Acyl Homoserine Lactone Signaling in Pseudomonas chlororaphis 06

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ACYL HOMOSERINE LACTONE SIGNALING IN
PSEUDOMONAS CHLORORAPHIS 06

by

Anna Hegsted

Thesis submitted in partial fulfillment
of the requirements for the degree
of
HONORS IN UNIVERSITY STUDIES
WITH DEPARTMENTAL HONORS
in
Biology
in the Department of Biology

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Spring 2010
Abstract

Pseudomonas chlororaphis (PcO6), a rhizosphere bacterium, is important to agriculture because of its use as a biological control against fungus and other pests. This biological control correlates with production of phenazines; a process regulated by acyl homoserine lactones (AHSLs). Long chain AHSLs might also have an affect on the production of phenazines. My research showed that PcO6 produced long chain AHSLs and that production is affected by nutrition with more long chain AHSLs produced in rich medium than minimal medium. The nutritional studies and findings with different mutants of PcO6 altered in phenazine production suggest that control of these antifungal compounds is not the major role of these long chain fatty acid signaling molecules. Long chain AHSLs production is also influenced by Pluronics in the media.
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Introduction

*Pseudomonas chlororaphis* O6 (*PcO6*)

colonizes the plant root surface and the area immediately around the root termed the rhizosphere (Figure 1). This region has a higher nutrient level than the surrounding soil because of exudates from the plant. Colonization by *PcO6* provides several benefits to the plant, including stimulation of the plant’s defense against leaf pathogens, increasing tolerance to drought and salinity, as well as direct inhibition of the growth of fungal pathogens (Spencer et al. 2003; Kang et al. 2007; Han et al. 2006; Cho et al. 2006). Because of the ability of *PcO6* to facilitate the plant’s survival against various environmental stresses it is viewed an ideal biological control agent.

The ability of *PcO6* to hinder fungal pathogen growth is associated with the production of phenazines (Figure 2) and other secondary metabolites. *PcO6* produces three phenazines as seen the thin layer chromatographic analysis in Figure 3. They are

---

**Figure 1**: *PcO6* growing from roots of plants that were inoculated with the bacteria on the seeds.

**Figure 2**: Rich medium (LB) culture of O6 showing orange coloration due to phenazines.
phenazine-1-carboxylic acid (PCA), 2-hydroxy-PCA (2-OH-PCA) and 2-hydroxy-phenazine (2-OH-phenazine) (Radtke et al., 1994, Housley et al 2009) The main phenazine produced by Pco6 is 2-Hydroxyphenazine-1-carboxylic acid (2-OHPCA), a bright orange phenazine. Phenazines can shuttle electrons; if oxygen is the acceptor then reactive oxygen species are produced. This may impair the metabolism of the fungi (Price-Whelan et al, 2006).

Phenazine production in Pco6 is dependent on nutrient availability. When Pco6 is grown in a minimal medium (MM) no phenazines are produced (Figure

Figure 4: Phenazine production of Pco6. (a) No phenazines are produced by Pco6 or its mutants in minimal medium (MM). (b) Phenazines are produced by strains on rich Luria broth medium as shown by the stars. Pco6 wild type and mutants are shown.
4a). However, when \( Pco6 \) is grown in a rich medium, such as Luria-Broth (LB), phenazines cause the medium to become orange colored (Figure 4b as noted by stars) in the wild type strain.

Several mutations in \( Pco6 \) (Table 1) have been created to better understand the methods of control of gene expression for secondary products such as phenazines. There are two major categories of mutations: those in regulatory genes and those in biosynthetic genes.

Regulatory mutants have disruptions in genes encoding regulators of gene expression. A master regulator is GacS embedded in the cytoplasmic membrane that communicates with GacA, a transcriptional regulator. (Venturi 2006; Price-Whelan et al. 2006). A mutation in \( gacS \) in \( Pco6 \) eliminates production of phenazines (Spencer et al. 2003) and another antifungal secondary metabolite, pyrollnitrin (Kim unpublished).

Mutations in \( rpoS \) encoding an alternative sigma factor RpoS that operates during conditions of stress (Venturi 2005) also alters secondary metabolism in \( Pco6 \). Pyrollnitrin production requires RpoS in \( Pco6 \) (Kim et al. unpublished).

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**Figure 5:** Diagram of the *phz* operon illustrating the role of AHSLs in working with PhzR for gene regulation.
The biosynthetic mutants include a mutation in \textit{phzA}, the first gene in the phenazine operon. Expression from this operon requires the genes \textit{phzI} and \textit{phzR} in combination with cell signaling chemicals, termed acyl homoserine lactones (AHSLs), as shown in Figure 5. The synthesis of AHSLs is regulated by the sensor-kinase pair, encoded by \textit{gacS} and \textit{gacA} (Zhang et al. 2001). AHSLs bind to \textit{phzR} producing the protein PhzR; the PhzR protein binds to the regulator on the \textit{phz} operon to permit expression. PhzR recognizes the regulator of the \textit{phzI} gene; this signals the PhzI protein to be produced leading to the synthesis of more AHSLs. A second biosynthetic mutant used is in \textit{prnA}; this strain has mutation in the A gene of the pyrrollnitrin operon. Pyrrollnitrin is a secondary metabolite with anti-fungal activity. Production of both pyrrollnitrin and phenazines is highly regulated by the cell; thus, it is possible that there is a feedback loop regulating their accumulation. I will test whether such feedback involves AHSL cell signaling.

\textbf{Table 1: Mutants of PcO6 used in long chain acyl homoserine lactones studies.}

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{gacA} and \textit{gacS}</td>
<td>Master regulators of phenazine production; mutant lack phenazines—work as a sensor-kinase pair</td>
</tr>
<tr>
<td>\textit{rpoS}</td>
<td>Sigma factor; regulates RNA transcription through defining the binding site of RNA Polymerase</td>
</tr>
<tr>
<td>\textit{phzA}</td>
<td>Knock out of the first phenazine synthesis gene; mutants lack all phenazines</td>
</tr>
<tr>
<td>\textit{prnA}</td>
<td>Knock out of pyrrollnitrin; another anti-fungal compound</td>
</tr>
</tbody>
</table>

The AHSLs are signaling molecules that help control gene expression in Gram-negative cells such as \textit{PcO6}. AHSLs are composed of a lactone ring, and a fatty acid

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure6.png}
\caption{General structure for acyl homoserine lactone where \( n \) is chain length of the fatty acid and \( R \) can be a modification (Sy, 2007).}
\end{figure}
side chain as illustrated in Figure 6. The length of the acyl side chain denotes whether
the AHSL is long or short chained—short chains being less than or equal to six carbons;
long chain being more than or equal to eight carbons. However, most important to this
paper is a potential association between AHSLs and phenazines. Both short and long
chain AHSLs regulate phenazine production in a related microbe *Pseudomonas
aeruginosa* (Venturi 2005).

Khan et al. (2007) demonstrated that *Pseudomonas chlororaphis* strains 30-84, and
PCL1391 as well as *P. fluorescens* 2-79 produce both long and short chain AHSLs.
Their research showed that these bacterial strains produced AHSLs with side chains
ranging from four to ten carbons (Khan et al. 2007). Although it is known that *PcO6*
produces short chain AHSLs (C4 and C6), my honors work centers on determining
whether this strain also synthesizes long chain AHSLs.

Pearson et al. (1999) in their work with *Pseudomonas aeruginosa* showed that
long chain AHSLs freely diffuse into cells. However, an active efflux mechanism
appears to promote the release of long chain AHSLs from the cells. Their research
showed that at equilibrium, long chain AHSL concentration was three times higher in the
cell than in the surrounding medium (Pearson et al. 1999). Is an active transport
mechanism also involved for *PcO6*?

Work with *PcO6* shows that phenazine production is regulated by environmental
factors. For instance surfactants called Pluronics activate or reduce phenazine production
according to their structure (Housely et al. 2009). Pluronics are surfactants composed of
the hydrophobic chain polypropylene oxide (PPO) and the more hydrophilic chain
polyethylene oxide (PEO). Pluronic 25R2 increased the amount of phenazines produced
in rich and minimal media whereas P123 reduced the amount of phenazine produced in rich medium (Figure 3 above). Pluronic P123 has the structure PEO-PPO-PEO whereas 25R2 has the reverse configuration PPO-PEO-PPO. It is possible that this regulation correlates with alterations in AHSL production. Han et al. (unpublished) found 25R2 enhanced and P123 reduced short chain AHSL levels. Do these Pluronics also effect long chain AHSL production?

Based on this information I had several objectives for my honors research with \( \text{PcO6} \)

1. Does \( \text{PcO6} \) produce long chain AHSLs?
2. Does nutrition affect levels of long chain AHSLs produced?
3. Does removal of \( \text{PcO6} \) cells by filtration change the level of long chain AHSL detected?
4. Do mutations in the regulatory genes \( \text{gacS} \) and \( \text{rpoS} \) effect production of long chain AHSLs?
5. Do mutations in the biosynthetic genes \( \text{phzA} \) or \( \text{pynA} \) affect long chain AHSL formation?
6. Do Pluronics affect production of long chain AHSLs?

**Materials and Methods**

**Bacterial strains:** Bacterial strains used in this work are listed in Table 2. All strains of *Pseudomonas chlororaphis O6* were grown in Luria-Broth (LB) without sodium chloride or in minimal medium (MM) containing in 1 L: \( \text{K}_2\text{HPO}_4 \), 10.5 g; \( \text{KH}_2\text{PO}_4 \), 4.5 g; Na-citrate-\( 2\text{H}_2\text{O} \), 0.5 g; \( \text{(NH}_4)_2\text{SO}_4 \), 1.0 g; \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \), 0.25 g; and sucrose, 2.0 g, with shaking at 28° C. All strains of *Agrobacterium tumefaciens* were
grown in MM or Nutrient Agar (Difco). *Agrobacterium* stock cultures were grown at 28°C. Cells on plate medium were grown at 26 °C. When needed, antibiotics were used in the following concentrations (μg/mL): kanamycin 20 for *P. chlororaphis* O6 mutants *gacA, gacS, rpoS, phzA* and *prnA*; gentamycin 20-30 for *A. tumefaciens*.

### Table 2. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. chlororaphis</em> O6</td>
<td>Wild type</td>
<td></td>
</tr>
<tr>
<td><em>gacS</em></td>
<td><em>PcO6</em> mutant <em>kn</em>R</td>
<td></td>
</tr>
<tr>
<td><em>gacA</em></td>
<td><em>PcO6</em> mutant <em>kn</em>R</td>
<td></td>
</tr>
<tr>
<td><em>rpoS</em></td>
<td><em>PcO6</em> mutant <em>kn</em>R</td>
<td></td>
</tr>
<tr>
<td><em>phzA</em></td>
<td><em>PcO6</em> mutant <em>kn</em>R</td>
<td></td>
</tr>
<tr>
<td><em>pynA</em></td>
<td><em>PcO6</em> mutant <em>kn</em>R</td>
<td></td>
</tr>
<tr>
<td><em>A. tumefaciens</em></td>
<td>Synthesizes <em>Agrobacterium</em></td>
<td>Khan et al. 2007</td>
</tr>
<tr>
<td>NTL4(pTiC58ΔaccR)</td>
<td>AutoInducer (AAI)</td>
<td></td>
</tr>
<tr>
<td>Gentamycin</td>
<td>Does not produce long chain AHSL; negative control</td>
<td>Khan et al. 2007</td>
</tr>
<tr>
<td>NTL4(pZLR4)</td>
<td>Detector strain for identifying long chain AHSLs</td>
<td>Khan et al. 2007</td>
</tr>
</tbody>
</table>

**Long Chain AHSLs Detection Assay:** An *Agrobacterium* detector strain was used to determine the presence and levels of long chain AHSLs by *PcO6*. This strain was used according to Khan et al. (2007). This engineered detector strain senses long chain AHSLs showing their presence with the production of the enzyme, beta-galactosidase that generates a blue product by hydrolysis of X-gal.

AHSL production was assessed from *PcO6* cultures grown in 20 mL LB or MM for 24 h. Cultures were centrifuged at 10,000g for 20 minutes to pellet the cells and the supernatant was decanted off. This culture filtrate was filtered through a 0.22 μm or 0.2 μm sterile filter. The filtered product was mixed at a 1:1 ratio with ethyl acetate. A portion (10 mL) of the ethyl acetate was removed and allowed to evaporate overnight.
The ethyl acetate-extracted material was dissolved in 1 mL of water. These extracts were diluted 10-, 100- and 1000 fold before being bioassayed. Assays were performed as soon as possible because studies showed the material was not stable to freezing at -20 C.

A 20 mL overlay of MM with 1.5% agar, 1 mL of detector strain in MM, and 200 μL of X-gal (stock solution: 4 mg/mL in DMSO) was poured over a 1.5% agar-MM plate. The surface of the agar was treated with 10 μL of undiluted and a series of ten-fold diluted extracts. The plates were incubated at 22 C for 24 h to allow the samples to dry followed by further growth at 28°C.

Two positive controls were performed. The first was a stab of the engineered strain Agrobacterium NTL4(pTiC58ΔaccR) that generates large amounts of long chain AHSLs; these—with the X-gal in the overlay—provide the substrate needed for the blue halo to appear. The second control was the addition of 10 μL of culture filtrate obtained from growing Agrobacterium NTL4(pTiC58ΔaccR) in MM with mannitol producing long chain fatty acid AHSLs. This supernatant also has large amounts of long chain AHSLs present; blue coloration also occurs. These controls both assured the integrity of the overlay by forming blue halos. A stab of Agrobacterium NTL4 acted as a negative control; it does not change in the coloration

![Control Plate](image)

**Figure 7:** Bioassay controls. Supernatant of the positive control contains long chain AHSLs from A. accR. Inoculum of A. accR strain produces long chain AHSLs. Both controls react to produce blue coloration. Inoculum from A. NTL4 does not produce any long chain AHSLs; coloration of the overlay does not occur.
of the surrounding overlay (see Figure 7). The overlayed plates were examined for blue coloration and quantified by measuring the diameter of the blue halos in two different directions.

To test the effect of Pluronics on production of long chain AHSLs, cells were grown in MM or LB amended with Pluronics. In the first experiment, 5% Pluronics were added; in the second and third experiments, 10% Pluronics were added. These were based on a stock preparation of 5% Pluronic in sterile water.

Results

**PcO6 produces long chain AHSLs.** To determine whether long chain AHSLs were produced by *PcO6*, cells were added to a bioassay plate. *PcO6* produced long chain AHSLs because a blue halo formed around the growing colony on the detector plate as shown in Figure 8. This finding shows that long chain AHSLs are present in the cell and that it has diffused out into the surrounding environment.

**Long chain AHSLs are present in culture filtrate.** To confirm that long chain AHSLs are released from the cell, experiments were done with culture filtrates. Figure 9 shows that the diameter of the blue zone around the
treatment site was dose dependent. Activity was noted even with x1000 fold diluted culture filtrate.

Can the presence of cells contribute to the levels of AHSLs? The concept that an efflux pump was required for export of long chain AHSLs was explored by extraction from cultures retaining cells versus filtrates from which all cells had been removed by filtration through a 0.2 micron filter. The data shown in Figure 10 showed that 0.2 micron filtration did not reduce the amount of long chain AHSLs detected from wild type cells whether they were grown on minimal or LB medium.

**Figure 10:** The amount of long chain AHSLs is not reduced by filtration.

**Figure 11:** Equal levels of long chain AHSLs present in rich and minimal media when samples are undiluted. (A) Diameter of halo is not significantly different. (B) There is no significant difference in the halo size of *PcO6* grown in rich or minimal media for the undiluted culture filtrate.
Effect of Nutrition for wildtype *PcO6*.

Equal levels of long chain AHSLs produced on rich and minimal media for *PcO6* wildtype when samples are undiluted. Using culture filtrate prepared from cells grown in minimal medium (MM) and Luria-Broth (LB) we assayed for the effect of nutrients. Figure 11 A shows that the culture filtrates applied to the detector plates produced equal diameters of blue halos when undiluted samples were used. In Fig 10 B the diameter of the blue halos is plotted. These results show that for the x10-fold diluted sample there was a significant difference between media; the rich medium promoted more production see Table 3. This trend was observed over seven independent studies.

<table>
<thead>
<tr>
<th>Strain</th>
<th>MM:LB Ratio</th>
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<th>100</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>O6</td>
<td>0.99</td>
<td>0.77</td>
<td>0.53</td>
<td>0</td>
</tr>
<tr>
<td>gacA</td>
<td>0.45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gacS</td>
<td>0.51</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rpoS</td>
<td>0.81</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>phzA</td>
<td>0.81</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>prnA</td>
<td>0.83</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Production in the Gac mutants. Extracts from the *gacS* and *gacA* mutants

**Figure 12:** Production of long chain AHSLs in *gac* mutants A and C plate assays, B graphical representation of halo diameters from undiluted samples.
reacted positively in the bioassay although only the undiluted samples gave measurable
coloration (Figure 12 A). Extracts diluted x10 and x100 from the gac mutants produced
no blue coloration in the bioassay. The levels of production were 34-38% that of the wild
type cells (Figure 12 A, B and C). There was a trend for the level of production of the
long chain AHSLs from the gac mutants being greater in LB than in MM, although when
results from all of seven studies were averaged there was no significant difference
(Figure 12 B).

**Production in the rpoS mutant.** When tested in rich and minimal media, rpoS produced
similar levels of long chain AHSLs to the wild type, being detected in a dose-dependent
manner to x1000-fold dilution (Figure 13a b and Appendix Figure 1). The trend for less
production in MM than LB medium was observed.

**Effect of mutations in biosynthetic genes.** The biosynthetic mutants phzA and prnA
produced wild type levels of the long chain AHSLs. For both mutants there was a trend
of higher production in LB medium than in MM (Figure 14 and Appendix).

![Figure 13: Production of long chain AHSLs by the rpoS mutant (A, images of bioassay plates B diameter of blue halos ) undiluted samples.](image-url)
Figure 14: Biosynthetic mutants in \textit{phzA} and \textit{prnA} produce equal amounts of long chain AHSL as the wildtype.

Figure 15: Shows the affect of Pluronics on long chain AHSL production in \textit{PcO6} in rich and minimal media.
Effects of Pluronics on long chain AHSL formation in wild type.

Pluronic P123 did not affect levels of long chain AHSL produced in rich or minimal media when the wild type cells were grown in the presence of 5 or 10% from a stock solution prepared at 5% (Figure 15). With Pluronic 25R2 the effect was media dependent; Pluronic 25R2 increased levels in MM but reduced levels in LB (Fig 15).

Discussion

PcO6 produced long chain AHSLs and, thus, has a metabolism that is similar to the related strains *Pseudomonas chlororaphis* 30-84 and PLC1391 as well as *Pseudomonas fluorescens* 2-79 (Khan et al. 2007). This finding suggested that the long chain AHSLs have a function in PcO6 as signaling compounds.

Mutations in both *gacS* and *gacA* similarly reduced by about 60% the production of long chain AHSLs. This finding is consistent with regulation by the Gac system operating through GacA rather than other factors. In contrast, mutation in another regulatory gene *rpoS* did not affect the level of production. Thus, this differential effect distinguishes the regulons controlled by *gac* and by *rpoS*. Mutations in biosynthetic genes *phzA* and *prnA* also did not change level from that of the wild type showing that the production of the long chain AHSLs was not influenced by the presence antifungal compounds, phenazine and pyrollnitrin.

Production of the long chain AHSLs was affected by nutrients with rich medium supporting higher synthesis than a defined medium. This trend was stronger with the *gac* mutants than with the wild type, the *rpoS* and the biosynthetic mutants. The fact that considerable levels of long chain AHSLs were produced even in minimal medium was not anticipated. Bioassays of short chain AHSLs suggested that their levels were severely
reduced in the defined medium. This finding correlated with the almost complete elimination of phenazine production in the minimal medium. Also puzzling is the finding that the *gacS* mutant although it produces long chain AHSL to 40% the level of the wild type does not generate phenazines. Possibly there are other control mechanisms active. The *rpoS* gene and long chain AHSLs function in the control phenazine production in *P. aeruginosa* (Venturi 2005), however, *rpoS* mutants produce similar levels of long chain AHSLs as the wildtype for *PcO6*. An hypothesis for future testing is that long chain (>C8) AHSLs are not directly involved in the synthesis of phenazines in *PcO6*.

The results with the Pluronics also were not anticipated. The presence of Pluronic P123 did not affect long chain AHSLs level in either rich or minimal media. However, Pluronic P123 caused a decrease in phenazine production in rich medium, Luria-Broth (Housley et al. 2009) and, thus, a decrease might have been anticipated. However, the Pluronic 25R2 produced a decrease in long chain AHSLs in rich medium but an increase in minimal medium. This finding is consistent with the increase in phenazine production caused by 25R2 in minimal medium (Housley et al. 2009). These findings suggest that more work is needed to understand the role of phenolics in conditioning both long chain AHSLs and phenazines synthesis.
Literature Cited


Appendix I

Figure APPENDIX 1: Effect of dilution of extracts from culture filtrates from the *rpoS*, *phzA* and *prnA* mutants on levels of long chain AHSLs. (A) *rpoS*. (B) *phzA*. (C) *prnA*.

DATA are means with the standard deviations of 7 different studies.
Author’s Biography

Anna Hegsted was raised in Pocatello, Idaho and graduated from Highland Senior High School in 2006. She started Utah State University in the fall of 2006 as a Presidential Non-Resident Scholar and an Undergraduate Research Fellow. Anna knew she wanted to study biology and added a chemistry minor once she was began mapping out her plans. Once Anna was involved in her major, she began searching for a post-graduate degree of interest to her. She settled on pursuing a Masters of Physician Assistant Studies and considers going on to get a Ph.D in Physician Assistant Studies in order to teach.

While at Utah State, Anna worked to fulfill her requirements for a degree in Honors in University Studies with Departmental Honors. She also participated in Undergraduate Research in Dr. Frank Messina’s Insect Behavior Lab, followed by research in Dr. Anne Anderson’s lab, studying molecular level plant-microbe interactions. Anna also worked for Utah State Residence Life Program; a year and a half working as the Peer Mentor of the Pre-Health Professional Theme, a summer as a summer resident assistant and a year and a half as the resident assistant over Moen Hall.