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hcnABC Gene Expression in the Soil Bacterium Pseudomonas putida ATH2-IRI/9 Under Various Culture and Rhizosphere Conditions

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ABSTRACT

*hcn*ABC GENE EXPRESSION IN THE SOIL BACTERIUM *PSEUDOMONAS PUTIDA* ATH2-1RI/9 UNDER VARIOUS CULTURE AND RHIZOSPHERE CONDITIONS

by

Myrna May Biswas

Chair: Robert Zdor

ABSTRACT OF GRADUATE STUDENT RESEARCH

Thesis

Andrews University

College of Arts and Sciences

TITLE: *hcn*ABC GENE EXPRESSION IN THE SOIL BACTERIUM *PSEUDOMONAS PUTIDA* ATH2-1RI/9 UNDER VARIOUS CULTURE AND RHIZOSPHERE CONDITIONS

Name of researcher: Myrna May Biswas

Name and degree of faculty chair: Robert Zdor, Ph.D.

Date completed: September 2010

Pseudomonas putida ATH2-1RI/9, a bacterium that colonizes plant roots, can suppress weed seedling growth of velvetleaf by producing hydrogen cyanide in the rhizosphere. The operon *hcn*ABC constitutively expresses HCN synthase, an enzyme which catalyzes glycine to form carbon dioxide and hydrogen cyanide, a secondary metabolite. Cyanogenesis is influenced by several environmental factors including iron, phosphate, and oxygen-limiting conditions. This study used a *hcn*ABC::*lux*AB gene fusion in *Pseudomonas putida* ATH2-1RI/9 involving the insertion of a promoterless *lux*AB gene into the *hcn*ABC operon, which results in bacterial luciferase expression instead of HCN synthase. Bioluminescence by this reporter strain was compared to

cyanide production by the wild-type *P. putida* ATH2-1RI/9.

The reporter strain, *Pseudomonas putida* ATH2-1RI/9, was grown in four different cultures as well as rhizosphere conditions: aerobic +$FeCl_3$, microaerobic +$FeCl_3$, aerobic –$FeCl_3$, and microaerobic –$FeCl_3$. In two of three experiments, aerobic+$FeCl_3$ stationary phase cultures had statistically higher levels of HCN in comparison to log phase cultures. In two of three of the culture experiments, microaerobic+$FeCl_3$ log phase cultures had statistically higher levels of gene expression in comparison to the other three treatment groups. A possible explanation for this result is that the anaerobic regulator ANR, which acts as an iron sensor in the *hcn*ABC operon, converts to its active form under low oxygen supply. The effect of iron and oxygen levels on bioluminescence and cyanide production in the rhizosphere was less clear.

Further research in the rhizosphere environment will clarify some of the unknown variables that could have been involved in triggering the *hcn*ABC gene expression of this soil microbe *Pseudomonas putida* ATH2-1RI/9.

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A Thesis

Presented in Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Myrna May Biswas

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APPROVAL BY THE COMMITTEE:

_____
Robert Zdor, Ph.D., Chair

_____
Tom Goodwin, Ph.D.

_____ _____
Marlene Murray-Nsuela, Ph.D. Date approved

This thesis is dedicated to the One who deserves all glory and honor, to the One who gave me strength and support when I needed it the most, to the One who is ever more passionate about nature and life than I can ever be. This thesis is dedicated to God the Father, the omnipotent One.

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First, I would like to acknowledge Dr. Robert Zdor, the chair of my thesis committee, for all the assistance he has provided me during the time I worked on my thesis. I learned some very important things from him, one being patience, the other optimism, that will hopefully make me a better scientist someday. I thank him for also providing opportunities so I could present my work at the International Bioherbicide Workshop that was held at the Walt Disney Resort in Orlando, Florida, in February 2009.

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CHAPTER 1

INTRODUCTION

Pseudomonas putida ATH2-1RI/9 as a Potential Weed Biocontrol Organism

Velvetleaf (*Abutilon theophrasti*) is a very competitive plant that is a major weed problem in crops grown in the United States. After being introduced to North America in the 1700s, velvetleaf has become an invasive in agricultural regions of the eastern and midwestern United States. It is one of the most detrimental weeds to corn, causing decreases of up to 34% of crop yield if not controlled and costing hundreds of millions of dollars per year in control and damage (Spencer, 1984; Warwick & Black, 1988). Velvetleaf is an extremely competitive plant, so much so that it can steal nutrients and water away from crops.

Pseudomonas putida ATH2-1RI/9, a gram negative, rod-shaped rhizobacterium, has the ability to suppress weed seedling growth of velvetleaf, and hydrogen cyanide production in the rhizosphere (cyanogenesis) is implicated in this effect on plant growth (Owen & Zdor, 2001). Owen and Zdor (2001) reported that the weed deleterious rhizobacteria (WDR) *P. putida* ATH2-1RI/9 and *Acidovarax delafieldii* ATH2-2RS/1 significantly inhibited velvetleaf shoot and root growth (40-80% of control) but did not reduce corn growth even if populations of each bacterial strain recovered from the corn rhizosphere were higher than the velvetleaf rhizosphere. Introduction of *P. putida*

ATH2-1RI/9 into the rhizosphere may therefore be a way to improve agricultural production in an environmentally acceptable way. However, knowledge of the factors that may affect bacterial growth and survival, and bacterial genetic and physiological responses to these conditions, is scarce.

Transcriptional Activity of the Hydrogen Cyanide Synthase-Encoding Operon *hcn*ABC

The *hcn*ABC operon has been studied in *P. fluorescens* CHA0 and *P. aeruginosa* PAO1 but not in *P. putida*. The operon *hcn*ABC constitutively expresses HCN synthase, an enzyme which catalyzes the conversion of glycine to carbon dioxide and hydrogen cyanide. The enzyme is sensitive to molecular oxygen, but little is known about the biochemistry of the enzymatic reaction. The enzyme becomes rapidly inactivated in the presence of oxygen and depends on ANR (anaerobic regulator of arginine deiminase and nitrate reductase), a transcriptional regulator of *hcn*ABC operon (Castric, 1981, 1983, 1994).

Several environmental conditions can influence the production of cyanide by cyanogenic bacteria. The biosynthesis of the secondary metabolite hydrogen cyanide (HCN) is maximal during the transition from exponential to stationary phase (Blumer & Haas, 2000). This expression pattern is dependent on quorum sensing, which relies on signals that are part of an autoinduction mechanism tied to increasing cell density. In *P. aeruginosa*, increasing cell density increases secondary metabolites via N-acyl-homeserine lactone signals which mediate quorum sensing (Pessi & Hass, 2000). Work from Blumer and Haas (2000) showed that the anaerobic regulator ANR also acts as an

iron sensor in the *hcn*ABC operon of *P. fluorescens* CHA0 stimulating *hcn*ABC expression in the presence of iron.

Using a Reporter to Study Gene Expression in Culture and Rhizosphere

For successful biological control of weeds, the introduced microbial inocula must be able to develop metabolically active populations on the developing plant root or within the soil environment (Baker, 1987; Bull, Weller, & Thomashow, 1991; Elad & Chet, 1987). To understand how the *hcn*ABC operon is expressed in varying rhizosphere and culture conditions, I used a luminescence-based marker system which may be easily tracked through detection of light emitted by marked cells, without the requirement of cell extraction. Extraction of cells from soil in a form suitable for probing and PCR amplification is particularly difficult because of problems of contamination by soil clay and humic components. Bacterial luciferase genes, originally cloned from the marine bacteria *Vibrio fischeri* or *Vibrio harveyi*, are widely used as reporters of gene expression because of the high sensitivity of chemiluminescence detection and the possibility of monitoring light production in intact cells (Gonalez-Flecha & Demple, 1994).

This *Lux*-marker technology has been used previously with considerable success to monitor population dynamics of introduced bacterial inocula in response to environmental perturbation. Light (590 nm) is produced from the action of a luciferase enzyme by oxidizing the substrate (tetradecanal) and reducing flavin ($FMNH_2$) (Glover, Killham, Prosser, & Rattray, 1996). The reporter strain used in the current study involved an engineered fusion of the promoterless luciferase structural genes (*lux*AB) to the *hcn*ABC operon of *P. putida* ATH2-1RI/9.

This study will identify how environmental conditions, such as iron and oxygen, influence cyanide production in the bacterium *P. putida* ATH2-1RI/9 in culture and the rhizosphere using *hcn*ABC::*lux*AB gene fusion and cyanide quantification.

CHAPTER 2

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Rifampicin-resistant isolates of *P. putida* ATH2-1RI/9 wild-type and ATH2-1RI/9 reporter strain with gene fusion *hcn*ABC:*lux*AB were used in this study. Bacterial strains for soil conditions were routinely grown in half-strength tryptic soy broth (Acumedia) for 24 hours with vigorous shaking (150 rpm) or on half-strength tryptic soy agar (TSA) plates at 28^0C. Bacterial strains for culture conditions were routinely grown in half-strength King's medium B (KB) (King, Ward, & Raney, 1954) for 24 hours with vigorous shaking (150 rpm) or on half-strength tryptic soy agar (Acumedia) plates at 28^0C . When required, 100 µg ml^{-1} rifampicin (wild-type and mutant) plus 50 µg ml^{-1} kanamycin (mutant only) was added to the media.

Culture Conditions

To determine HCN production for aerobic growth in culture conditions, *P. putida* ATH2-1RI/9 was subcultured from KB media into the outer wells of a 250 ml Erlenmeyer flasks containing 100 ml synthetic glycine minimal medium (MMC) described by Castric (1975). For microaerobic (oxygen-limited) growth in culture conditions, the 250 ml Erlenmeyer flasks had rubber stoppers to limit oxygen entry into the flask. The culture flask was gently shaken (100 rpm) at 28^0C with or without 20 µM $FeCl_3$ for 18 and 30 hours.

To measure light production, the reporter bacteria were subcultured from KB into MMC medium, gently shaking at 28^0C for 30 hours. Samples were taken out for analysis after 18 and 30 hours of incubation.

Rhizosphere Conditions

Field soil recovered from the Andrews University Farm, Berrien Springs, Berrien County, Michigan, that had not been treated with herbicides during the previous growing season was autoclaved for 60 min, cooled overnight, and autoclaved again for 60 min (Owen & Zdor, 2001). Velvetleaf (*Abutilon theophrasti*) seeds were collected from the Andrews University dairy corn fields, Berrien Springs, Berrien County, Michigan.

Velvetleaf seed was surface disinfected in 50% ethanol followed by 6% sodium hypochlorite (5 min each treatment), heat-shocked at 65^0C for 10 min and pre-germinated in 1% agar for 2 days at 28^0C. Pre-germinated velvetleaf seeds were planted in sterile plastic tubes (length: 115 mm X diameter: 30 mm) with 50 g of autoclaved soil/tube (3 seeds/tube), and inoculated with approximately 10^9 cells/seed of ATH2-1RI/9 wild-type or reporter *hcn*ABC::*lux*AB strain. Inoculum for the rhizosphere was prepared by washing half-strength TSB-grown cells in 0.1M $MgSO_4$ twice, then resuspending in 0.1M $MgSO_4$. For microaerobic (oxygen-limiting) conditions, autoclaved soil had the moisture level adjusted to 100% water-holding capacity while aerobic conditions had moisture adjusted to 70% water-holding capacity. Iron was added to the soil at a concentration of 8 µg $FeCl_3$/g dry soil when appropriate. Inoculated plants were grown under 12/12 hours of light/dark cycle for 7 days at 25^0C in a growth chamber. Water was added to the plants every day in order to maintain the 70% or 100% moisture level.

Measurements of Bioluminescence

Bioluminescence of the reporter strain growing in MMC liquid cultures was measured by luminometry (Turner Designs 20/20 model) after 18 and 30 hours incubation at 28^{o}C. To measure bioluminescence in soil, the samples were prepared by harvesting roots from 7-day-old velvetleaf plants. The freshly harvested roots were chopped, weighed, and thoroughly shaken in 1 ml of 0.1M $MgSO_4$ solution and vortexed for 60 seconds. The luciferase substrate n-decanal (1% (v/v) suspension in ethanol) was added to culture or rhizosphere samples (10 µl substrate in 500 µl sample). The cumulative light production over 180 seconds was expressed in relative light units (RLU). Diluted samples were plated on tryptic soy agar (TSA) plates and incubated at 28^{0}C to determine the total $CFUg^{-1}$ root basis or total CFU ml^{-1} culture.

Measurements of Cyanide

At plant harvest, roots plus adhering soil were weighed then added to the outer well of a flask containing 20ml of sterile distilled water, and placed on a rotary shaker (150 rpm) for 10 min. Appropriate dilutions of the resultant root suspension were plated on one-half strength TSA supplemented with 100 µg ml^{-1} rifampicin. To capture HCN from the root suspension, 3ml of 1M NaOH was added to the inner well of the flask while 10ml of 5M HCl was added to the outer well, vigorously shaking (150 rpm) the suspension at 45^{0}C for 6 hours. In a similar manner, HCN from the bacterial cultures was captured in 5ml of 1M NaOH (inner well) using 100ml of MMC media (outer well) as previously mentioned. HCN from culture and rhizosphere trapped in 1M NaOH was then quantified using N-chlorosuccinimide/succinimide and hydantoin-pyridine reagents (Lambert, Ramasamy, & Paukstelle, 1975).

Statistics

All treatments were performed in triplicate, and each experiment was repeated at least three times; means of the triplicates in each experiment are presented. Means of the different treatment groups within each experiment were compared with the one-way ANOVA for statistical significance. To determine which treatment groups gene expression significantly differed from the other, the Bonferroni and Scheffé Post hoc tests were performed on each experiment. A *t* test was used to determine significant differences in bioluminescence and hydrogen cyanide levels between stationary phase vs. log phase cultures.

CHAPTER 3

RESULTS

Culture Experiment

Bioluminescence of *P. putida* ATH2-1RI/9 cultures containing the gene fusion *hcn*ABC::*lux*AB was strongest in the presence of iron (Figure 1A-1C). In the culture experiments the range of bioluminescence measured in relative light units (RLU) of the reporter strain was from 5,500 RLU/CFUml^{-1} in experiment 3 to 2 RLU/CFUml^{-1} in experiment 1 (Figure 1A, 1C). In one of the three experiments, the presence of iron in the log phase microaerophilic culture significantly increased bioluminescence (ANOVA, $p < 0.05$) sixfold compared to the iron-limited microaerophilic culture (Figure 1B). We see similar trends in Figure 1C for the stationary and the log phase cultures; adding iron to the microaerobic conditions significantly increased bioluminescence (ANOVA, $p < 0.05$). In stationary phase, the presence of iron in microaerobic cultures gave a 35-fold increase in bioluminescence compared to the microaerobic-iron-depleted culture, while a sevenfold increase in bioluminescence is seen for aerobic culture conditions (Figure 1C). In Figure 1A we see similar increasing trends for stationary phase cultures, the addition of iron gave significantly higher gene expression in both aerobic and microaerobic conditions. When comparing stationary phase and log phase cultures (Figure 1B), my study reveals that iron-containing microaerophilic condition has a significantly higher gene expression at the log phase in 1 experiment (*t* test, $p < 0.05$). However, the two

other experiments did not show any significance in increasing bioluminescence for the iron-containing microaerophilic condition in the log phase when compared to the stationary phase with a *t* test (Figure 1A, 1C).

In two out of three experiments shown (Figure 2A, 2B), the iron-supplemented stationary phase cultures of the wild-type strain *P. putida* ATH2-1RI/9 produced significantly more hydrogen cyanide under microaerobic conditions in comparison to log phase cultures (*t* test, $p < 0.05$). In experiment 3 (Figure 2C), the iron-supplemented stationary phase culture significantly produced fourfold more hydrogen cyanide under aerobic conditions in comparison to log phase cultures (*t* test, $p < 0.05$). The opposite trend is seen in the iron-depleted aerobic culture of experiment 1 (Figure 2A), the log phase gave a significantly higher cyanide level than stationary phase cultures (*t* test, $p < 0.05$). In experiment 1, the iron-supplemented microaerobic log phase cultures gave a twofold significantly higher HCN levels than iron-supplemented aerobic culture conditions (Figure 2A) (ANOVA, $p < 0.05$). In experiment 3 of the stationary phase cultures, the iron-supplemented aerobic culture had a fourfold increase in HCN production reaching 5000 µM/CFUml^{-1} compared to 1200 µM/CFUml^{-1} in the iron-limited aerobic culture condition (Figure 2C). The highest HCN levels recorded were in the stationary phase of experiment 2; the iron-supplemented microaerobic culture condition reached 11,000 µM/CFUml^{-1}, which is significantly higher when compared to iron-limiting aerobic culture conditions (Figure 2B) (ANOVA, $p < 0.05$).

The average populations of the wild-type and reporter strains in culture and soil of *P. putida* ATH2-1RI/9 are presented in the appendix section (see Appendix Table 1, 2, & 3). In Tables 1 and 2, the lowest population was seen in the log phase culture of the wild-

type strain reaching approximately $1.83 \pm 1.4 \times 10^{8}$ CFU/ml and the highest population being $1.34 \pm 1.03 \times 10^{10}$ CFU/ml in the wild-type strain of the stationary phase culture.

Rhizosphere Experiment

The influence of rhizosphere iron levels on bioluminescence of *P. putida* ATH2-1RI/9 containing the gene fusion *hcn*ABC::*lux*AB was not as strong as when it was in culture. In the rhizosphere experiments the range of bioluminescence measured in relative light units (RLU) of the reporter strain was from 550 RLU/CFUg root^{-1} in experiment 2 to 7 RLU/CFUg root^{-1} in experiment 1 (Figure 3A, 3B). With iron supplementation in the rhizosphere, bioluminescence under microaerobic conditions was significantly higher ($p < 0.05$) in experiment 1, from 10 RLU/CFUg root^{-1} in the aerobic condition to 170 RLU/CFUg root^{-1} in iron-supplemented microaerobic condition (Figure 3A). In the iron-limiting conditions of experiment 3, bioluminescence was significantly higher ($p < 0.05$) in the aerobic condition compared to the microaerobic condition (Figure 3C). The highest bioluminescence (500 RLU/CFUg root^{-1}) was obtained with cells in microaerobic iron-limiting rhizosphere conditions (Figure 3B). However, this increasing trend in experiment 2 was not significant.

Bioluminescence results from the *P. putida* ATH2-1RI/9 reporter strain in soil rhizosphere were quite variable. In two of the three experiments significant changes were seen in bioluminescence due to iron and oxygen conditions. In experiment 1, bioluminescence in iron-supplemented microaerobic conditions was significantly greater than in both aerobic conditions. In experiment 3, bioluminescence in aerobic conditions lacking iron supplementation was significantly higher than in both microaerobic

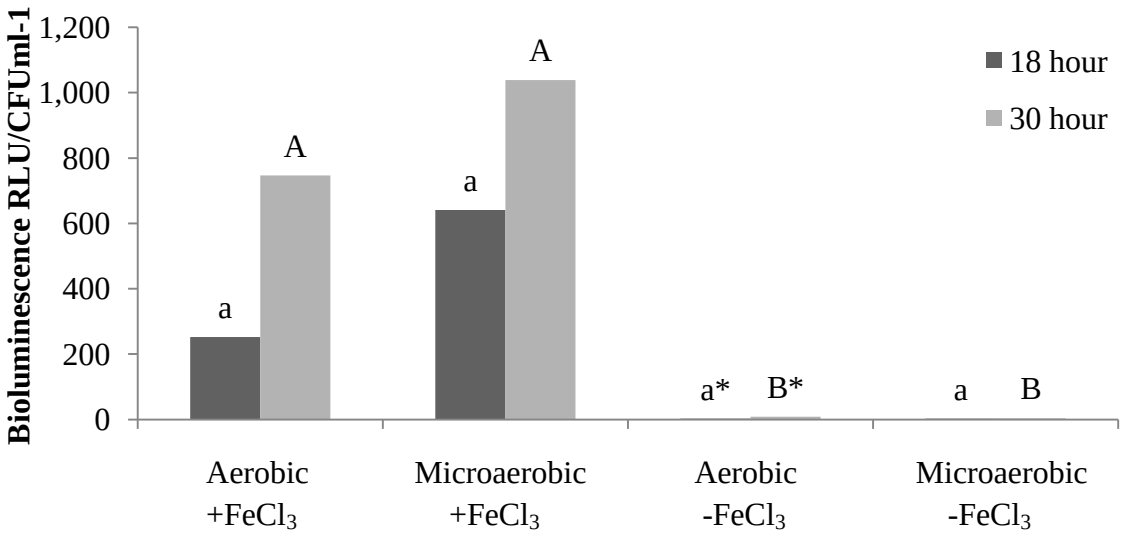
conditions. The overall trend for bioluminescence in iron-supplemented rhizosphere condition is not consistent among the three different experiments.

In one of the three experiments, HCN production significantly increased tenfold in the iron-limiting aerobic rhizosphere condition compared to iron-limiting microaerobic conditions (Figure 4B). In experiment 2, HCN production significantly increased twentyfold in the iron-limiting aerobic rhizosphere condition compared to iron-supplemented microaerobic rhizosphere conditions (Figure 4B). In experiment 3, the aerobic iron-supplemented rhizosphere condition significantly increased HCN production tenfold compared to the microaerobic iron-limiting conditions (Figure 4C). In the rhizosphere, microaerobic conditions decreased HCN production dramatically in comparison to aerobic conditions in two of the three experiments (Figure 4B, 4C).

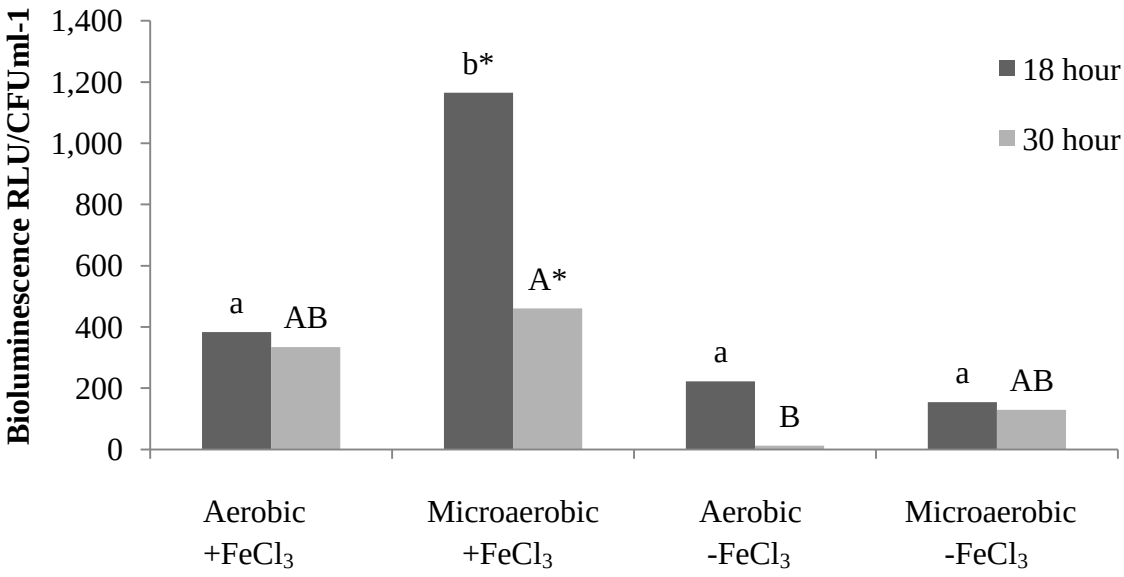
In the rhizosphere condition, the overall trend for HCN production was highest in the aerobic conditions under both iron-supplemented and non-supplemented conditions. However, this increase was significant only in two of the three experiments (Figure 4B, 4C).

The average populations of the wild-type and reporter strains in the rhizosphere of *P. putida* ATH2-1RI/9 are presented in the appendix section (see Appendix Table 3). In Table 3, the lowest population was seen in iron-limiting aerobic rhizosphere conditions with the wild-type strain (Experiment 1), reaching $5.56 \pm 1.9 \times 10^6$ CFU/g root and the highest population of the reporter strain being $9.08 \pm 2.8 \times 10^8$ CFU/g root (Experiment 2) under microaerobic iron-supplemented rhizosphere conditions.

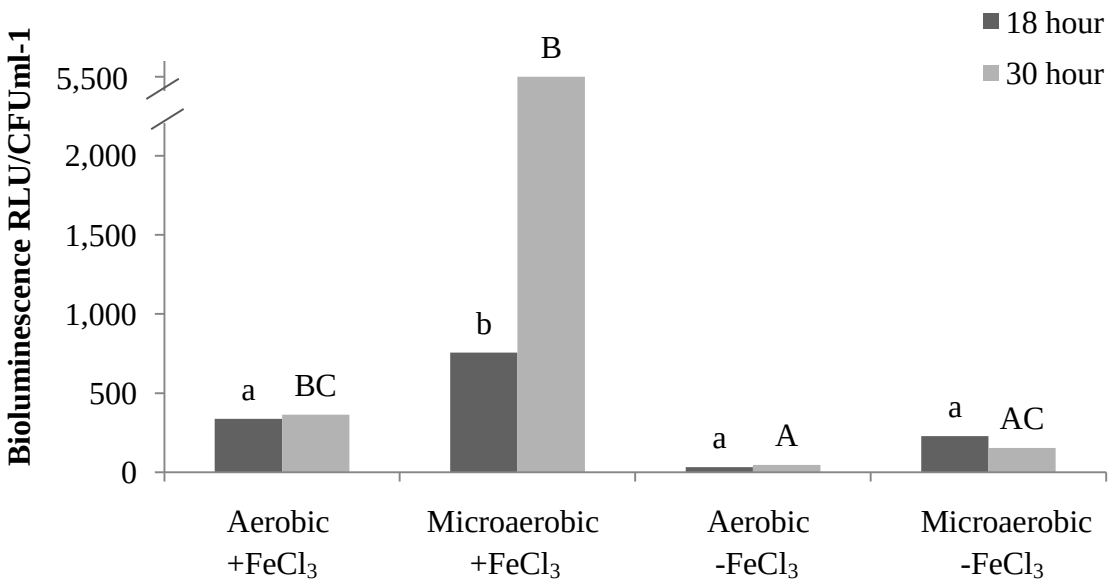
Figure 1. Bioluminescence activity of reporter strain *Pseudomonas putida* ATH2-1RI/9 in log phase (18 hour) and stationary phase (30 hour) cultures from three individual experiments performed at three different times. Mean values from the triplicates in each experiment are presented. (A) Experiment 1 (B) Experiment 2 (C) Experiment 3; Darker shade bars, 18 hour sample; Lighter shade bars, 30 hour sample. +$FeCl_3$: iron-supplemented culture; -$FeCl_3$: no iron supplementation; Aerobic: with oxygen; microaerobic: with limited oxygen. RLU: relative light units. Between the four treatment groups within each age culture, values followed by a different letter are significantly different using Scheffé's post hoc test, while significance as a result of *t* tests between 18 and 30 hour culture is shown with *. (p value < 0.05).


Bioluminescence RLU/CFUml-1
1,200
1,000
800
600
400
200
0
18 hour
30 hour
a
A
a
A
a*
B*
a
B
Aerobic +FeCl3
Microaerobic +FeCl3
Aerobic -FeCl3
Microaerobic -FeCl3

A)

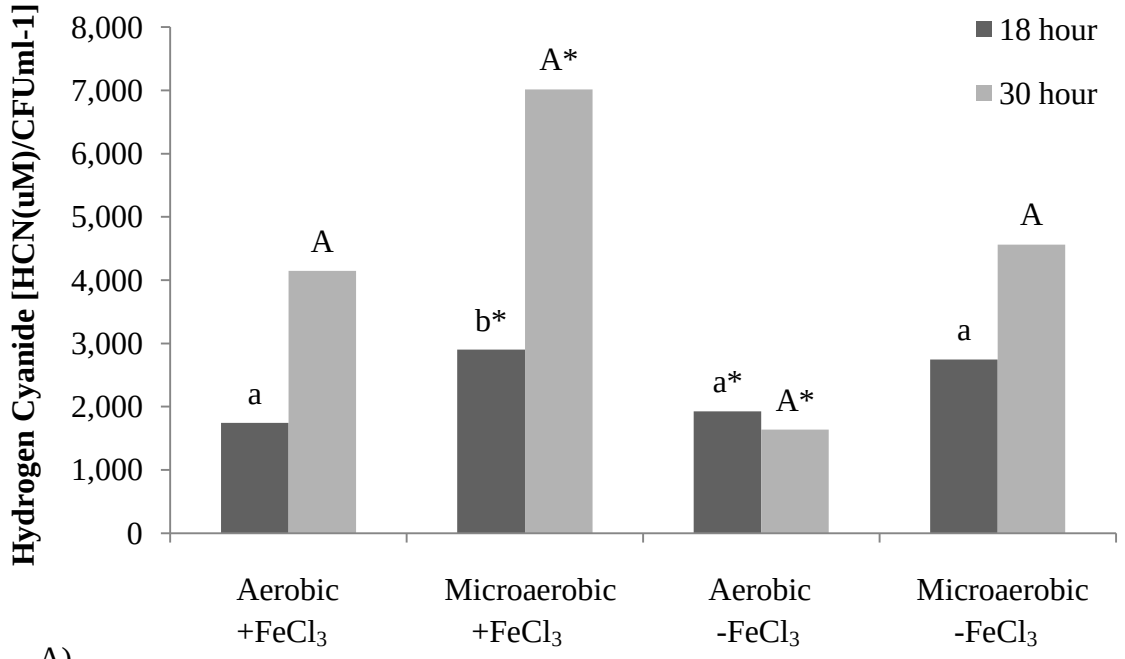

Bioluminescence RLU/CFUml-1
1,400
1,200
1,000
800
600
400
200
0
18 hour
30 hour
a
AB
b*
A*
a
B
a
AB
Aerobic +FeCl3
Microaerobic +FeCl3
Aerobic -FeCl3
Microaerobic -FeCl3

B)

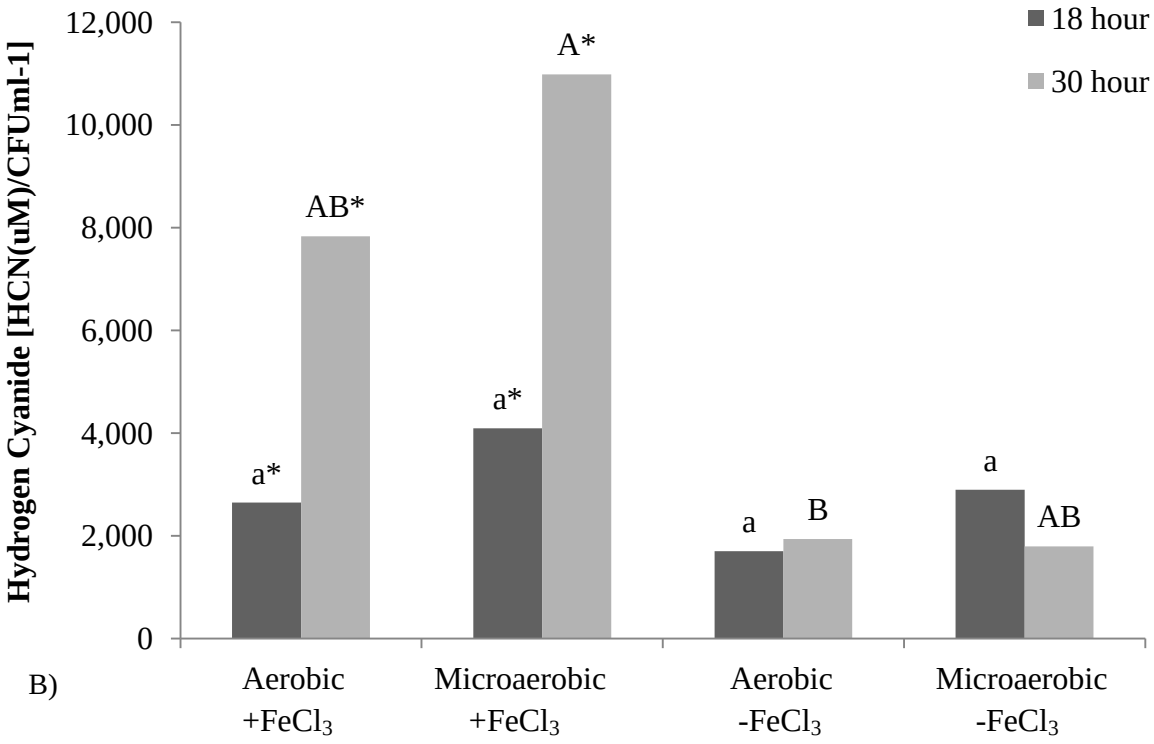

Bioluminescence RLU/CFUml-1
5,500
2,000
1,500
1,000
500
0
18 hour
30 hour
a
BC
b
B
a
A
a
AC
Aerobic +FeCl3
Microaerobic +FeCl3
Aerobic -FeCl3
Microaerobic -FeCl3

C)

Figure 2. Hydrogen cyanide production of the wild-type strain *Pseudomonas putida* ATH2-1RI/9 in log phase (18 hour) and stationary phase (30 hour) cultures from three individual experiments performed at three different times. Mean values from the triplicates in each experiment are presented. (A) Experiment 1 (B) Experiment 2 (C) Experiment 3; Darker shade bars, 18 hour sample; Lighter shade bars, 30 hour sample. +$FeCl_3$: iron-supplemented culture; -$FeCl_3$: no iron supplementation; Aerobic: with oxygen; microaerobic: with limited oxygen. Between the four treatment groups within the same age culture, values followed by a different letter are significantly different using Scheffé's post hoc test, while significance as a result of *t* tests between 18 and 30 hour culture is shown with *. (p value < 0.05).


Hydrogen Cyanide [HCN(uM)/CFUml-1]
8,000
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0
18 hour
30 hour
a
A
b*
A*
a*
A*
a
A
Aerobic +FeCl3
Microaerobic +FeCl3
Aerobic -FeCl3
Microaerobic -FeCl3

A)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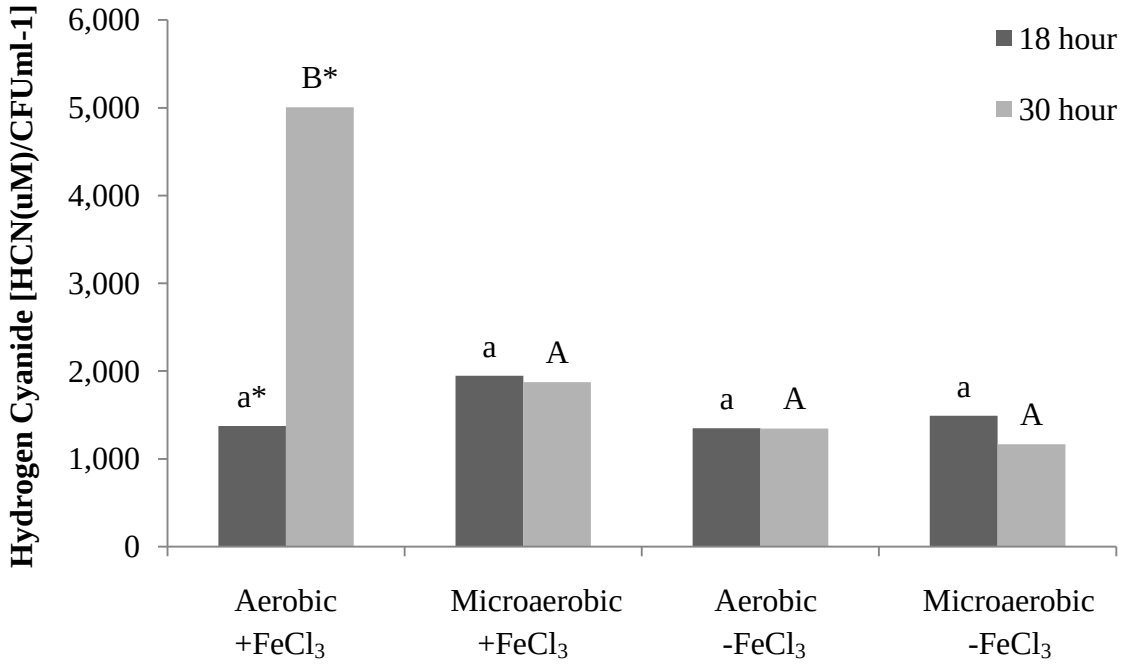
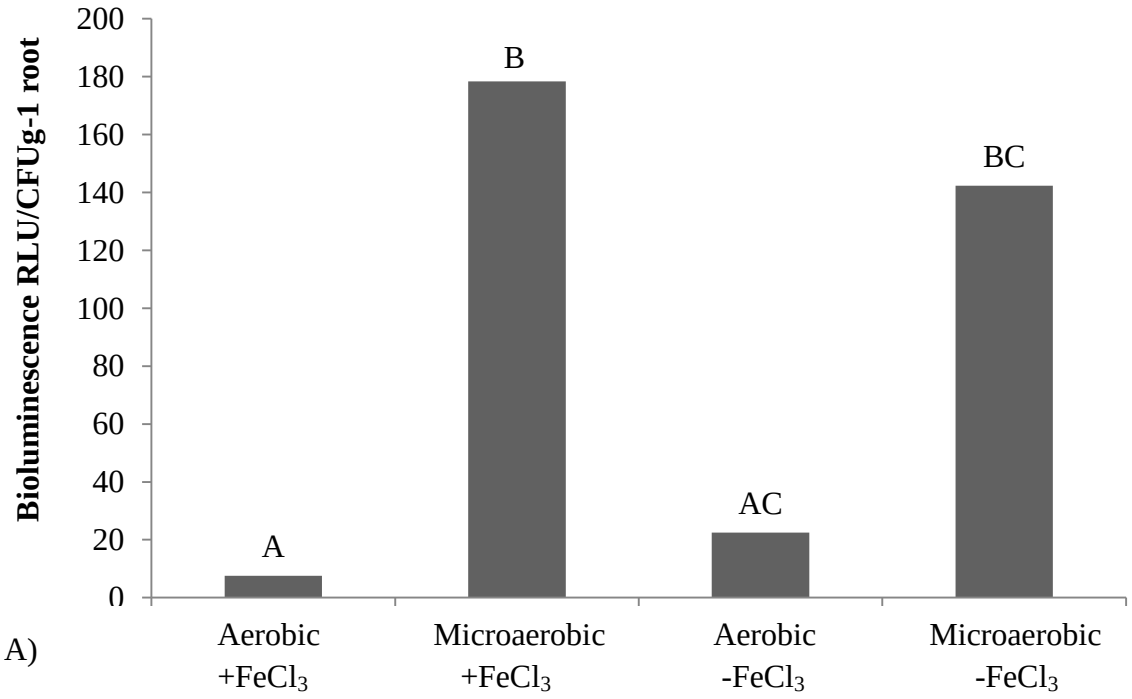
10,000
8,000
6,000
4,000
2,000
0
18 hour
30 hour
a*
AB*
a*
A*
a
B
a
AB
Aerobic +FeCl3
Microaerobic +FeCl3
Aerobic -FeCl3
Microaerobic -FeCl3

B)

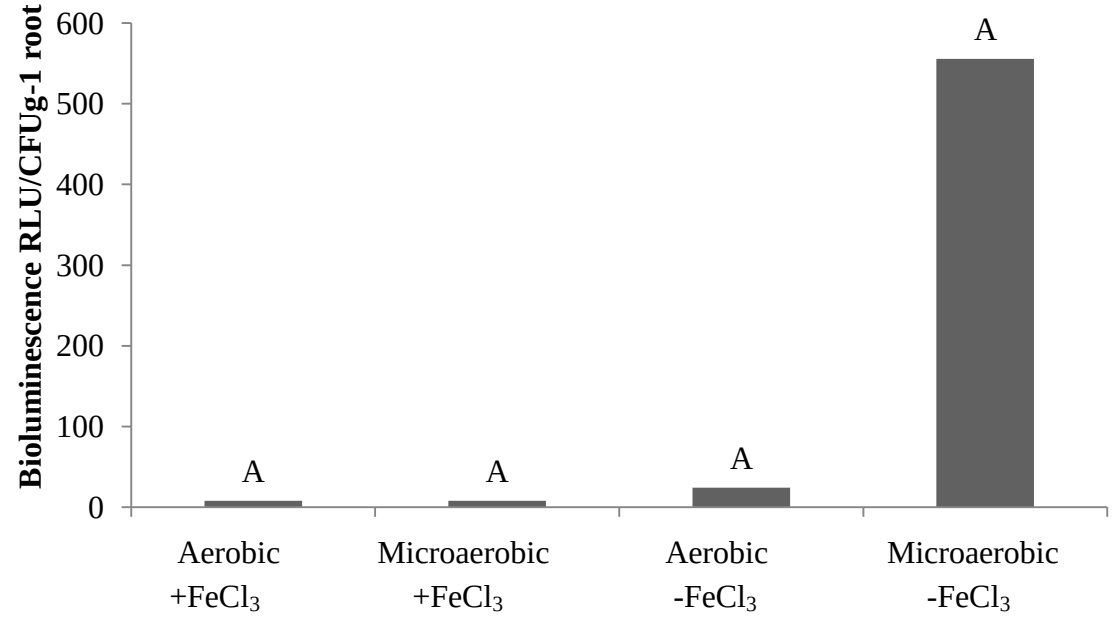

Hydrogen Cyanide [HCN(uM)/CFUml-1]
6,000
5,000
4,000
3,000
2,000
1,000
0
18 hour
30 hour
a*
B*
a
A
a
A
a
A
Aerobic +FeCl3
Microaerobic +FeCl3
Aerobic -FeCl3
Microaerobic -FeCl3

C)

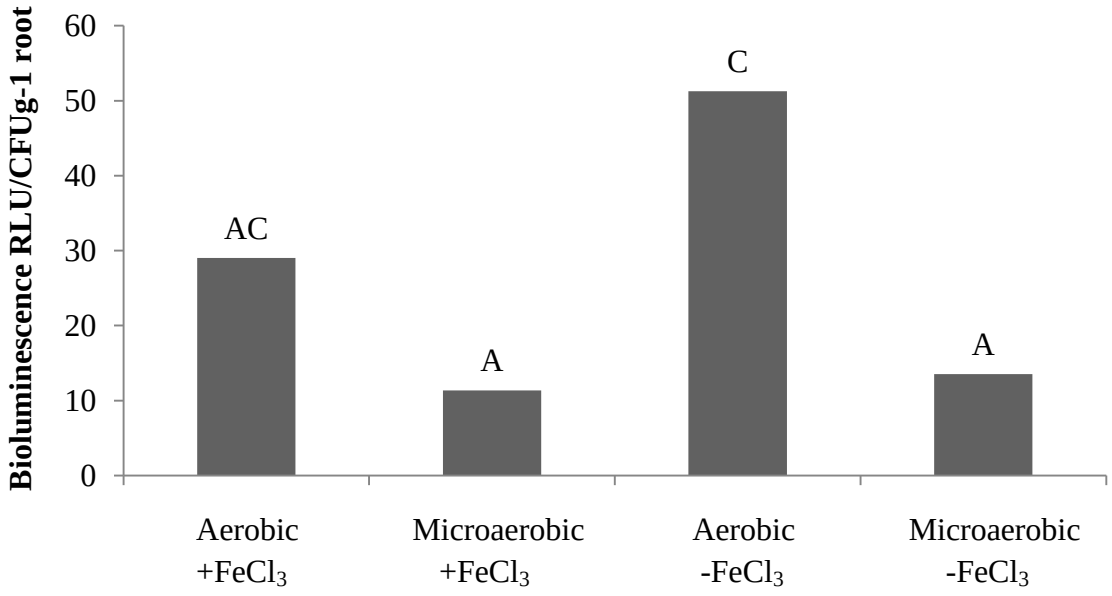
Figure 3. Bioluminescence activity of the reporter strain *Pseudomonas putida* ATH2-IRI/9 in the rhizosphere from three individual experiments performed at three different times. Mean values from the triplicates in each experiment are presented. (A) Experiment 1 (B) Experiment 2 (C) Experiment 3; +$FeCl_3$: iron-supplemented culture; -$FeCl_3$: no iron supplementation; Aerobic: with oxygen; microaerobic: with limited oxygen. RLU: relative light units. Between the four treatment groups, values followed by a different letter are significantly different using Scheffé's post hoc test. (p value < 0.05).


Bioluminescence RLU/CFUg-1 root
200
180
160
140
120
100
80
60
40
20
0
A
B
AC
BC
Aerobic +FeCl3
Microaerobic +FeCl3
Aerobic -FeCl3
Microaerobic -FeCl3

A)

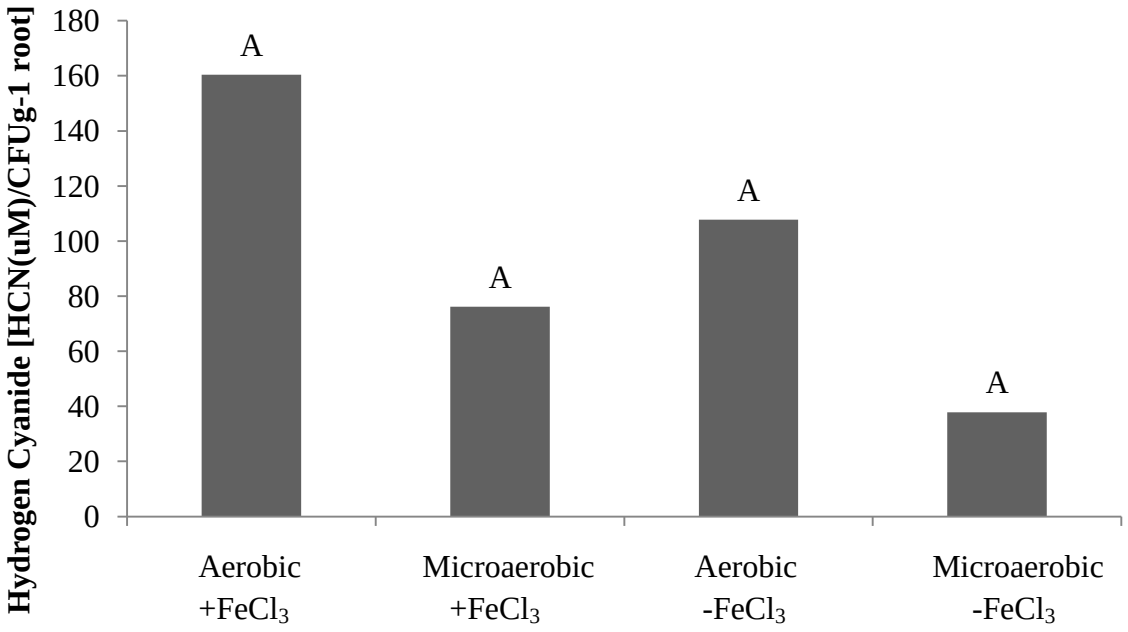

Bioluminescence RLU/CFUg-1 root
600
500
400
300
200
100
0
A
A
A
A
Aerobic +FeCl3
Microaerobic +FeCl3
Aerobic -FeCl3
Microaerobic -FeCl3

B)

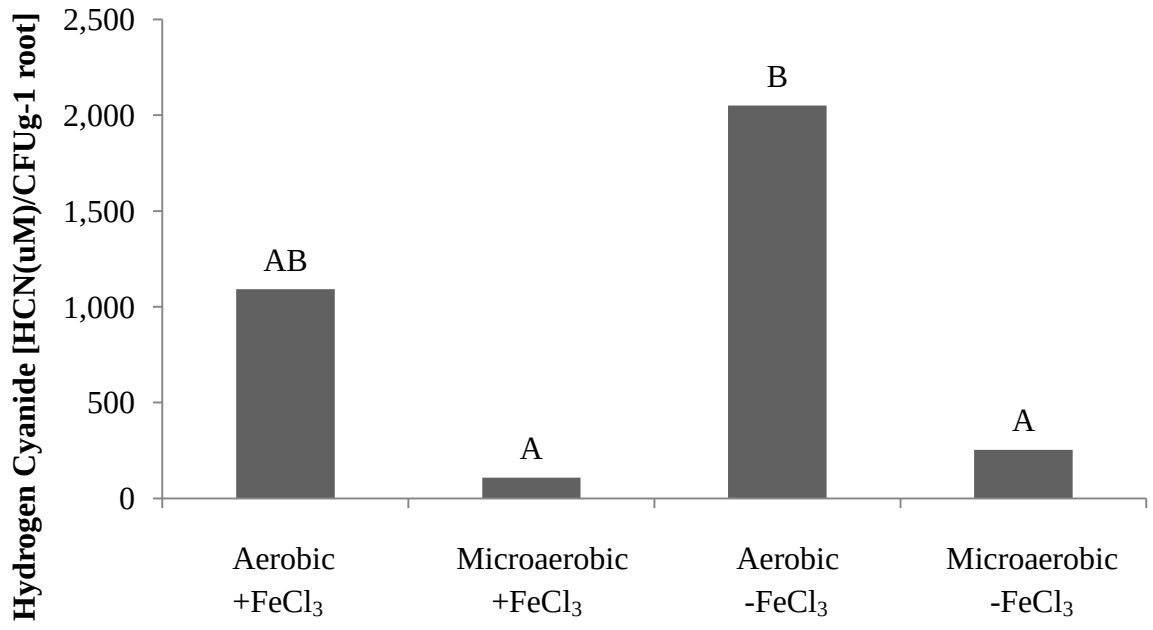

Bioluminescence RLU/CFUg-1 root
60
50
40
30
20
10
0
AC
A
C
A
Aerobic +FeCl3
Microaerobic +FeCl3
Aerobic -FeCl3
Microaerobic -FeCl3

C)

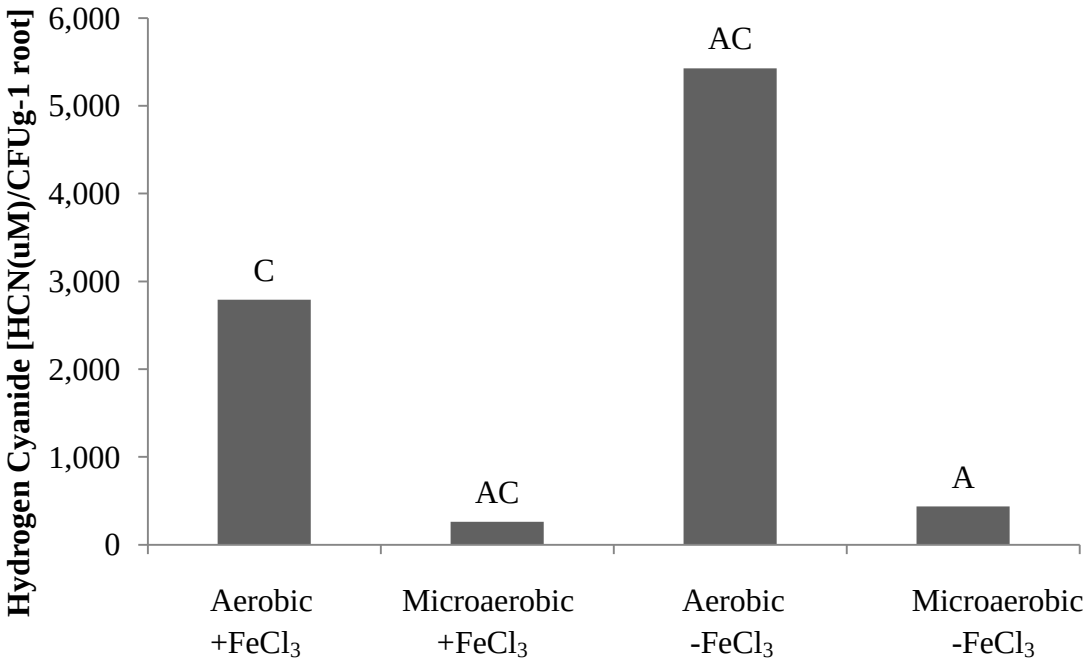
Figure 4. Hydrogen cyanide production of the wild-type strain *Pseudomonas putida* ATH2-1RI/9 in the rhizosphere from three individual experiments performed at three different times. Mean values from the triplicates in each experiment are presented. (A) Experiment 1 (B) Experiment 2 (C) Experiment 3; +$FeCl_3$: iron-supplemented culture; -$FeCl_3$: no iron supplementation; Aerobic: with oxygen; microaerobic: with limited oxygen. Between the four treatment groups, values followed by a different letter are significantly different using Scheffé's post hoc test. (p value < 0.05).


Hydrogen Cyanide [HCN(uM)/CFUg-1 root]
180
160
140
120
100
80
60
40
20
0
A
A
A
A
Aerobic +FeCl3
Microaerobic +FeCl3
Aerobic -FeCl3
Microaerobic -FeCl3

A)


Hydrogen Cyanide [HCN(uM)/CFUg-1 root]
2,500
2,000
1,500
1,000
500
0
AB
A
B
A
Aerobic +FeCl3
Microaerobic +FeCl3
Aerobic -FeCl3
Microaerobic -FeCl3

B)


Hydrogen Cyanide [HCN(uM)/CFUg-1 root]
6,000
5,000
4,000
3,000
2,000
1,000
0
C
AC
AC
A
Aerobic +FeCl3
Microaerobic +FeCl3
Aerobic -FeCl3
Microaerobic -FeCl3

C)

CHAPTER 4

DISCUSSION

In this study, a reporter strain of *Pseudomonas putida* ATH-1RI/9, with the gene fusion *hcn*ABC::*lux*AB, was used to monitor gene expression under varying iron and oxygen levels.

I predicted from the work of Castric et al. (1975) that the bioluminescence in culture would be greatest in stationary phase cultures. Although two experiments (Figure 1A, Figure 1C) confirm that assumption, one experiment (Figure 1B) gave the opposite result, log phase (18 hours), giving the highest bioluminescence. The increase in bioluminescence for reporter strain culture in stationary phase is statistically significant from cells in log phase in one of the treatment groups (Aerobic, -$FeCl_3$; Figure 1A). The reporter strain in culture is behaving as predicted in two experiments out of a total of three experiments performed. The reporter strain is a reliable indicator of *hcn*ABC gene expression due to two reasons: first, because of the lack of HCN production with the insertion of *lux*AB into the *hcn*ABC operon, and, second, because the maximal bioluminescence occurs at the culture age where HCN production is maximal.

Gene expression of reporter bacterium *P. putida* ATH2-1RI/9 in microaerobic cultures was strongest in the presence of iron, supporting the work of Blumer and Haas (2000) with *Pseudomonas fluorescens* CHA0 who found that a conserved FNR/ANR recognition sequence in the -40 promoter region was necessary and sufficient for the

regulation by ANR in response to oxygen limitation in *P. fluorescens* CHA0. They also suggested that ANR responds to iron availability by stimulating *hcn*A'-*lac*Z' expression in the presence of iron (Blumer & Haas, 2000). Pessi and Haas (2000) found in *P. aeruginosa* PAO1 that the *hcn*A promoter was controlled by the FNR-like anaerobic regulator ANR, which activates transcription from start site T2 at low oxygen levels. The basis for variability in culture bioluminescence in the *P. putida* reporter strain is not known, but unknown culture conditions may influence *hcn*ABC expression. Paulin et al. (2009) studied the transcriptional activity of *hcn* genes in KB culture media and found that factors such as pathogen presence and media composition can alter patterns of gene expression in *Pseudomonas* strains over time.

Even though bioluminescence of the reporter bacterium in the rhizosphere was highest under microaerobic conditions in two of three experiments, iron supplementation generally did not stimulate bioluminescence in the rhizosphere. Soil conditions without iron supplementation seemed to have higher *hcn*ABC::*lux*AB expression. Previously Voisard, Keel, Haas, and Defago (1989) showed from their work that better protection of the disease black root rot of tobacco mediated by HCN by strain CHA0 was obtained when using $FeCl_3$ in artificial soil containing illite as clay mineral. These data suggest that soil iron status influences cyanogenesis. It is a possible that the soil used in this present work had trace amounts of iron present and thus adding 8µg $FeCl_3$/g dry soil did not affect the *hcn*ABC operon like it did in culture. Recent work by Jamali, Sharifi-Tehrani, Luzt, and Maurhofer (2009) showed an increase in *P. fluorescens* CHA0 *hcn*A-*lac*Z gene expression in the rhizosphere of bean plants in response to the genotype of the host cultivar used. These data suggest that HCN production by rhizobacteria can be

altered by association with specific plant roots. My data with the reporter strain suggest that the *hcn*ABC operon is expressed in the velvetleaf rhizosphere, but no consistent trends were seen in all three experiments for the soil conditions tested.

In two of the experiments, HCN production in culture was highest in microaerobic conditions (Figure 2A, 2B) with iron supplementation reflecting the bioluminescence results obtained with the reporter strain. HCN levels in stationary phase cultures were generally higher than log phase cultures HCN levels under iron-supplemented conditions. The trend in soil for HCN production was different from *hcn*ABC::*lux*AB gene expression. HCN production in soil was highest in aerobic conditions, which is opposite from the culture data. However, the difference was not significant in one experiment (Figure 4A). Microaerobic conditions in soil were achieved by saturating the soil with water, with the results showing lower HCN production. It could be that oxygen levels were low enough to suppress the metabolic activity and thus cyanogenesis of this aerobic organism. Soil is a very complicated biomaterial on the planet, and there are many factors that can affect the gene expression of the soil microbe that we do not know about.

We know from previous culture work done on *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* that HCN production is dependent on quorum sensing (Blumer & Haas, 2000; Pessi & Haas, 2000). From my results (see Appendix, Table 1, 2, & 3), bacterial populations did not have a major effect on gene expression levels. Table 1 shows highest cell density for the wild-type strain in treatment 3 of the stationary phase cultures, which does not give the highest gene expression in Figure 2C. Treatment 3 of Figure 3C shows that the bioluminescence reaches 10 RLU/CFUg root^{-1} when the cell

density in the rhizosphere is the highest, reaching $6.57 \pm 2.1 \times 10^8$ CFU. The population in the rhizosphere ranges from $5.56 \pm 1.9 \times 10^6$ CFU to $6.57 \pm 2.1 \times 10^8$ CFU which corresponds to bioluminescence levels of 7 to 550 RLU/CFUg root^{-1} (Figure 3). We know from the work done by Wood et al. (1997) that the expression of phenazine in wheat rhizosphere colonizing *Pseudomonas aureofaciens* 30-84 is controlled by an interpopulation signal molecule N-hexanoyl-homoserine lactone (HHL). To test if the population density of *Pseudomonas putida* ATH-1RI/9 is mediating *hcn*ABC gene expression through production of some signal molecule further research has to be done. We know from previous work done on *P. fluorescens* CHAO that quorum sensing acts post-transcriptionally to regulate cyanogenesis (Blumer & Haas, 2000).

In conclusion, the results from both techniques gave variable results from experiment to experiment but showed general trends where the presence of iron (under microaerophilic conditions) for both early and late growth stage cultures was the main variable that stimulated cyanogenesis resulting in cyanide and bioluminescence levels greater than levels from cells grown in the absence of iron. These results support similar findings obtained with the plant-associated bacterium *P. fluorescens* CHA0 (Blumer & Haas, 2000). Further work must be done to test the effect of iron in promoting cyanogenesis in the rhizosphere and its possible role in optimizing weed-control strategies that involve cyanogenic WDR.

APPENDIX

Figure 5. *hcn*ABC operon with the *lux*AB insertion in the genome of *Pseudomonas putida* ATH2-1RI/9.

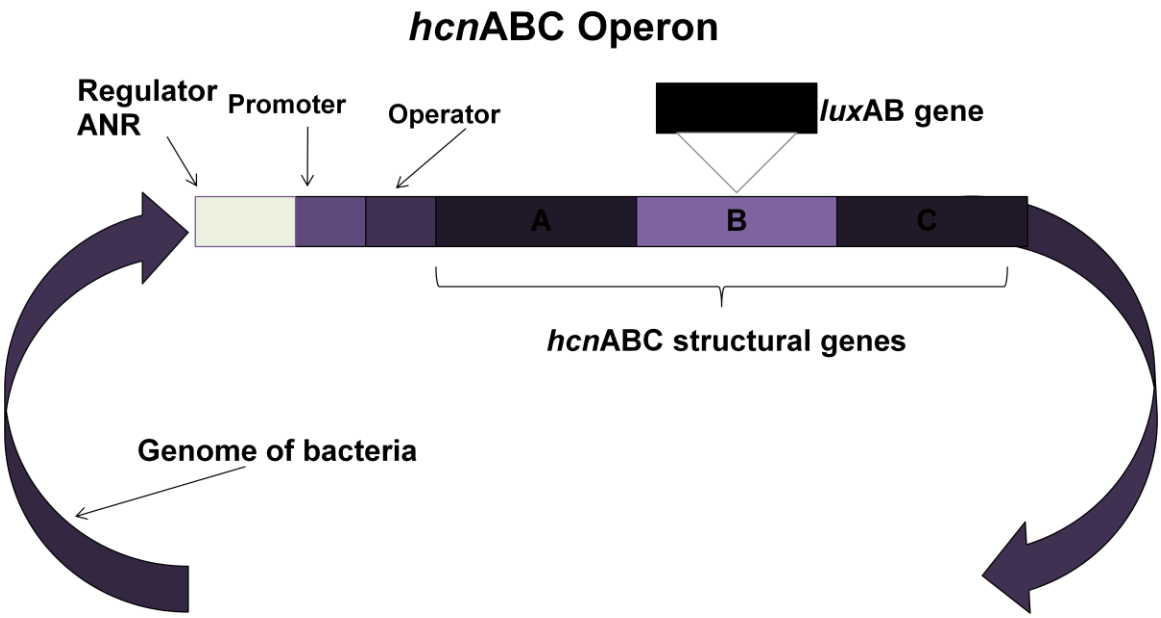

*hcn*ABC Operon
Regulator ANR
Promoter
Operator
*lux*AB gene
A
B
C
*hcn*ABC structural genes
Genome of bacteria

Table 1. Average (±1 S.D.) populations of wild-type and reporter strains of *Pseudomonas putida* ATH2-1RI/9 in culture conditions from Experiment 1 and 2. Each mean is based on triplicate dilution plate samples from two to three replicates.

Culture (CFU/ml)	Experiment 1	
	18 hour	30 hour
Reporter Strain		
Aerobic+$FeCl_3$	$1.85\pm1.07x10^9$	$1.35\pm1.2x10^9$
Microaerobic+$FeCl_3$	$1.43\pm0.88x10^9$	$5.97\pm0.86x10^8$
Aerobic -$FeCl_3$	$1.47\pm0.36x10^9$	$5.8\pm0.97x10^8$
Microaerobic -$FeCl_3$	$1.22\pm0.83x10^9$	$1.11\pm0.21x10^9$
Wild-type strain		
Aerobic+$FeCl_3$	$1.99\pm1.8x10^9$	$5.83\pm2.9x10^9$
Microaerobic+$FeCl_3$	$1.83\pm1.4x10^8$	$5.44\pm2.8x10^9$
Aerobic -$FeCl_3$	$1.44\pm0.58x10^9$	$6.93\pm2.1x10^9$
Microaerobic -$FeCl_3$	$1.83\pm1.1x10^9$	$8.23\pm2.5x10^9$

Culture (CFU/ml)	Experiment 2	
	18 hour	30 hour
Reporter Strain		
Aerobic+$FeCl_3$	$7.3\pm1.4x10^9$	$1.05\pm0.42x10^{10}$
Microaerobic+$FeCl_3$	$3.07\pm0.42x10^9$	$7.57\pm2.1x10^9$
Aerobic -$FeCl_3$	$9.43\pm3.8x10^9$	$1.03\pm0.28x10^{10}$
Microaerobic -$FeCl_3$	$8.52\pm1.8x10^9$	$4.03\pm1.2x10^9$
Wild-type strain		
Aerobic+$FeCl_3$	$7.56\pm2.3x10^9$	$7.87\pm3.6x10^9$
Microaerobic+$FeCl_3$	$4.52\pm1.3x10^9$	$5.01\pm1.05x10^9$
Aerobic -$FeCl_3$	$4.59\pm1.03x10^9$	$7.19\pm0.4x10^9$
Microaerobic -$FeCl_3$	$4.17\pm1.4x10^9$	$5.11\pm1.1x10^9$

Table 2. Average (±1 S.D.) populations of wild-type and reporter strains of *Pseudomonas putida* ATH2-1RI/9 in culture conditions from Experiment 3. Each mean is based on triplicate dilution plate samples from two to three replicates.

Culture (CFU/ml)	Experiment 3	
	18 hour	30 hour
Reporter Strain		
Aerobic+$FeCl_3$	$9.04 \pm 2.9 \times 10^9$	$5.51 \pm 1.6 \times 10^9$
Microaerobic+$FeCl_3$	$5.04 \pm 2.09 \times 10^9$	$3.68 \pm 0.83 \times 10^9$
Aerobic -$FeCl_3$	$7.56 \pm 3.4 \times 10^9$	$9.54 \pm 10.04 \times 10^9$
Microaerobic -$FeCl_3$	$8.07 \pm 1.5 \times 10^9$	$3.57 \pm 0.85 \times 10^9$
Wild-type strain		
Aerobic+$FeCl_3$	$7.95 \pm 0.45 \times 10^9$	$3.53 \pm 0 \times 10^9$
Microaerobic+$FeCl_3$	$5.88 \pm 0.23 \times 10^9$	$1.04 \pm 0.25 \times 10^{10}$
Aerobic -$FeCl_3$	$6.46 \pm 0.99 \times 10^9$	$1.34 \pm 1.03 \times 10^{10}$
Microaerobic -$FeCl_3$	$6.58 \pm 2.7 \times 10^9$	$8.07 \pm 2.7 \times 10^9$

Table 3. Average (±1 S.D.) populations of wild-type and reporter strains of *Pseudomonas putida* ATH2-1RI/9 in soil conditions. Each mean is based on triplicate dilution plate samples from two to three replicates.

Soil (CFU/g root)	Experiment 1	Experiment 2	Experiment 3
Reporter strain			
Aerobic+$FeCl_3$	$9.97\pm4.8x10^7$	$2.61\pm2.4x10^8$	$2.33\pm1.2x10^8$
Microaerobic+$FeCl_3$	$2.14\pm0.34x10^8$	$9.08\pm2.8x10^8$	$6.57\pm2.1x10^8$
Aerobic -$FeCl_3$	$7.67\pm2.7x10^7$	$3.64\pm3.7x10^8$	$1.41\pm0.62x10^8$
Microaerobic - $FeCl_3$	$2.69\pm0.202x10^8$	$6.26\pm1.05x10^8$	$4.9\pm0.93x10^8$
Wild-type strain			
Aerobic+ $FeCl_3$	$2.33\pm2.6x10^7$	$6.44\pm6.3x10^7$	$6.67\pm3.3x10^6$
Microaerobic+ $FeCl_3$	$9.78\pm5.9x10^7$	$2.49\pm2.5x10^8$	$3.44\pm1.67x10^7$
Aerobic - $FeCl_3$	$5.56\pm1.9x10^6$	$2.33\pm0.94x10^7$	$8.89\pm1.9x10^6$
Microaerobic - $FeCl_3$	$4.57\pm1.2x10^7$	$1.62\pm1.02x10^8$	$3.33\pm0.88x10^7$

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