Environmental Effect of anr Gene on Nitrogen Fixation and Biofilm Formation in Pseudomonas putzeri 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$_2$O$_2$ 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

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 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)$_2$ 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
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.

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

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Genotype or phenotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. stutzeri</em> A1501</td>
<td>Wild type strain, Chinese culture</td>
<td>Hughes, 1988</td>
</tr>
<tr>
<td>pRK-2013</td>
<td>Helper plasmid for conjugation into <em>P. stutzeri</em> A1501, Km</td>
<td>Figurski DH, Helinski DR</td>
</tr>
<tr>
<td>pLAFR-3</td>
<td>Mobilizable vector, Tcr</td>
<td>Staskawicz B, Dahlbeck D, Keen N, Napoli C</td>
</tr>
<tr>
<td>Δanr, Km, insertional mutant</td>
<td>Δanr-Km insertion mutant, Km</td>
<td>This Study</td>
</tr>
</tbody>
</table>

**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).

<table>
<thead>
<tr>
<th>Composition</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yest extract</td>
<td>5 g</td>
</tr>
<tr>
<td>Tryptone</td>
<td>10 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>10 g</td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th>Composition</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.4 g</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>0.1 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.1 g</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.2 g</td>
</tr>
<tr>
<td>MnSO$_4$.H$_2$O</td>
<td>0.01 g</td>
</tr>
<tr>
<td>Fe$_2$(SO$_4$)$_3$.H$_2$O</td>
<td>0.01 g</td>
</tr>
<tr>
<td>Na$_2$MoO$_4$.H$_2$O</td>
<td>0.01 g</td>
</tr>
<tr>
<td>C$_3$H$_5$NaO$_3$</td>
<td>6 ml</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>0.4 g</td>
</tr>
</tbody>
</table>

In order to prevent H$_3$PO$_4$, HPO$_4$ 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 OD₆₀₀ 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 OD₆₀₀. For Biofilm biomass quantification, the wells were washed with ddH₂O 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 ddH₂O 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 OD₅₄₀ 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 H₂O₂. Strains were grown overnight in LB broth at 30°C and were transferred into fresh LB broth up to an OD₆₀₀ of 0.1 again the broth was incubated at 30°C with 220 rpm shaking for about 3 hours so that the OD₆₀₀ may reach till 0.6. Then, 12 mM H₂O₂ 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 OD₆₆₀ 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 OD₆₆₀ 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 *Ceilvibrio, 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
changes are for the survival of the organism. From many 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 the 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 environments, 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 promotor 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 Δanr 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 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 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 the 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 environments, 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 promotor 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.

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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 Δanr 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$_2$O$_2$ together with the catalase activity. The anr gene showed no significant change when exposed to oxidative shock given by using 12mM of H$_2$O$_2$. 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 expression 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).

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**Figure 3**


**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, Δanr, 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, Δanr 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, Δanr and anr complimentary strain was observed were spotted on LB plates after being exposed to 12 mM H\textsubscript{2}O\textsubscript{2}. CK represents un-treated culture control.

Figure 7 Relative qPCR analysis under nitrogen fixation condition at 1% oxygen concentration, Δanr and Δanr+anr+pLAFR complimentary cells.

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

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.

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 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 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.
formation by regulating narL in the system. Since the gacA is positive and direct regulator for rsm XYZ, 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 H₂O₂ 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.

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Conflict of Interest
The authors declare that they have no conflict of interest.
References


