

## Environmental Effect of *anr* Gene on Nitrogen Fixation and Biofilm Formation in *Pseudomonas stutzeri* A1501 at Low Levels of Oxygen

Guihua Hu and Min Lin\*

### Abstract

Nitrogenase activity is a very distinct property of *Pseudomonas stutzeri* A1501. It is carried out in the presence of a microaerophilic environment that is in oxygen-limited condition. The biofilm formation is at its best in minimal media at nitrogen depletion state. Anr, a global transcription regulator that promotes biofilm formation. Anr absence plays a negative role in nitrogen fixation by regulating *nifA*, *nifH* and *ntrC*. The nitrogenase activity in *anr* insertion mutant was significantly down-regulated. At a different concentration of oxygen, the nitrogenase activity was substantially differentiated. The qRT-PCR showed down-regulation of *nif* island genes at nitrogen fixation condition under the absence of *anr*. The finding suggested that different oxygen concentration in the environment play a differential role against the nitrogenase activity and biofilm formation. The qRT-PCR results for *narL* during biofilm formation was up-regulated showing that low level of oxygen has a signaling effect causing *anr* to express which in turns controls not only *rsmXYZ* but also regulate the biofilm formation. Effect of oxidative stress using H<sub>2</sub>O<sub>2</sub> showed no significant effect on the survival of *Pseudomonas stutzeri* A1501 that suggested no role in the survival of the organism under high oxygen concentration.

**Keywords:** *Pseudomonas stutzeri* A1501; Biofilm formation; Nitrogen fixation; Oxygen limitation; Nitrogenase activity

**Received:** September 13, 2019, **Accepted:** September 23, 2019, **Published:** September 26, 2019

### Introduction

Different conditions should be applied in response to act differently by different bacterial physiology, such as nutrients and oxygen availability, which changes with temperature and the presence of stress agents, these responses are coordinated by a number of different regulatory mechanisms, it includes global regulatory protein [1]. The expression of specific genes, belonging to a wide variety of regulons, responds to the overall physiological economy. One of the most significant environmental signals for bacteria is oxygen availability, which triggers several specific responses and the cellular redox balance. It is mostly studied in *Escherichia coli* that the redox state of cells occurs in the transition from aerobic to anaerobic extensively [2].

In *Escherichia coli* the DNA binding protein Fnr responds differently during various concentration of cellular oxygen status by activating or repressing the expression of respiratory enzymes During several concentration of cellular oxygen status response

in *Escherichia coli* the DNA binding protein Fnr acts differently by activating or repressing the expression of respiratory enzymes [3,4]. *P. aeruginosa* observes low oxygen tension through an anaerobic regulator Anr, a homologue of *E. coli* FNR, documented to be the general positive activator of the anaerobic response. Mutants defective in *anr* cannot increase in number anaerobically with nitrate, nitrite or nitrous oxide as terminal electron acceptor. *anr* has a significant role in anaerobic activation of the entire denitrification process [5-8].

As we are aware that oxygen tension is identified in *Pseudomonas* species by the *anr* regulator (anaerobic regulator of arginine deiminase and nitrate reductase) [5,9]. For this purpose dimeric Anr carries a (4Fe-4S)<sup>2+</sup> clusters [10]. For the regulation of transcription, the active form of Anr protein binds with a conserved DNA binding site at the promoter region [11]. Partially destructions occur in the transcription factor on losing its DNA binding and gene regulation ability when there is an exposure to

Chinese Academy of Agricultural Science,  
Biotechnology Research Institute, Beijing,  
100081, China

\*Corresponding author:  
Min Lin

✉ linmin@caas.com

Biotechnology Research Institute, Chinese  
Academy of Agricultural Science, Beijing,  
100081, China

Tel: +0086-10-82109868

**Citation:** S Baftft, Hu G, Zhang H, Lfui Y, Lu W, et al. (2019) Environmental Effect of *anr* Gene on Nitrogen Fixation and Biofilm Formation in *Pseudomonas stutzeri* A1501 at Low Levels of Oxygen. Br Biomed Bull Vol.6 No.2: 47.

oxygen or NO the iron-sulfur cluster [10]. *Pseudomonas stutzeri* A1501 reacts rapidly with the changing free oxygen present in the environment thus is considered to be an oxygen-sensitive organism [12]. This strain received unique attention because of its specific metabolic properties, which include denitrification under anaerobic conditions, nitrification under aerobic conditions and nitrogen fixation under microaerophilic conditions [13]. *Pseudomonas stutzeri* A1501, like any other facultative anaerobe, has the ability to sense and adapt to changes in its redox environment and to select the energetically most efficient respiratory pathway for the prevailing condition.

In this study, we aimed to uncover the relationship between *anr* with reference to nitrogen fixation and biofilm formation. We explore Anr (transcription activator) regulating in a microaerophilic environment regulating the nitrogen fixation and allowing bacteria to adapt to different situations when living in a free form, during colonization of surfaces and mucoid or non-mucoid biofilm formation. We will also be going to focus on any impact occurring on *gacA* (global regulator) involved in biofilm formation and its relationship with nitrogen fixation. Using *Pseudomonas stutzeri* A1501, *anr* mutants, we investigated

whether micro-aerobic induction has some association between nitrogen fixation island and biofilm formation.

## Method and Materials

### Bacterial strains, culture media, plasmids and growth condition

Strains, plasmids, mutants strain construction **Table 1** (Supplementary file) showing the plasmids used in the following study. *Pseudomonas stutzeri* A1501 strain was grown in Luria Bertani (LB) medium or minimal lactate medium (medium K) at 30°C. The organism was grown at 30°C shaking on 220 rpm overnight. Antibiotics were used at the following concentrations, 50 µg/mL tetracycline (Tc), 50 µg/mL Kanamycin (Km). All the enzymes for DNA manipulation and endonuclease activity were purchased from New England Biolabs.

### Growth curve analysis

Growth curve analysis was done by using Luria Bertani (LB) medium and medium-K. After maintaining the initial OD600-0.1 in 20 mL medium incubated at 30°C, shanking at 220 rpm. After

**Table 1:** Showing the plasmids used in the following study.

Strains and plasmids	Genotype or phenotype	Source
<i>P. stutzeri</i> A1501	Wild type strain, Chinese culture	Hughes, 1988
pRK-2013	Helper plasmid for conjugation into <i>P. stutzeri</i> A1501, Km	Figurski DH, Helinski DR
pK-18 mob insertional plasmid	Mobilizable plasmid containing an <i>Escherichia coli</i> origin of replication, <i>kmr</i>	Schäfer A, et al.
pLAFR-3	Mobilizable vector, Tcr	Staskawicz B, Dahlbeck D, Keen N, Napoli C
$\Delta anr$ , Km, insertional mutant	$\Delta anr$ -Km insertion mutant, Km	This Study

**Medium:** Respectively, according to the amount of the above ingredients, dissolved in water to set to 1L, (**Table 1 A**) sterilization at 121°C high steam pressure for 20min. Solid medium contains 1.5% agar powder.

**Table 1(A):** LB medium: 1 L, pH 7.0 (solid medium containing 1.5% agar powder).

Composition	Weight (g)
Yeast extract	5 g
Tryptone	10 g
NaCl	10 g

Respectively, according to the amount of the above ingredients, dissolved in water to set to 1L, sterilization at 121°C high steam pressure for 20 min. Solid medium contains 1.5% agar powder (**Table 1(B)**).

**Table 1(B):** A15 Minimal K-Medium (Medium K) 1 L, pH 6.8 (1.5% agar powder in solid medium).

Composition	Weight (g)
$KH_2PO_4$	0.4 g
$K_2HPO_4$	0.1 g
NaCl	0.1 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$MnSO_4 \cdot H_2O$	0.01 g
$Fe_2(SO_4)_3 \cdot H_2O$	0.01 g
$Na_2MoO_4 \cdot H_2O$	0.01 g
$C_3H_5NaO_3$	6 ml
$(NH_4)_2SO_4$	0.4 g

In order to prevent  $H_2PO_4^-$ ,  $HPO_4^{2-}$  and  $Mg^{2+}$ ,  $Mn^{2+}$  form a precipitate, the reagent containing  $SO_4^{2-}$ -dissolved separately from the other reagents and then set to final volume 1L. NaOH solution was used to adjust the pH = 6.8, 121°C, high pressure steam sterilization for 20 minutes.

every two hours interval the growth changes were observed on spectrophotometer U-3010.

### Estimation of biofilm formation

Crystal violet assay of biofilm formation was performed after overnight culture of A1501 was washed with medium-K. After adjusting the OD<sub>600</sub> to 0.5. 1150 µl of washed culture was inoculated into corresponding media in 96 well PVC plates (Corning Co. New York, NY USA). Then the plates were incubated on understanding condition in air at 30°C for 48 hours. The growth of planktonic bacteria in microtiter wells used for biofilm assay was determined by measuring the OD<sub>600</sub> nm. For Biofilm biomass quantification, the wells were washed with ddH<sub>2</sub>O for three times. One hundred sixty (160 µL) of 0.1% crystal violet was added into each well and incubated for 10 minutes. The wells were washed with ddH<sub>2</sub>O for multiple times until no purple color remained in water. The crystal violet bound to biofilm was solubilized with 30% acetic acid and measured for absorbance at OD<sub>540</sub> nm using a spectrophotometer (FixStation 3, molecular device, USA). For observing the biofilm, also, the same test was performed in 5 ml test tubes, and after treating with Crystal violet, pictures of the biofilm was taken. Relative qPCR analysis was done at different oxygen concentration (0.5%, 1.0% and 1.5%).

### Oxidative stress analysis

The cells susceptibility of *Pseudomonas stutzeri* A1501 and its derivate was assayed to H<sub>2</sub>O<sub>2</sub>. Strains were grown overnight in LB broth at 30°C and were transferred into fresh LB broth up to an OD<sub>600</sub> of 0.1 again the broth was incubated at 30°C with 220 rpm shaking for about 3 hours so that the OD<sub>600</sub> may reach till 0.6. Then, 12 mM H<sub>2</sub>O<sub>2</sub> was added to the medium. The culture was incubated at 30°C with 220rpm for 10 minutes. Serial 10 fold dilutions of OD standardized cultures were spotted on LB plates. Plates were incubated at 30°C for 24 hours prior to colony enumeration. The survival rate was expressed as the percentage of the number of colonies in the treated samples compared with that in the untreated A1501 sample used as a control.

### Nitrogenase activity assays

To check the activity of nitrogenase, bacterial suspensions were incubated in N-free minimal medium at an OD<sub>600</sub> of 0.1 at 30°C under argon atmosphere containing oxygen at different concentrations and acetylene at 10% according in 110 ml bottle, shaking at 220 rpm to the protocol described [14]. After the concentration of oxygen was adjusted to 0.5, acetylene was injected, and gas samples were withdrawn periodically for gas chromatographic analysis of ethylene production. Under anaerobic conditions, the activity of nitrogenase was analyzed by incubating bacterial suspension in N-free minimal lactate medium supplemented with 1 mM nitrate at an OD<sub>600</sub> of 0.1. The specific activity of nitrogenase was expressed as nmol ethylene/min/mg protein. Protein concentrations were determined using standard protein assay (Bio-Rad Hercules, CA) with Bovine Serum Albumin (BSA) as the standard. Each experiment was repeated for three times.

### Quantitative real-time PCR analysis

Quantitative RT-PCR experiments were performed according to

the manufacturer's recommendations using the ABI PRISM 7200 Real time (Applied Biosystem). Data were analyzed using the ABI PRISM 7500 Sequence Detection System Software (Applied Biosystems). qPCR thermocycler and RNA isolation (by analytik Jena Kit) was used for the RNA isolation. The construction of cDNA, TaKaRa, Prime Script RT reagent Kit with gDNA Eraser (Perfect real-time) was used. Primers used for qRT-PCR are listed in the table (Appendix 2).

### Bioinformatics analysis

The *anr* regulon of *Pseudomonas stutzeri* A1501 was determined using the Virtual Footprint tool available in the PRODORIC software (<http://prodoric.tu-bs.de>) [15]. In addition, a phylogenetic tree was constructed by using MEGA 7.0 at bootstrap 1000 replicates.

### Statistical method

T-test was applied to find the significance between the numbers of colonies that appeared on the selective and non-selective plates of the stability test. The test was performed using GraphPad Prism version 7 for Windows, GraphPad Software, San Diego California USA.

## Results

### Bioinformatics analysis

*Pseudomonas* strains representing the different *anr* groups and several reference pseudomonads. The neighbor-joining tree was constructed using MEGA 7.0 and nodal supports of the inferred tree were evaluated by 1000 bootstrap replicates. The scale bar represents the number of substitutions per site. Phylogenetic analysis suggested that *anr* gene in pseudomonas species are closely related to other species of *Ceillvibrio*, *Halomonas* and *Rhodospirillum* **Figure 1**.

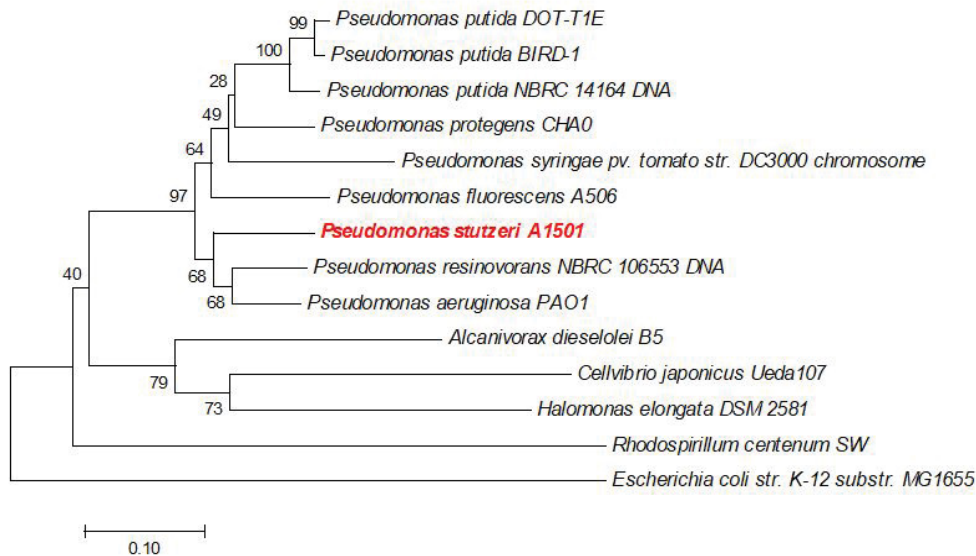
### Effect of different oxygen concentration on *anr*

On investigating the A1501 during different nitrogenase activity, it was observed that at an oxygen concentration of 1% the nitrogenase activity was at its highest **Figure 2**. Oxygen concentration is considered to be one of the most important elements when it comes to checking the biofilm formed on the surface of liquid-air contact area. *anr* mutant (transcriptional factor) absence showed non-reactive oxidative metabolism. This observation was performed to check the expressional change occurring with the presence of different concentration of oxygen (0.5%, 1.0% and 1.5%) in the system with *anr* genes. Oxygen was injected in gas form. In addition, relative qPCR analysis showed that there was an up-regulation of *anr* gene after 8 hour of exposure to 1% oxygen.

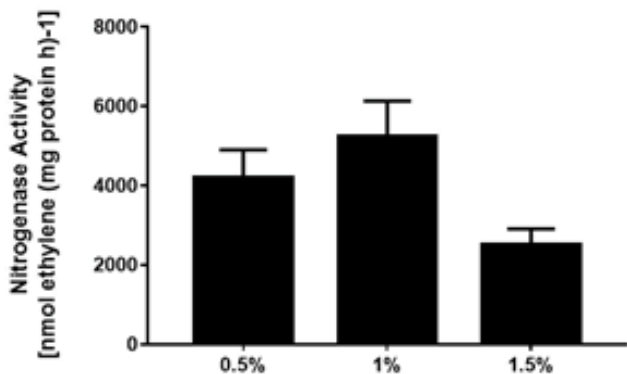
The observation places a foundation that in the presence of excessive amount of oxygen, *anr* expression is increased. The mutation of these genes will give us more insight into how they effect nitrogenase activity and biofilm formation.

### Growth curve analysis

The *Pseudomonas* strain growth was monitored to assess its metabolic capability in both LB as well as minimal media (medium-K). It can be observed from **Figure 3**. That there was no significant change observed in the growth pattern of mutants and



**Figure 1** Phylogenetic tree of A1501 *anr* gene. Bootstrap value on 1000 replication are listed as percentage at branching point bar, 0.10 substitutions per position.



**Figure 2** Showing effect of Oxygen in percentage Nitrogenase activity with different oxygen concentration.

complementary strains. This shows the growth of all the mutant at minimal nutrients availability can grow easily without any disruption. Because *anr* is oxygen-sensitive gene, their growth was needed to be observed in the normal condition the effect on genomic level may lead to abnormal growth. The difference of change could have pointed out the possible inability of intake of free oxygen within the system. The permeability of oxygen in the system ensures the smooth function of all the metabolic reactions occurring within the system. The growth curve analysis of the mutant strain and complimentary strain represents no difference in the cell generation number and there doubling in presence of minimal medium.

### Studying biofilm dispersal of *anr* genes

**Biofilm formation:** With the stressful environmental change in soil, it brings changes to the organism as well. Especially with reference to the formation of stuff which could protect the organism from the sudden changes occurring within. These

changes are for the survival of the organism. From many or such survival strategies, one of them is the formation of biofilm in the changing soil environment. Bacterial assemblies enclosed in a matrix, are found throughout many environmental and biological niches. Cells forming these communities have advantages over their planktonic counterparts with respect to protection against both physical and chemical stress. Bacteria in the form of biofilm are more resistant to antimicrobial agents and immune system surveillance. Biofilm formation and anaerobic metabolism have been linked in late stage of biofilms. Oxygen, in particular, can be in limited supply within the biofilm and oxygen gradients are detected within biofilms. The mechanisms whereby cells sense and respond to oxygen are complex and not fully understood. *Anr* activity is high in oxygen-limited environment, including biofilm and populations associated with soil. *Pseudomonas* species are reported to sense and respond to low levels of environmental oxygen through the activity of the transcription factor *anr*, due to *k* promoter to regulate transcription [11,16]. Upon exposure to the low levels of oxygen these other genes are activated which in response to it activates the cascade of pathway leading to biofilm formation within the anaerobic stress environment.

The 96 well plate method for the biofilm formation was analyzed to measure the biofilm formed in the following minimal conditions. The result showed  $\Delta anr$  single mutant, biofilm formation was reduced up to 33.52%. With oxygen levels contribution and changes keeping in mind, biofilm formation reaction was carried out by controlling the oxygen concentration within the system and then the expressional analysis was done by using relative qRT-PCR (Figure 4).

**Relative qPCR analysis for Biofilm Formation:** *anr* (homolog of Fnr in *E. coli*) shows significant relationship between *rsm* non-coding RNA. *rsm* (non coding RNA) are considered to play an important role in RsmA positive regulation in *Pseudomonas spp.* Therefore, it is evident to say that in the absence of *anr*



gene the regulation of non-coding genes are affected. The small non-coding RNAs *rsm* sequester and titrate RsmA to dictate the critical balance of this regulatory protein within the bacterial cells. Conversely, RsmA itself can also modulate sRNA production. Therefore, to assess the level at which low oxygen influences the *rsmX/Y/Z* network *anr*, *rsmX/Y/Z* promoter activity was examined in *Pseudomonas stutzeri* A1501 cultured in microaerobic (low oxygen). The result showed significant decrease in *gacA* expression levels in  $\Delta anr$  insertion mutant. Since it's known that defect in *gacA* leads to less or no expression of *rsmXYZ* which considered to be an important regulator for biofilm formation. The activity of *narL* was increased suggesting that *anr* regulate the activity of *narL* in the system (Figure 5).

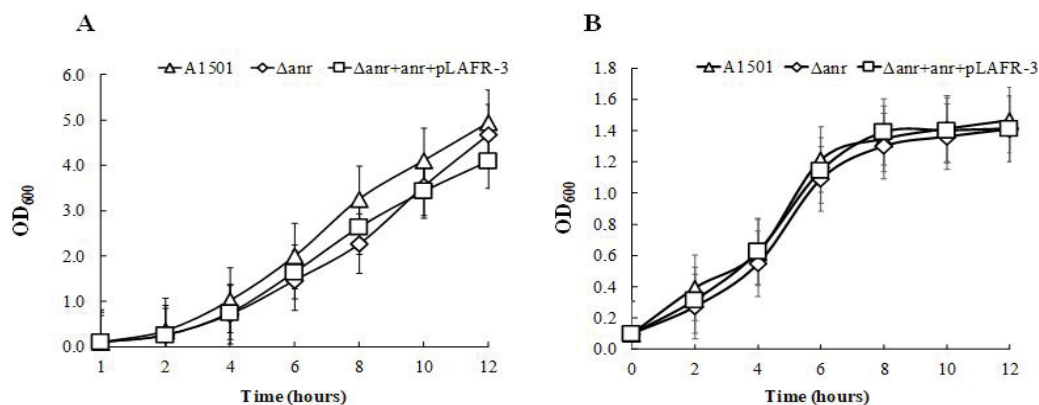
### Oxidative stress

In this test, no sensitivity was observed with the moderate concentration of  $H_2O_2$  together with the catalase activity. The *anr*

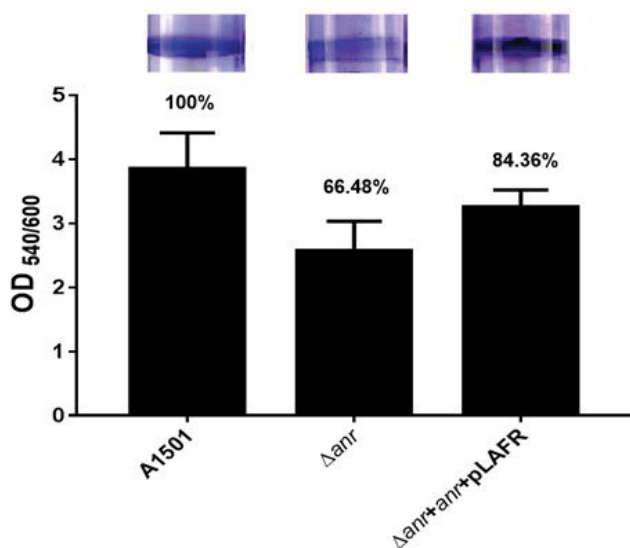
gene showed no significant change when exposed to oxidative shock given by using 12mM of  $H_2O_2$ . The results proof that increased amount of oxygen concentration has no effect on the functioning of *anr* gene (Figure 6).

### Nitrogenase activity *anr* genes

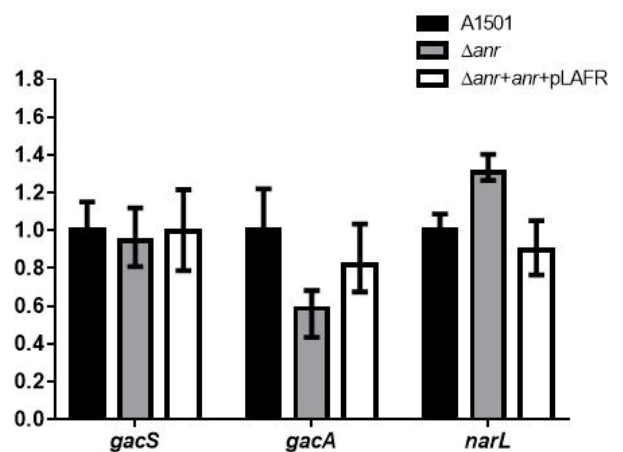
Nitrogen fixation (*nif*) genes expression is regulated in response to the environmental signals ammonium and oxygen depending on the transcriptional activators. *Pseudomonas stutzeri* A1501 fixes nitrogen under the micro-aerobic condition in the media. We observed the changes occurring in *anr* mutant and its complementary cells causing expressional changes in biological nitrogen fixation. The results at 1% oxygen concentration showed significant downregulation in the nitrogen fixation that is in *anr* insertion mutant (Figure 2). The gradual decline was observed during per hour observation of the nitrogen fixation (data not attached).



**Figure 3** (A): Growth pattern of the A1501 wild type  $\Delta anr$ , and complimentary strain, in LB- medium; (B): Growth pattern of the A1501 wild type  $\Delta anr$ , and complimentary strain in K- medium.



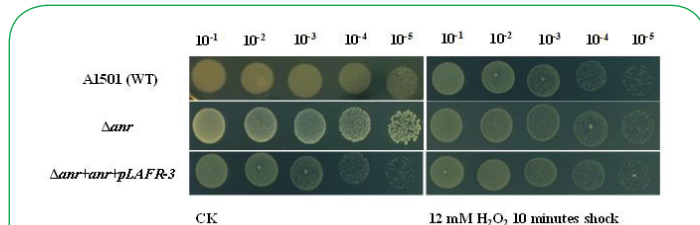
**Figure 4** Biofilm formation observed in 96 well plates and calculated the differential change occurred in the surface biofilm formation after 48 hours of incubation.



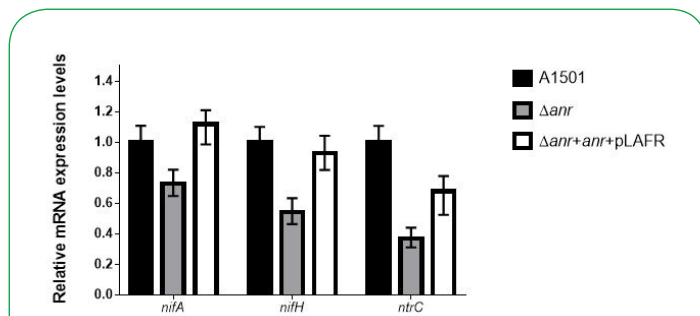
**Figure 5** Biofilm formation at oxygen concentration 1% was observed for,  $\Delta anr$ , and complimentary cells. *gacA* was down-regulated but *NarL* was up-regulated during the biofilm formation.

### Relative qRT-PCR analysis for nitrogenase activity

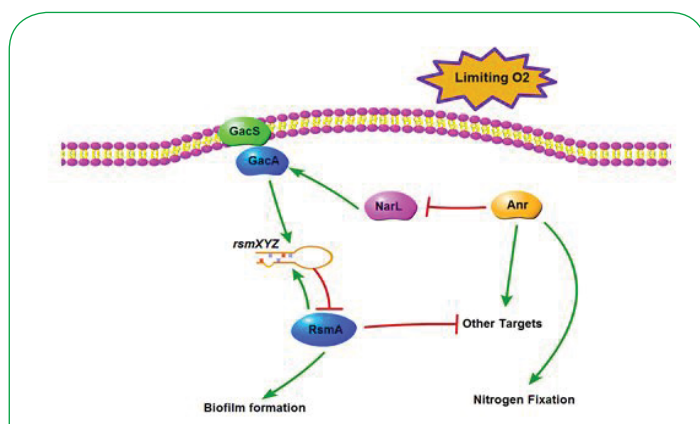
Relative qPCR analysis of nitrogenase activity of,  $\Delta anr$  showed down regulation of *gacA*, *nifA* and *nifH* along with *ntrC* (Figures 7-10).



**Figure 6** Growth upon oxidative stress. Serial 10 fold dilutions of OD-standardized WT A1501,  $\Delta anr$  and *anr* complimentary strain was observed were spotted on LB plates after being exposed to 12 mM H<sub>2</sub>O<sub>2</sub>, CK represents un-treated culture control.

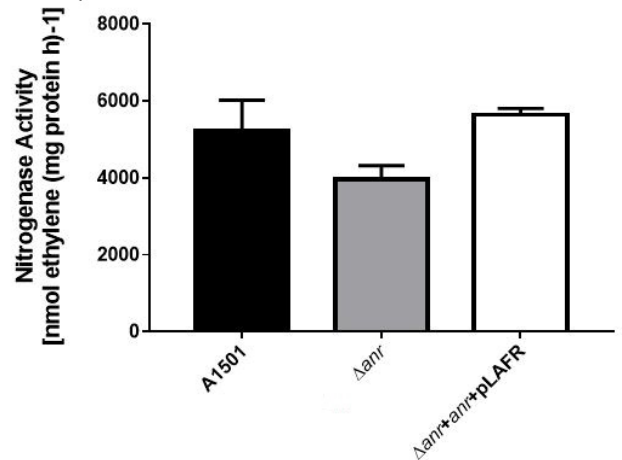


**Figure 7** Relative qPCR analysis under nitrogen fixation condition at 1% oxygen concentration,  $\Delta anr$  and  $\Delta anr+anr+pLAFR$  complementary cells.

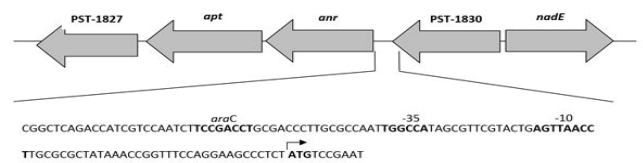


**Figure 8** Relative qPCR analysis showing *anr* expressional levels at different oxygen concentration.

Anr positively regulates target genes involved in the utilization of alternate electron acceptors in the absence of oxygen or low levels of oxygen. These target genes were regulated by secondary transcription factors that are active in the presence of their cognate electron acceptor. In the *anr* mutant the organism loses the ability to grow under anaerobic condition. Therefore, in microaerophilic condition the organism tries to consume as much



**Figure 9** Nitrogenase activity at 1% oxygen concentration. The figure shows less nitrogenase activity in *anr* mutant as compared to A1501 wild type and complementary cells.



**Figure 10** The promoter sequence analysis of *anr* in *Pseudomonas stutzeri* A1501.

oxygen as possible in order to survive in that environment. As nitrogenase activity require microaerophilic condition to perform its activity and in the absence of *anr* gene most of the essential metabolic pathways are affected, so that organism reduces the nitrogenase activity and decreases biofilm formation by down-regulating *rsmXYZ*, which leads to up-regulation of *rsmA*.

### Discussion and Conclusion

Anr is reported to be one of the genes responsible for their effect on biofilm formation during the micro-aerobic condition. Anr mediates the respiratory switch from normoxic to hypoxic and anoxic conditions [7,17].

As nitrogenase activity is also sensitive to microaerobic condition, the relationship in between could place a strong foundation between nitrogen fixation and biofilm formation in *Pseudomonas stutzeri* A1501. Anr insertion mutant showed a significant amount of down-regulation for *gacA* and during biofilm formation and nitrogenase activity.

The micro-aerophilic condition induces the A1501 to form biofilm formation along with that it requires sufficient amount of oxygen to carry out its metabolic reaction within the system [18,19]. Nitrogenase activity is best carried out in the presence of microaerophilic condition that is at 1% of oxygen [12,20]. The Anr inhibits the *gacA* which leads to the reduction of biofilm

formation by regulating *narL* in the system. Since the *gacA* is positive and direct regulator for *rsmXYZ*, downregulation of *gacA* gene causes significant reduction of biofilm formation that is, *gacA* leads to significant downregulation of *rsmXYZ* and biofilm formation within the system [21-28].

The results after exposing *anr* insertion mutant to 10 mM of  $H_2O_2$  concentration, it was observed that there was not significant change to the *anr* mutant as compared to the wild type strain [29-33] **Figures 8-10**.

## Acknowledgement

This work was supported by the National Basic Research Program of China (2015CB755700), the National Science Foundation of China (31470174, 31470205 and 31570081), and Guangdong Innovative and Entrepreneurial Research Team Program (No. 2013S033).

## Conflict of Interest

The authors declare that they have no conflict of interest.

## References

- Martinez-Antonio A, Collado-Vides J (2003) Identifying global regulators in transcriptional regulatory networks in bacteria. *Curr Opin Microbiol* 6: 482-489.
- Partridge JD, Sanguinetti G, Dibden DP, Roberts RE, Poole RK, et al. (2007) Transition of *Escherichia coli* from aerobic to micro-aerobic conditions involves fast and slow reacting regulatory components. *J Biol Chem* 282: 11230-11237.
- Iuchi S, Lin EJC (1991) Adaptation of *Escherichia coli* to respiratory conditions: regulation of gene expression. *Cell* 66: 5-7.
- Spiro S, Guest JR (1991) Adaptive responses to oxygen limitation in *Escherichia coli*. *Trends Biochem Sci* 16: 310-314.
- Galimand M, Gamper M, Zimmermann A, Haas D (1991) Positive FNR-like control of anaerobic arginine degradation and nitrate respiration in *Pseudomonas aeruginosa*. *J Bacteriol* 173: 1598-1606.
- Zimmermann A, Reimann C, Galimand M, Haas D (1991) Anaerobic growth and cyanide synthesis of *Pseudomonas aeruginosa* depend on *anr*, a regulatory gene homologous with *fnr* of *Escherichia coli*. *Mol Microbiol* 5: 1483-1490.
- Arai H, Igarashi Y, Kodama T (1994) Structure and ANR-dependent transcription of the *nir* genes for denitrification from *Pseudomonas aeruginosa*. *Biosci Biotechnol Biochem* 58: 1286-1291.
- Ye R, Haas D, Ka J, Krishnapillai V, Zimmermann A, et al. (1995) Anaerobic activation of the entire denitrification pathway in *Pseudomonas aeruginosa* requires *Anr*, an analog of *Fnr*. *J Bacteriol* 177: 3606-3609.
- Sawers RJM (1991) Identification and molecular characterization of a transcriptional regulator from *Pseudomonas aeruginosa* PAO1 exhibiting structural and functional similarity to the FNR protein of *Escherichia coli*. *Mol Microbiol* 5: 1469-1481.
- Yoon SS, Hennigan RF, Hilliard GM, Ochsner UA, Parvatiyar K, et al. (2002) *Pseudomonas aeruginosa* anaerobic respiration in biofilms: relationships to cystic fibrosis pathogenesis. *Dev Cell* 3: 593-603.
- Winteler HV, Haas D (1996) The homologous regulators ANR of *Pseudomonas aeruginosa* and FNR of *Escherichia coli* have overlapping but distinct specificities for anaerobically inducible promoters. *Microbiology* 142: 685-693.
- Yan Y (2010) Global transcriptional analysis of nitrogen fixation and ammonium repression in root-associated *Pseudomonas stutzeri* A1501. *BMC Genomics* 11: 11-12.
- Lalucat J, Bennisar A, Bosch R, García-Valdés E, Palleroni N (2006) Biology of *Pseudomonas stutzeri*. *Microbiol Mol Biol Rev* 70: 510-547.
- Desnoves N, Lin M, Guo X, Ma L, Carreño-Lopez R, et al. (2003) Nitrogen fixation genetics and regulation in a *Pseudomonas stutzeri* strain associated with rice. *Microbiology* 149: 2251-2262.
- Münch R, Hiller K, Grote A, Scheer M, Klein J, et al. (2005) Virtual Footprint and PRODORIC: an integrative framework for regulon prediction in prokaryotes. *Bioinformatics* 21: 4187-4189.
- Yoon SS, Karabulut AC, Lipscomb JD, Hennigan RF, Lyman SV, et al. (2007) Two-pronged survival strategy for the major cystic fibrosis pathogen, *Pseudomonas aeruginosa*, lacking the capacity to degrade nitric oxide during anaerobic respiration. *EMBO J* 26: 3662-3672.
- Schreiber K, Krieger R, Benkert B, Eschbach M, Arai H, et al. (2007) The anaerobic regulatory network required for *Pseudomonas aeruginosa* nitrate respiration. *J Bacteriol* 189: 4310-4314.
- Brencic A, Lory S (2009) Determination of the regulon and identification of novel mRNA targets of *Pseudomonas aeruginosa* RsmA. *Mol Microbiol* 72: 612-632.
- Hassett DJ (2002) Anaerobic metabolism and quorum sensing by *Pseudomonas aeruginosa* biofilms in chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets. *Adv Drug Deliv Rev* 54: 1425-1443.
- Kinniment S, Wimpenny J (1992) Measurements of the distribution of adenylate concentrations and adenylate energy charge across *Pseudomonas aeruginosa* biofilms. *Appl Environ Microbiol* 58: 1629-1635.
- Humair B, Wackwitz B, Haas D (2010) GacA-controlled activation of promoters for small RNA genes in *Pseudomonas fluorescens*. *Appl Environ Microbiol* 76: 1497-1506.
- Mulcahy H, O'Callaghan J, O'Grady EP, Adams C, O'Gara F (2006) The posttranscriptional regulator RsmA plays a role in the interaction between *Pseudomonas aeruginosa* and human airway epithelial cells by positively regulating the type III secretion system. *Infect Immun* 74: 3012-3015.
- Blumer C, Haas D (2000) Iron regulation of the *hcnABC* genes encoding hydrogen cyanide synthase depends on the anaerobic regulator ANR rather than on the global activator GacA in *Pseudomonas fluorescens* CHA0. *Microbiology* 146: 2417-2424.
- Arai H, Igarashi Y, Kodama T (1995) Expression of the *nir* and *nor* genes for denitrification of *Pseudomonas aeruginosa* requires a novel CRP/FNR-related transcriptional regulator, DNR, in addition to ANR. *FEBS Lett* 371: 73-76.
- Arai H, Kodama T, Igarashi Y (1997) Cascade regulation of the two CRP/FNR-related transcriptional regulators (ANR and DNR) and the denitrification enzymes in *Pseudomonas aeruginosa*. *Mol Microbiol* 25: 1141-1148.
- Chen F, Xia Q, Ju LK (2006) Competition between oxygen and nitrate respirations in continuous culture of *Pseudomonas aeruginosa* performing aerobic denitrification. *Biotechnol Bioeng* 93: 1069-1078.
- Comolli JC, Donohue T (2004) Differences in two *Pseudomonas aeruginosa* *cbb3* cytochrome oxidases. *Mol Microbiol* 51: 1193-1203.
- Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* 280: 295-298.
- Heeb S, Valverde C, Gigot-Bonnefoy C, Haas DJF (2005) Role of the stress sigma factor RpoS in GacA/RsmA-controlled secondary metabolism and resistance to oxidative stress in *Pseudomonas fluorescens* CHA0. *FEMS Microbiol Lett* 243: 251-258.
- Jackson AA, Gross MJ, Daniels EF, Hampton TH, Hammond JH, et al. (2013) *anr* and its activation by PlcH activity in *Pseudomonas aeruginosa* host colonization and virulence. *J Bacteriol* 195: 3093-3104.
- Lazazzera BA, Beinert H, Khoroshilova N, Kennedy MC, Kiley PJ (1996) DNA binding and dimerization of the Fe-S-containing FNR protein from *Escherichia coli* are regulated by oxygen. *J Biol Chem* 271: 2762-2768.
- Wessel AK, Arshad TA, Fitzpatrick M, Connell JL, Bonnecaze RT, et al. (2014) Oxygen limitation within a bacterial aggregate. *M Bio* 5: e00992-00914.
- Xu KD, Stewart PS, Xia F, Huang C-T, McFeters GA (1998) Spatial physiological heterogeneity in *Pseudomonas aeruginosa* biofilm is determined by oxygen availability. *Appl Environ Microbiol* 64: 4035-4039.