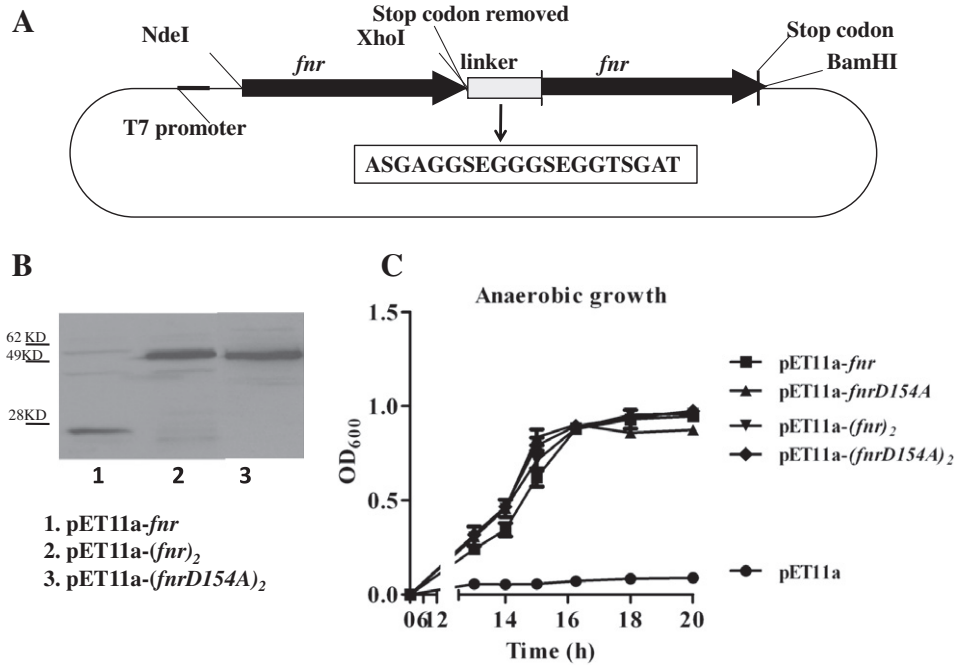

Fig. 1. Construction and expression of covalently linked tandem FNR. (A) A schematic diagram of the tandemly-expressed FNR construct. The two copies of *fnr* were transcribed and translated as a single polypeptide connected by a flexible and protease resistant linker under the control of T7 promoter. (B) Expression of covalently linked $(FNR)_2$ and $(FNRD154A)_2$. Wild type or covalently linked tandem FNR was expressed from pET11a without the IPTG induction. The protein expressed from $(FNR)_2$ and $(FNRD154A)_2$ was about twice (~56 KD) as that of native single copy FNR (~28 KD). (C) $(FNR)_2$ and $(FNRD154A)_2$ can support the anaerobic growth of *E. coli* as the native FNR protein. Strains which genomic *fnr* was deleted but transformed with either vector control (pET11a) or plasmids containing wild type FNR, covalently linked FNR variants of (pET11a-$(fnr)_2$) or pET11a-$(fnrD154A)_2$) were inoculated in M9 medium supplemented with 0.4% glycerol, 0.2% casamino acids and 20 mM nitrate under anaerobic conditions. A_{600} was recorded and plot the growth curve.

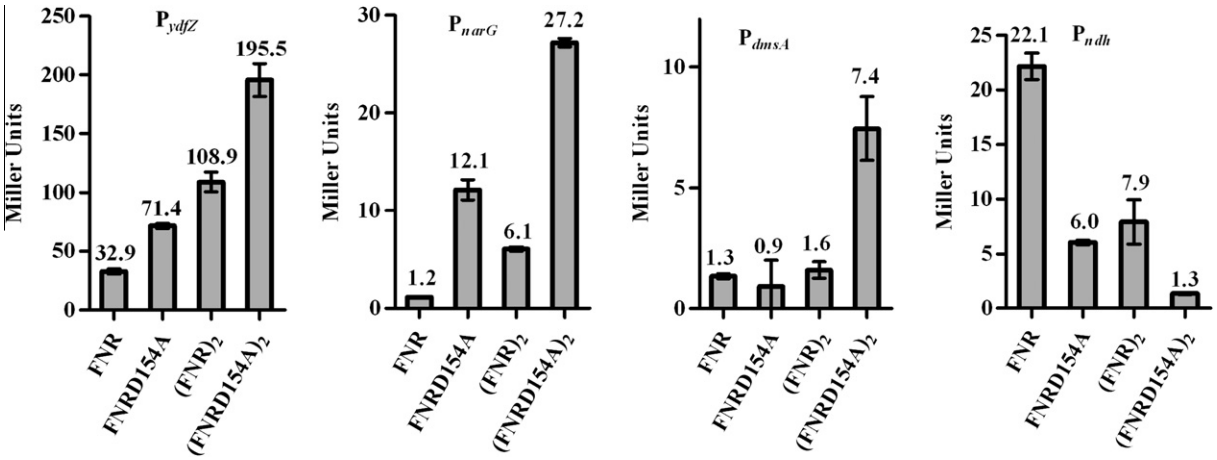

Fig. 2. *In vivo* transcription assay of FNR variants under aerobic conditions from FNR-dependent *promoter-lacZ* fusions. β-Galactosidase activity assay from several FNR dependent promoters including *ydfZ*, *narG*, *dmsA* and *ndh* showed that $(FNRD154A)_2$ displays higher activity than that of $(FNR)_2$ and FNRD154A variants under aerobic conditions. Cells grown to A_{600} as ~0.3 were terminated by addition of Chloramphenicol (final concentration: 20 μg/mL). Following cell lysis using chloroform–SDS, 200 μL of cell suspension was mixed with 800 μL Z-buffer and 200 μL of 4 mg/mL ONPG to assay for β-galactosidase activities. β-gal activities were measured and calculated from absorbance at 420 and 550 nm using the equation mentioned in Section 2.

which is spontaneous *in vivo*. Together, these results suggest that we have isolated an FNR variant which displays similar promoter binding capability and specificity as native FNR dimer but has increased oxygen stability than that of wild type FNR and FNRD154A.

3.4. $(FNRD154A)_2$ displays elevated DNA binding affinity under aerobic conditions

To test the *in vitro* promoter binding capability of $(FNRD154A)_2$, we first performed electrophoresis gel mobility shift assay (EMSA) to test the binding of $(FNRD154A)_2$ to the promoter of *ydfZ* gene and compare with that of FNRD154A under aerobic conditions. We utilized DIG-labeled promoter DNA so that the free DNA and protein-DNA complex can be readily detected with high sensitivity and DNA binding reaction can be conducted in pseudo-first order conditions in order to determine the dissociation constant of the FNR variants. EMSA assay demonstrated that $(FNRD154A)_2$ binds specifically to the DIG-labeled *ydfZ* promoter (Fig. 3) with a dissociation constant (K_d) of 3×10^{-9} M. Comparing with the K_d of FNRD154A to the same promoter which was 7×10^{-8} M,

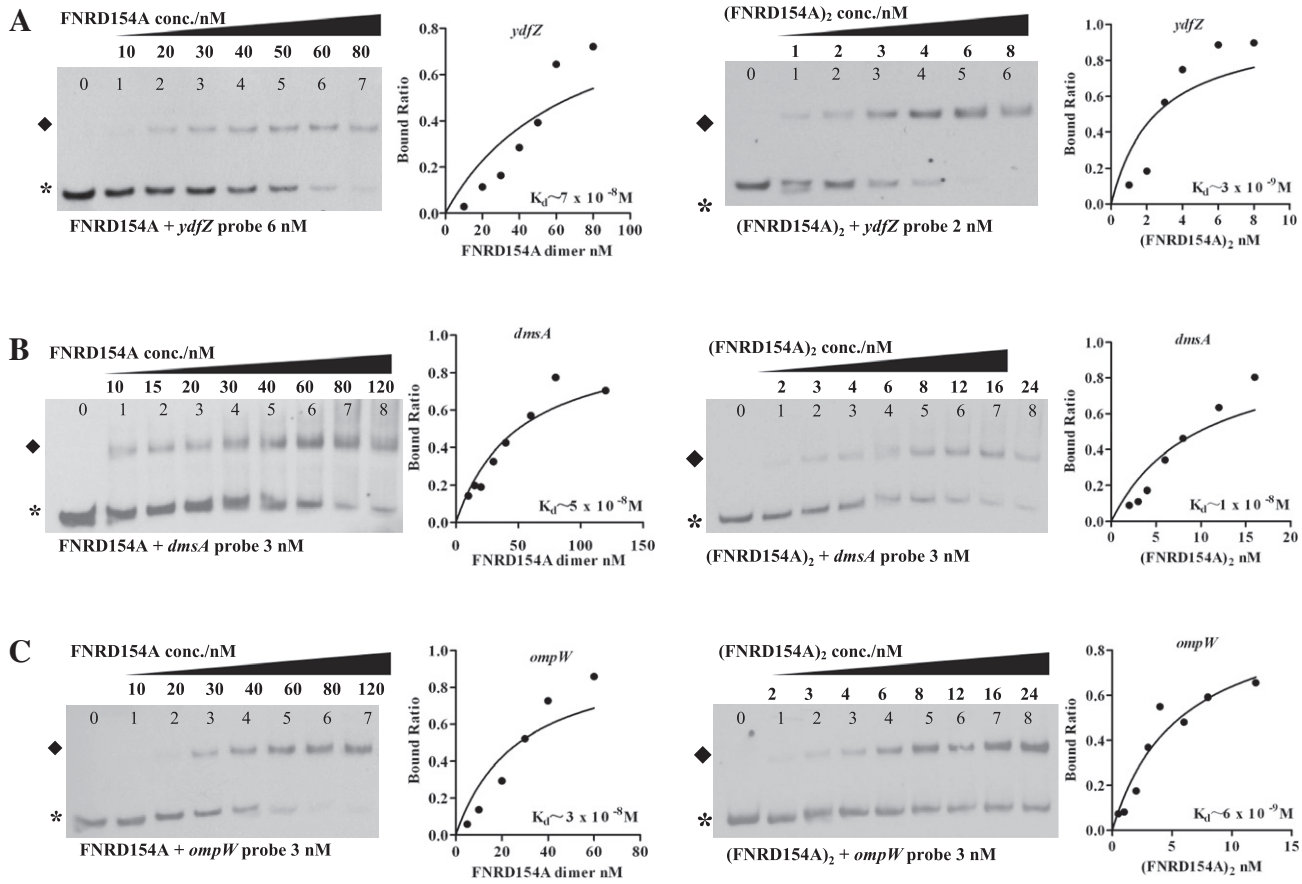

Fig. 3. Comparison of the binding of $(FNRD154A)_2$ and FNRD154A to FNR dependent promoters using DIG-labeled EMSA assay. Various amounts of purified FNRD154A protein (left) or $(FNRD154A)_2$ protein (right) were incubated with DIG-labeled *ydfZ*(A), *dmsA* (B), and *ompW* (C) promoter, respectively for EMSA and calculation of dissociation constant (K_d). *Asterisk* indicates free probe and *diamond* indicates protein–DNA complex. Equilibrium DNA binding curve for FNRD154A and $(FNRD154A)_2$ with different promoters were derived by non-linear fitting. K_d of FNRD154A-DNA was determined to be $\sim 7 \times 10^{-8}$ M for *ydfZ* promoter, $\sim 5 \times 10^{-8}$ M for *dmsA* promoter, and $\sim 3 \times 10^{-8}$ M for *ompW* promoter. K_d of $(FNRD154A)_2$-DNA was determined to be $\sim 3 \times 10^{-9}$ M for *ydfZ* promoter, $\sim 1 \times 10^{-8}$ M for *dmsA* promoter, and $\sim 6 \times 10^{-9}$ M for *ompW* promoter.

$(FNRD154A)_2$ displays ~20-fold higher affinity to *ydfZ* promoter than that of FNRD154A under aerobic conditions (Fig. 3A). It is noteworthy that the range of dissociation constant of FNRD154A determined in this assay is consistent with previous report, which was determined to be $\sim 3 \times 10^{-8}$ M^{-1} for a synthetic FNR promoter [9].

To examine whether the enhanced DNA binding and oxygen stability also observed in the cases of other FNR dependent promoters, we also examined its binding to *dmsA* promoter and compared with that of FNRD154A (Fig. 3B). The dissociation constant (K_d) of $(FNRD154A)_2$ to *dmsA* promoter was determined as 1×10^{-8} M, which is ~5-fold lower comparing to the FNRD154A under the same condition (5×10^{-8} M). This further confirmed that $(FNRD154A)_2$ exhibited enhanced DNA binding affinities than the currently widely used FNRD154A.

To expand the application of this oxygen stable FNR variant, we also examined its binding to a newly identified FNR dependent promoter, *PowpW*, which was indicated by genome-wide CHIP-chip assay [18], but has not been verified by *in vitro* DNA binding. We found that both $(FNRD154A)_2$ and single FNRD154A can bind to the *ompW* promoter region (Fig. 3C), but the dissociation constant for $(FNRD154A)_2$ is ~5-fold lower (6×10^{-9} M) comparing with FNRD154A (3×10^{-8} M), demonstrating that $(FNRD154A)_2$ exhibits enhanced DNA binding on a broad range of FNR dependent promoters than FNRD154A.

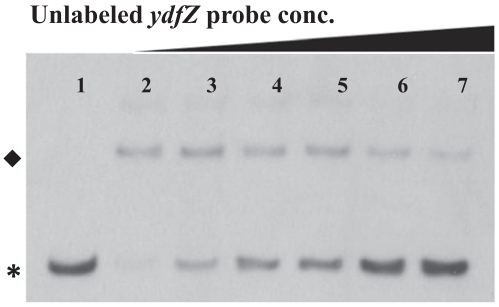

Fig. 4. Competition of DIG-labeled *ydfZ* promoter DNA by non-labeled promoter DNA to the binding of $(FNRD154A)_2$. Gel mobility shift assay of tandem $(FNRD154A)_2$ binds to DIG-labeled and non-labeled *ydfZ* promoter. DNA binding reaction of $(FNRD154A)_2$ (final concentration 30 nM) with DIG-labeled *ydfZ* promoter (final concentration 6 nM) was added with increasing amount of non-labeled DNA probe. Lane 1: free DIG-labeled probe; lane 2: DIG-labeled *ydfZ* probe and $(FNRD154A)_2$ protein; lane 3-lane 7: reaction from lane 2 was added with 1.5 nM, 3 nM, 6 nM, 12 nM and 24 nM y*dfZ* promoter DNA, respectively. Binding of $(FNRD154A)_2$ to the DIG-labeled *ydfZ* promoter was competed specifically by the non-labeled *ydfZ* promoter DNA probe. *Asterisk* indicates DIG-labeled free probe and *diamond* indicates the protein-DNA complex between covalent $(FNRD154A)_2$ and DIG-labeled *ydfZ* promoter DNA.

To confirm the DNA binding specificity of $(FNRD154A)_2$ to FNR dependent promoter, we performed competition assay in which unlabeled *ydfZ* probe was added with increasing concentrations

to the binding reaction of DIG-labeled *ydtZ* promoter with $(FNRD154A)_2$ to examine if it will compete with the DIG-labeled promoter. As shown in Fig. 4, with the increase of unlabeled probe, decrease of protein-DNA-probe complex was observed and it is concomitant with the increase of DIG-labeled free probe, indicating that the unlabeled probe competes with the DIG-labeled DNA probe to bind to $(FNRD154A)_2$. This result demonstrates that tandem $(FNRD154A)_2$ binds *ydfZ* promoter with high specificity (Fig. 4).

4. Discussion

In summary, in this study we have designed an FNR construct that retains the promoter specificity, O_2 sensing capacity, and similar structure as the native FNR dimer but with enhanced stability to O_2. Hence it can be widely used in *in vitro* DNA binding assay to study FNR regulated genes and to characterize the structural details of FNR. This variant is especially useful in functional genomics studies to verify the vast numbers of previously unrecognized genes in FNR regulon and their functions in the anaerobic adaptation of *E. coli*. Furthermore, the tandem expression system we used in this study also has a general application for the study of protein dimer or oligomers. For instance, since most transcription regulators function as dimers, the tandemly expressed system can be applied to other transcription factors for functional and mechanistic studies. Finally, given its significantly enhanced *in vivo* and *in vitro* activities under aerobic conditions than the currently used FNRD154A variant, we conclude that $(FNRD154A)_2$ can be used in a broad range of biochemical and structural characterizations of the FNR protein.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2012.01.121.

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