Analysis of the *hex* regulon in *Pseudomonas aeruginosa*

by
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June, 2012

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Pseudomonas aeruginosa is an opportunistic pathogen whose defense mechanisms include biofilm production through carbohydrate metabolism. The activity of the enzymes in the *hex* regulon are required for catabolism of glucose and these activities are coordinately regulated. Previous work has shown that these enzymes are induced when grown on carbohydrates and catabolite repressed when grown on TCA cycle intermediates. The HexR protein is a negative transcriptional repressor and the Crc protein participates in regulation of these genes and has been shown to be necessary for biofilm formation. The main objective of this study was to analyze control of these genes in different growth mediums and determine if Crc is a transcriptional protein or a post-transcriptional controller of the regulon. The transcriptional start sites for *zwf* and *glk* were mapped by primer extension assays. Comparisons of *glk* between wild type and a *hexR* mutant revealed the possibility of transcription without HexR control. Analysis of *zwf* mRNA showed that Crc affects mRNA stability and is acting at a post-transcriptional level.

Analysis of the *hex* regulon in *Pseudomonas aeruginosa*

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by
Melinda Williams
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INTRODUCTION

Pseudomonas aeruginosa is an important pathogen that is causing great concern in the medical field due to its ability to cause chronic and lethal infections. Its natural antibiotic resistance and ability to produce biofilm barriers make it a prominent organism without effective treatments for eradication. The versatility of this bacterium and especially the metabolic processes that allow for its defenses make it a prime subject for researchers in the microbioloy field. In addition to the infections and hospital pneumonias, this bacteria is responsible for a large number of deaths in cystic fibrosis (CF) patients. This work will add more depth into the research of genes involved in metabolic pathways

P. aeruginosa is proposed to be the most abundant organism on earth. This rod-shaped ubiquitous bacterium is found in soil, water, plants and animals and has the remarkable ability to propagate in conditions too extreme for other common bacteria (Costerton 1980). While most bacteria utilize a particular set of carbon sources and grow within a narrow range of temperatures, *P. aeruginosa* grows between 10ºC and 42ºC on more than 80 different compounds and is able to live on metals, plastics and in distilled water (Montie 1998).

Pseudomonas is phenotypically similar to gram negative enteric bacteria and like most enteric bacteria, it is a non-sporulating facultative anaerobe that uses flagella for mobility (Todar 2012). One important difference is that all enteric bacteria are capable

of fermenting glucose to acid end products but pseudomonas does not ferment glucose (Pitt 2006).

Most pseudomonads do not infect humans but *P. aeruginosa* is the most frequently isolated strain in hospitals. This pathogen is a frequent cause of nosocomial infections, such as pneumonias and UTIs (Bitsori 2012). Although controllable in healthy individuals, it is highly virulent in immunocompromised patients such as burn victims, transplant recipients, chemotherapy and AIDS patients, and people with cystic fibrosis. Even after antibiotic treatment, life threatening infections still arise from the previously secreted but still active endotoxins and exotoxins (Costerton 1999).

This high level of virulence is mainly attributed to its innate resistance to many antibiotics, including penicillin, along with its abilities to change components of the host's immune system (Lambert 2002). This bacterium secretes enzymes and compounds such as quinolones and cyanide that degrade cell membranes, organ tissue and immune cells (Gallagher 2011). *P. aeruginosa* also has type IV pili that allow attachment to its host's surfaces and epithelial cells (Lederberg 2000).

CF is a genetic disorder but it is estimated that between 70% and 80% of CF related deaths are attributed to this bacterium. The mucosal environment created by the CF-induced electrolyte imbalance is an ideal haven for *P. aeruginosa*, which enters through the respiratory tract and uses its flagellum to enter the anaerobic area, causing chronic lung infections (Govan et al 1996). It produces a highly elastic alginate that serves as a

barrier against antibiotics and host immune cells (Henry 1992). Through quorum sensing, the cells are able to recognize antibiotics and immune activity and act to maintain their own biofilm barrier within the host (Dong et al 2001) Although not all *P.aeruginosa* infections are mucosal, CF patients acquire infections with this characteristic mucoid presence (Costerton 1999).

Due to its ability to thrive in nutrient depleted conditions and its unique metabolic capabilities, *P. aeruginosa* is used in several industrial settings. The ability to degrade polycyclic aromatic hydrocarbons allows *P. aeruginosa* to be used for alleviating spills and pollution (Botzenhardt 1993). While most plants do not survive contact with *P. aeruginosa*, some strains support growth, thereby making it a candidate for the inactivation of pesticides (Botzenhardt 1993). Despite its beneficial use in these applications, this bacterium is still a highly pathogenic organism to immunocompromised people and efforts have increased in the past decade to identify an effective treatment. In January of 2012, Novartis Vaccines and Diagnostics released the OprF/1 fusion-generated IC43 vaccine into phase II/III efficacy clinical trials (Intercell 2012).

The Embden-Meyerhof and Entner-Doudoroff pathways for glucose utilization are important to researchers as most of the virulence factors can be attributed to one of these areas (Figure 1). This bacterium is able to undergo catabolite repression in order to survive in minimal growth conditions and in contrast to other common bacteria that prefer glucose, *P. aeruginosa* prefers acetate or tricarboxylic acid cycle (TCA cycle)

intermediates over glucose and other carbohydrates. This catabolite repression system is also independent of the classic *E. coli* cyclic AMP (cAMP) regulation (Macgregor et al 1992). An important group of enzymes for the catabolism of glucose is the *hex* regulon. Although much is known about glucose metabolism and the genes involved in alginate synthesis, little is known about each mRNA and the regulatory proteins involved in catabolite repression. It is important for each of these genes to be analyzed in order to fully understand the regulation of processes involved in glucose metabolism. Identifying the mechanisms and levels of regulation by local proteins will also be important to understanding virulence and identifying future drug targets.

This study will focus on the genes that comprise the *hex* regulon (Figure 2). The first objective is to identify transcriptional start sites for *zwf* and *glk*. The second objective is to analyze catabolite repression and determine if the Catabolite Repression Control protein (Crc) is acting transcriptionally or post-transcriptionally. A first step will be to look at steady state levels of *zwf* mRNA to make an accurate assessment of the effects of Crc on mRNA levels. After looking at steady state levels of *zwf* mRNA, the next step will be to look at *zwf* mRNA with a *crc* mutant and *crc* complemented mutant. These data will provide more insight into the level of control of Crc on the *hex* regulon and show that the control by Crc is post-transcriptional. This information along with the operon characterization will give a more complete picture of the *hex* regulon and its regulatory proteins.

Carbohydrate Metabolism in *P. aeruginosa*

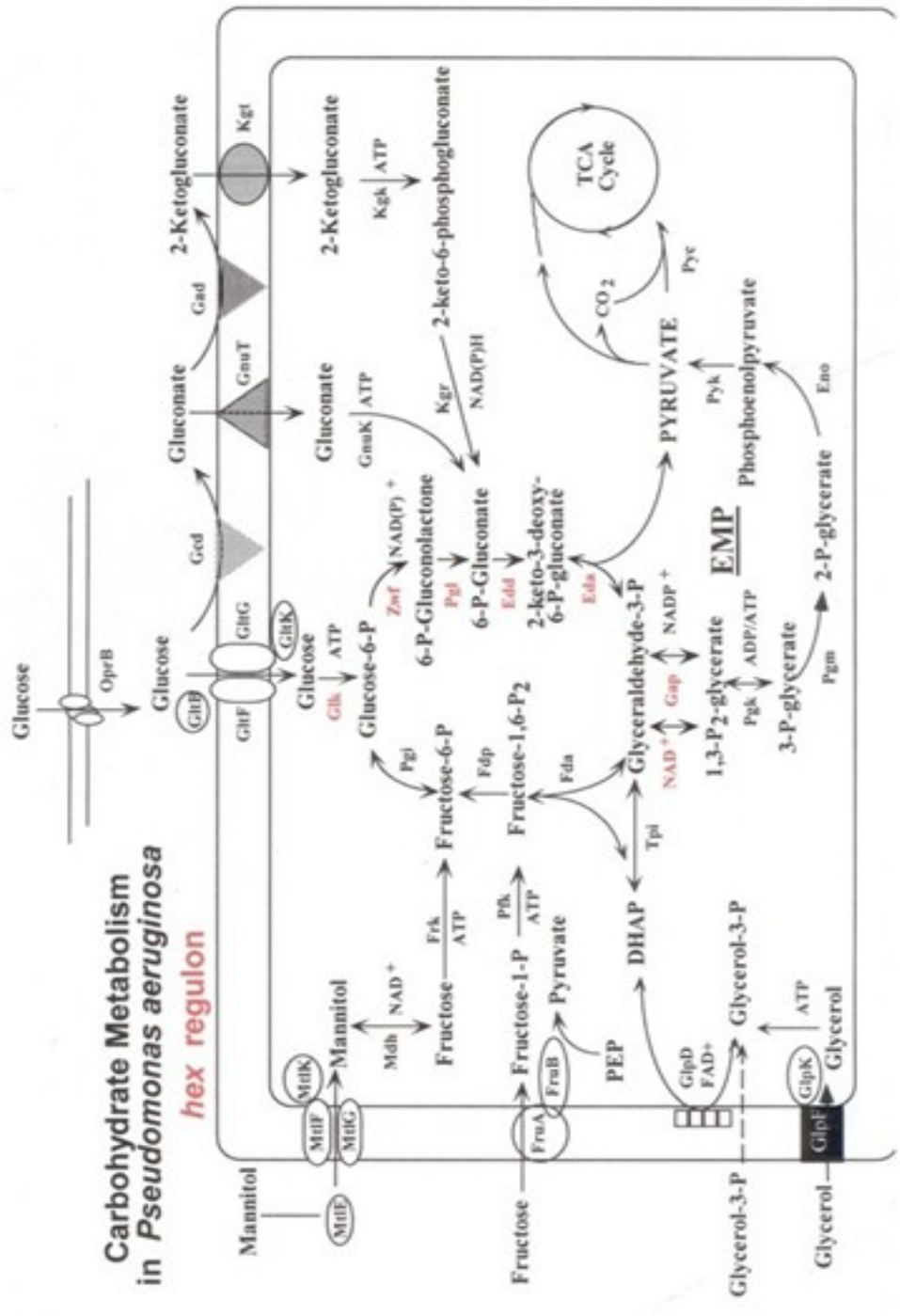

Figure 1. Carbohydrate metabolism (Hager et al, 2000)

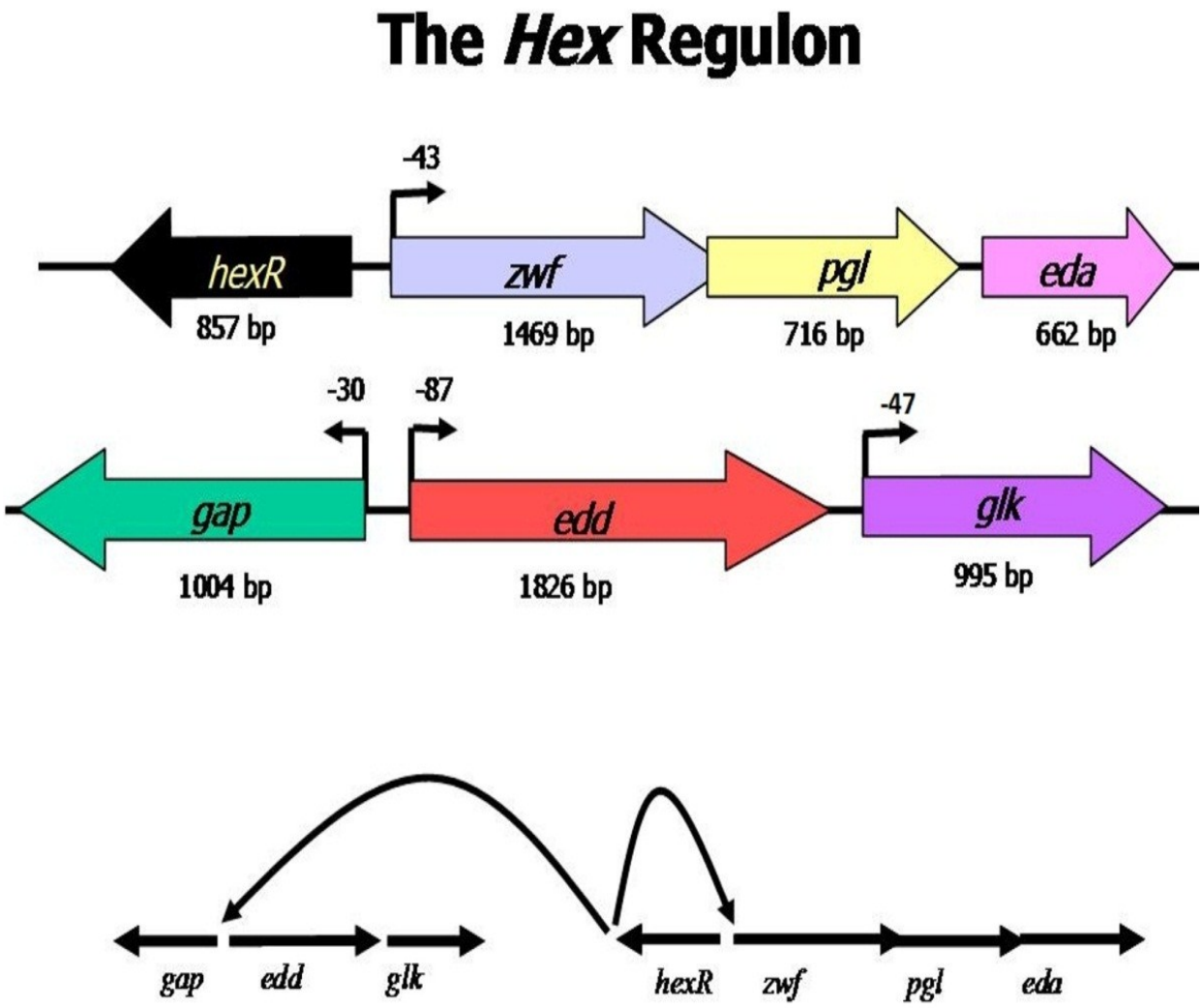

Figure 2. The *hex* regulon with start sites of transcription (top) and known HexR binding areas (bottom)

LITERATURE REVIEW

Pseudomonas aeruginosa was first characterized in the late 1800s and continues to be a widely studied organism. Its unique preferences for TCA cycle acids and its pathways of carbohydrate metabolism are focus areas for researchers; one reason being that carbon source has a direct impact on biofilm formation. One tremendous contribution to research was the sequencing of the entire genome and identification of 5570 predicted genes on a 6.3Mbp chromosome of PAO1 in 2000 (Stover 2000). Characterizations of its pathways for glucose utilization revealed the use of the central Entner-Doudoroff pathway, the absence of a complete Embden-Meyerhof pathway, and the ability to produce alginate through the precursor, fructose-6-phosphate (Ramsey 2005). A mutation in the protein responsible for catabolite repression control resulted in the loss of biofilm formation. This all shows that carbohydrate metabolism is an important system for the bacteria's virulence (Govan 1996).

This bacterium is able to undergo catabolite repression and prefers acetate or TCA cycle intermediates over glucose and other carbohydrates (Collier 1996). This repression occurs independently of cAMP as addition or elimination of cAMP does not significantly affect enzyme levels when subjected to conditions optimal for catabolite repression (Rojo 2010). This is very different from the system used by *E.coli* for catabolite repression whereby Catabolite Activator Protein (CAP) binds cAMP and allows RNA polymerase to bind. The presence of glucose causes cAMP levels to

decrease, reducing the binding to CAP, and therefore repressing transcription (Siegel et al 1977, MacGregor et al 1992)

In *P. aeruginosa*, glucose can be utilized via a direct oxidative pathway or glucose can be utilized anaerobically using nitrate as a terminal electron acceptor (Hunt et al 1981, Hunt et al 1983). Carbohydrates are then metabolized through a central cycle that includes the Entner-Doudoroff pathway (Figure 1). Unlike *E. coli*, *P. aeruginosa* lacks 6-phosphofructokinase, a key enzyme in the Embdem-Meyerhoff pathway, disabling it from using this pathway for glucose utilization (Tiwari et al 1969).

The *hex* regulon is a group of 6 genes that catabolize intracellular glucose: glucose-6-phosphate dehydrogenase (*zwf*), 6-phosphogluconolactonase (*pgl*), 2-keto-3-deoxy-6-phosphogluconate aldolase (*eda*), glyceraldehyde-3-phosphate dehydrogenase (*gap*), 6-phosphoglyconate dehydratase (*edd*), and glucokinase (*glk*) (Figure 2). These genes are coordinately regulated (Sage 1996) and map to 39 minutes on the PAO1 chromosome (Roehl et al 1983). They are transcribed from at least two operons, due to their locations on two separate chromosomal loci; the first consisting of *gap*, *edd*, and *glk*, and the second consisting of *zwf*, *pgl*, and *eda* (Cuskey et al 1985).

This regulon is sensitive to catabolite repression control when TCA cycle intermediates are added to growth media (Ma et al 1988). Addition of succinate to cells already

growing in glucose causes the cells to preferentially start using succinate as the primary carbon source and causes suppression of the enzymes of the *hex* regulon.

The transcriptional start sites of *edd* and *gap* were mapped by primer extension assays and S1 nuclease assays and the promoters are located 9 bp apart within the divergent intergenic region (Temple et al 1994). The proposed *zwf-pgl-eda* operon is divergently transcribed from *hexr* (Proctor, 1998). Since the 5' end of *pgl* overlaps the 3' region of *zwf*, it is likely that *pgl* does not have its own promoter and it is also likely that *eda* does not have its own promoter because only 9 bp separate *eda* and *pgl*. There are 187 bp between the 3' end of *hexR* and the 5' end of *zwf* and two binding sites for the HexR dimer are located in this region (Proctor 1998). The *zwf* gene is 1469 bp and known to be controlled by Crc and subject to catabolite repression. It encodes the enzyme glucose-6-P-dehydrogenase which catalyzes the conversion of glucose-6-P to 6-P-gluconolactone. The *glk* gene encodes the enzyme glucokinase, which is responsible for the conversion of glucose to glucose-6-P. It is transcribed 97 bp downstream of *edd* and *gap* is transcribed divergently from *edd* (Figure 2). Previous assays indicate the possibility of a weak promoter for *glk* (Cuskey 1985).

The *hex* regulon was cloned from a *Pseudomonas aeruginosa* library cosmid pPZ201. The promoter for the divergently expressed *edd* and *gap* genes was subcloned and found to cause a two to nine fold increase in the activities of all members of the *hex* regulon; a phenotype referred to as the hexC effect (Temple et al 1994). This phenotype suggested that hexC, on a multicopy plasmid, is titrating a putative

repressor away from its normal regulatory sites within the *hex* regulon. A hexC DNA-binding activity was further characterized by Proctor (Proctor 1998). He identified this hexC DNA-binding activity as HexR by showing that the binding activity is abrogated by 2-keto-3-deoxy-6-phosphogluconate (KDPG), the product of *edd*. He subsequently cloned and expressed the *hexR* gene in *E. coli* and showed that the purifed protein protects DNA upstream of *zwf* and within hexC from DNase I digestion. DNase footprint assays showed HexR binding sites upstream of *zwf, gap* and *edd.* Finally he inactivated HexR and showed that HexR mutants are constitutive for the expression of the enzymes of the *hex* regulon. Addition of DNA containing the HexR binding locus resulted in increased *hex* enzyme activities (Proctor 1998). Therefore, HexR is a negative repressor of the *hex* regulon genes.

The significance of the *hex* regulon in carbohydrate metabolism combined with the identification of such a strong repressor leads to the question of how this protein fits in with the function of Crc. One study confirmed that HexR activity is not affected by Crc (Temple et al 1994). The *crc* gene produces a 30 kda protein and is responsible for catabolite repression in *P. aeruginosa* (Macgregor 1996). It plays a regulatory function and is required for repression of carbohydrate metabolism in the presence of TCA cycle intermediates. The *crc* gene was mapped to 11 minutes on the PAO1 chromosome and its protein amino acid sequence shows high homology to a family of apurinic / apyrimidimic nucleases found in *E.coli* and other bacterial species (Macgregor et al 1996). Studies have shown Crc is negative for any DNA repair activity associated with the endonucleases or exonucleases (Macgregor et al 1996). Previous studies also

show that Crc does not bind any region of DNA upstream of *zwf*, *gap* or *edd*, eliminating the possibility that it regulates the *hex* regulon by directly binding DNA (Collier et al 1996).

Galli used a *crc* mutant to show that *crc* is essential to catabolite repression. The addition of glucose to cells already growing on succinate caused an induction of the *hex* genes and synthesis resumed when the succinate was depleted from the media. This is in contrast to the wild type that lacks gene synthesis in the presence of succinate (Galli et al 1992).

MacGregor identified and cloned the *crc* gene product from *P. aeruginosa* and later purified and sequenced *crc*. This same group verified the lack of DNA binding activity through gel shift assays and the nonexistence of exonuclease or endonuclease activity. The PAO8020 *crc* mutant was constructed along with a complementing plasmid pPZ354. This pPZ354 restored wild type activity of *zwf* under repressive conditions, further showing that *crc* is a player in catabolite repression (MacGregor et al 1991, Macgregor et al 1996). The PAO8020 *crc* mutant along with another mutant, PAO8007, were both used to further analyze *crc* mediated catabolite repression and the plasmid pPZ448 also restored wild type activity with both of the *crc* mutants (Collier et al 96).

The Pseudomonas species *P. putida* has been widely researched along with *P. aeruginosa*. The HexR repressor protein was identified in *P. putida* along with two

binding sites for the dimer in a similar area upstream of *zwf* (Daddaoua 2009). Thus we can also look at Crc results from this strain to gain an understanding of its role in *P. aeruginosa*. Hester showed that Crc does affect the levels of mRNA in *P.putida* but this is not a function of the mRNA half life and is not a result of transcriptional control (Hester et al 2000).

In the early 2000s, small RNAs were first being described in the literature as actual controllers of gene expression, adding depth to the traditional gene-to-protein story (Couzin-Frankel 2004). Since the completion of this research, a group of researchers published that Crc is indeed operating at a post-transcriptional level. They identified an sRNA molecule, *crcZ*, that is controlled by the upstream CbrA/CbrB system. This sRNA binds the Crc protein, inhibits Crc from binding to its target mRNA and ultimately represses translation. When culturing cells in glucose or succinate, it is the concentration of *crcZ* that changes in response to varying conditions, and this entire signal transduction pathway allows for a gradual mode of catabolite repression (Sonnleitner 2009). The further characterization of *crc*Z by Moreno and Wei-Li indicates that *crc*Z transcription is initiated by CbrAB, a member of the NtrBC that participates in nitrogen control and carbon utilization (Moreno et al 2009, Wei-Li et al 2007).

Although the mechanism for Crc control is now attributed to post-transcriptional sRNA binding, it is still important to acknowledge the primer extension assay results because they show that Crc repression does directly affect *zwf*, and the results indicate that this control is post-transcriptional, as shown by the Sonnleitner et al. Sonnleitner et al

determined that global regulation is post-transcriptional but did not look at any genes in the *hex* regulon. This investigation of the *zwf* promoter and with a Crc mutant further validates the findings of that group.

It is now more apparent that Crc plays a crucial role in the virulence and antibiotic resistance of pseudomonads because a decrease in antibiotic resistance and virulence is seen after the inactivation of Crc protein (Linares et al 2010). The FRD1 strain isolated from a CF patient exerts no catabolite repression on *zwf* and this lack of repression is not a consequence of Crc inactivity. This *zwf* mutated strain produced 90% less alginate than wild type *P. aeruginosa* (Silo-Suh 2005).

The results in this thesis will give more transcriptional details about the *hex* regulon and provide more supporting data for how this regulon is controlled by Crc.

MATERIALS AND METHODS

Strains and growth conditions

Strains and plasmids are listed in Table 1. PAO1 are wild type *P. aeruginosa* cells. PAO8020 is a *crc* mutant created by 3' end truncation. Cells were grown in a rotary incubator with aeration at 37ºC in basal salts medium (BSM). BSM contains 50 mM KPi pH7 and 0.002 w/v $(NH_4)_2SO_4$. After autoclaving for 20 min, 1% volume of 0.1 M $MgCl_2$, 0.2% 1 mM $FeSO_4$, and appropriate carbon sources or antibiotics are added to the BSM. After overnight growth, the stationary phase cells were diluted to a Klett value of 20 in fresh media and allowed to reach mid log phase at a Klett of 130, which corresponds to approximately 2.5 generations. Final concentrations for carbon sources were 20 mM glucose, 40 mM lactate, and 30 mM succinate. Rifampicin was added to a final concentration of 50 ug/mL to end RNA synthesis. For strains with plasmids, 100 ug/ml carbenicillin or 50 ug/ml gentamycin was added to the media.

RNA isolations for primer extensions

PAO1 cells were grown in BSM containing 40 mM lactate and 20 mM glucose to induce activity. After reaching a growth Klett of 130, 2 mls of cells were removed to chilled 14 ml eppendorf tubes. Cells were centrifuged at 4ºC for min at 6000 rpm in a table top centrifuge. RNA was isolated from whole cell extracts using the RNeasy Midi kit from Qiagen. This kit uses guanidine hydrochloride to disrupt cell walls. RNA is eluted off the column with water then formamide is added to a 50:50 ratio with water to prevent degradation. RNA solutions were stored at -70ºC. RNA was quantitated by measuring

a 1:20 dilution of total RNA in a quartz cuvette at a wavelength of 260 nm in a Beckman spectrophotometer.

Conditions for strain comparisons

PAO1 and PAO8020 cells were grown in BSM containing either 40 mM lactate alone, 40 mM lactate with 20 mM glucose, or 40 mM lactate with 20 mM glucose and 30 mM succinate. At a growth Klett of 130, 100 ul of cells were removed to chilled microcentrifuge tubes. Cells were centrifuged at 4ºC for 2 min at 14000 rpm in a microcentrifuge and cell pellets were stored at -70ºC for later use. Total RNA was isolated using the hot-phenol method. Pellets were dissolved in AE-SDS buffer containing 20 mM NaAc, 0.5% SDS, and 1mM EDTA at pH 5. Phenol, equilibrated overnight with AE buffer, 20 mM NaAc and 1 mM EDTA at pH 5, was added at 65˚C and tubes were shaken for 10 min at 65ºC. After microcentrifuging for 10 min at 4˚C and 14000 rpm, the aqueous upper phase was removed and the phenol extraction was performed twice more. The aqueous solution was precipitated twice with 95% Ethanol and RNA pellets were resuspended in 50% ddH2O / 50 % formamide and stored at -70ºC. RNA was quantitated by measuring a 1:20 dilution of total RNA in a quartz cuvette at a wavelength of 260 nm in a Beckman spectrophotometer.

Collection of time points

Three flasks each of PAO1 and PAO8020 cells were grown in BSM containing 40 mM lactate and 20 mM glucose. At a Klett of 130, for both strains, 50 ug/ml rifampicin was added to one flask, succinate to 30 mM to a second flask, 50 ug/ml rifampicin and 30

mM succinate in a third. A fixed amount of cells corresponding to 13000 Klett units (Klett x volume ml = 13000 units) were removed at various time points over time. These cells were transferred to chilled microcentrifuge tubes, microcentrifuged for 2 min, and pellets stored at -70ºC until RNA extractions were performed using the hot-phenol method.

Primer extension assays

For *zwf,* the 16 bp pH64 primer used to identify the start site extends 11 bp into the 5' end of *zwf* and 7 bp upstream of the coding region. To create more copies of *glk* and facilitate the identification of the possible weak promoter, a plasmid containing *gap*, *edd*, and *glk* was electroporated into PAO1 and PAO8026 before extracting total RNA. For the *glk* primer extension assay, the bp pH primer used to identify the start site extended bp into the 5' end of *glk* and bp upstream of the coding region.

The oligo nucleotide was kinased in a 40 ul reaction volume containing 8 ul 5X T4 polynucleotide forward reaction buffer, 1 ul T4 polynucleotide kinase, 24 ul water, 2 ul of 25 uM primer in water, and 5 ul of 3.3 uM, 3000 Ci/mmol, 10 uCi/uL γ ^{32}P·ATP. After incubating at 37˚C for 45 min, and 70˚C for 10 min in heat blocks, a 2 ul sample was diluted 1:50 and 10 ul was used to perform a TCA precipitation. One ml of TCA was added to the dilution along with 10 ul of 10 ug/ul tRNA for a co-precipitant. The mixture was placed onto a Whatman filter that had been rinsed with TCA. After vacuuming the sample onto the filter, the filter was washed with EtOH and placed into a scintillation vial. The filter and 5 ul of the original diluted material were placed in separate scintillation vials and counted on the ^{32}P program to determine total number of

radioactive counts. Total incorporation of ATP was calculated by dividing TCA counts by total reaction counts and adjusting for dilutions. Percent incorporation was always around 60%. Specific activity of the primer was determined by dividing number of cpms (counts per min) incorporated by pmol of primer to obtain a consistent activity of approximately $3x10^4$ cpm/pmol. The final kinase reaction (now 38 ul) was precipitated in 95% EtOH for at least 30 min at -70ºC and microcentrifuged at 14k rpm for 15 min at 4˚C. The pellets were rinsed with 70% EtOH in water and microcentrifuged for 5 min at 4˚C and 14k rpm. The air dried pellet was resuspended in 200 ul of 100 mM TE buffer (50 mM Tris-HCl, pH 8.0 1 mM EDTA). A 2 ul sample was removed for a final count by the scintillation counter and cpm/ul was determined. Ten ug of total RNA was combined with $5x10^5$ cpm of end-labeled primer in hybridization buffer and annealed overnight at 42ºC. Reactions were EtOH precipitated as described above and pellets were resuspended in 20 uL of a primer extension mixture containing 1000 units of either Superscript II or Superscript III reverse transcriptase. Extension reactions were incubated in a water bath for one hour at 50ºC then one hour at 55ºC. Loading dye from the T7 sequenase kit was added to a 30% volume and final product was heated to 75ºC to denature. Tubes were immediately chilled and loaded onto a 6% denaturing sequencing gel consisting of 7 M urea and 40% 19:1 acrylamide:bis–acrylamide. For a 100 mL gel, 75 ul of TEMED and 750 ul of 10% ammonium persulfate were used as solidifying agents. The T7 Sequenase DNA sequencing kit from US Biochemical was used to generate a sequencing ladder using a plasmid containing the target gene, end-labeled primer, cold dATP, reagents supplied by kit and its sequence ladder protocol. This kit is based on the dideoxy chain termination method. Extension reactions were

electrophoresed beside the sequencing ladder in a large sequencing apparatus connected to an electrical unit at 75 watts for one hour in 0.5X TBE buffer (10X dilution of solution containing 108 g Tris base, 55 g boric acid, 20 ml 0.5M EDTA, water to 1L). The gel was transferred to a sheet of Whatman filter paper, covered with Saran wrap, and dried for 1.5 hours at 80˚C under vacuum in a Biorad gel dryer. Gels were placed in autoradiography cassettes with Kodak film or placed in a phosphorimaging cassette with white screen overnight at room temperature. Films were developed in a Minolta Kamika SRX-101A film processor and phosphorimaging screens were scanned into the phosphorimager manufactured by Molecular Dynamics, Inc.

Quantitation

Gel images from the phosphorimager were analyzed using the ImageQuant software by Molecular Dynamics, Inc. The areas for analysis were selected and background was determined for each lane on the gel separately. The program integrates volume by measuring the amount of pixels in each selected area. The time zero for each time point experiment was correlated to 100% mRNA expression and each time point value after was recorded as percent of the original material. In straight comparison studies not performed over time, wild type induced activity was recorded as 100% expression and each value for other media or strains was recorded as percent of wild type induced expression.

RESULTS

The *zwf* transcriptional start site is located within the HexR binding domain

Before looking at the effects of Crc on the *zwf* promoter, the exact start site for transcription was mapped for *zwf* and the autoradiograph showing the primer extension product is shown in Figure 3. The position of the gel product corresponded to a guanine nucleotide located 43 bps upstream of the *zwf* ATG coding sequence and this start site is located between the two HexR binding sites.

The *glk* gene may have its own weak promoter

The product band for the *glk* transcriptional start site corresponds to a thymine base located at 47 bases upstream of the *glk* coding sequence and a scan of the autoradiogram is shown in Figure 4.

In the primer extension assay, HexR mutant PAO8026 was compared to wild-type under induced and non-induced conditions. In PAO1, the signal was higher from mRNA extracted from cells growing in glucose compared to lactate. For PAO8026, the signal was greater for cells grown in lactate than glucose.

To determine whether *glk* is also transcribed from the *edd* promoter, S1 nuclease assays were performed on the region between *edd* and *glk*. The plasmid was not electroporated into the cells for this assay. This method showed a single transcript containing both genes, but did not reveal a transcript for *glk* alone.

Effects of carbon source on *zwf* mRNA levels

Quantitation of primer extension products revealed that PAO8020 cells have a 35% increase in *zwf* levels relative to PAO1 when both are grown in media containing glucose. After growth in succinate, PAO8020 cells had a seven fold higher amount of *zwf* expression than PAO1. Comparisons between wild type and *crc* mutants show that growth in succinate does result in lower amounts of *zwf* mRNA in wild type cells (Figure 5).

Effects of succinate on *zwf* mRNA decay

Rifampicin inhibits further transcription and is used to analyze mRNA decay. The addition of succinate and rifampicin to wild type cells resulted in a longer mRNA half life than cells with rifampicin alone. The half life increased from 7 min in rifampicin alone to 12 min in rifampicin with succinate (Figure 6). This effect was not seen with the Crc mutant. In the Crc mutant, the plots of rifampicin alone are very similar to rifampicin with succinate; both half lives are around 25 sec (Figure 7). Crc is responsible for an increased half life with succinate.

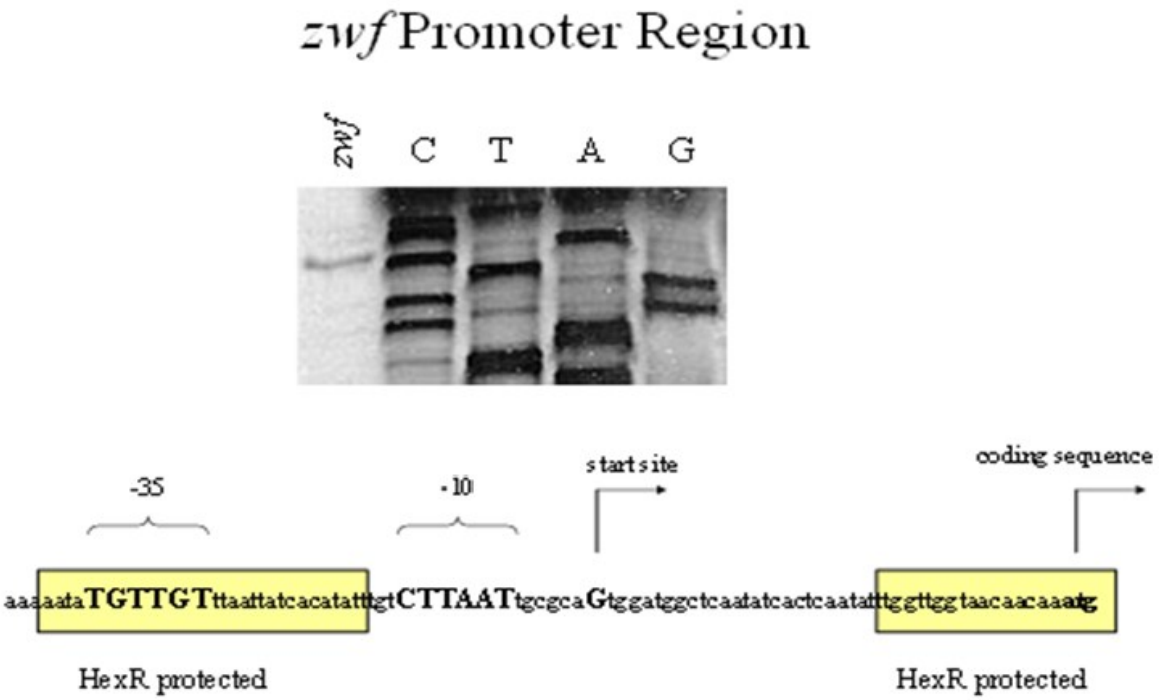

Figure 3. Identification of the *zwf* transcription start site in PAO1

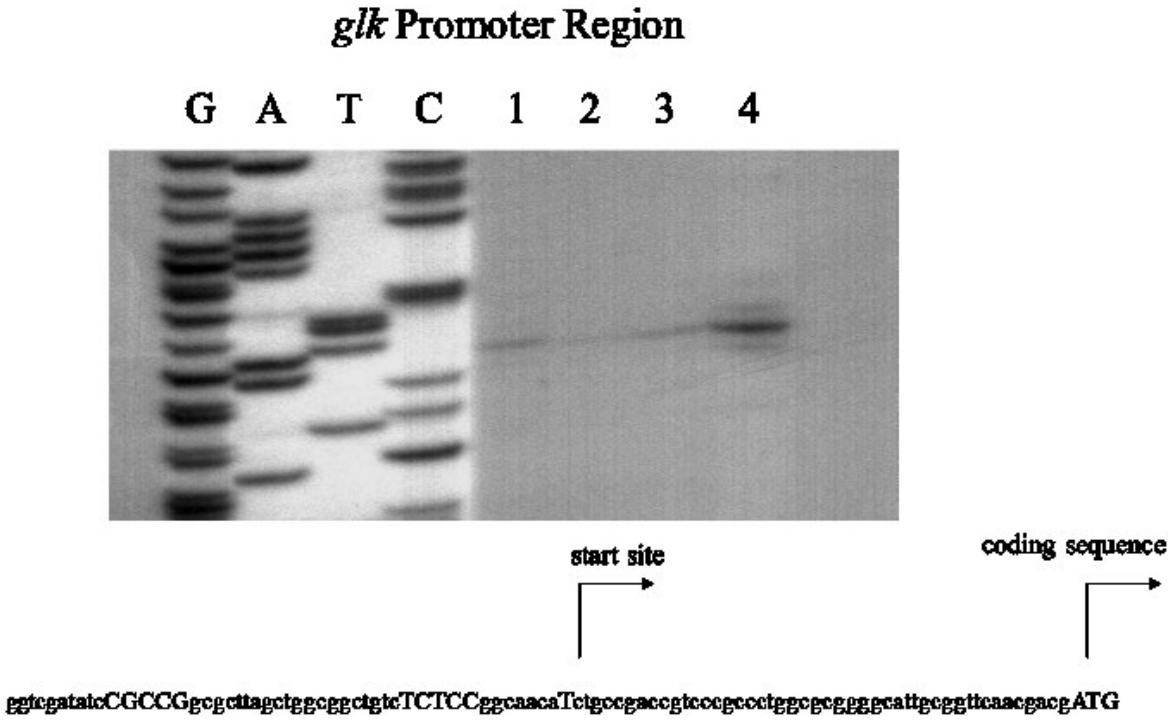

Figure 4. Identification of the *glk* transcription start site in PAO1

Lane 1 – Wild type induced, Lane 2 – Wild type not induced,

Lane 3 – HexR- induced, Lane 4 – HexR- not induced

.Effects of carbon source on *zwf* mRNA levels

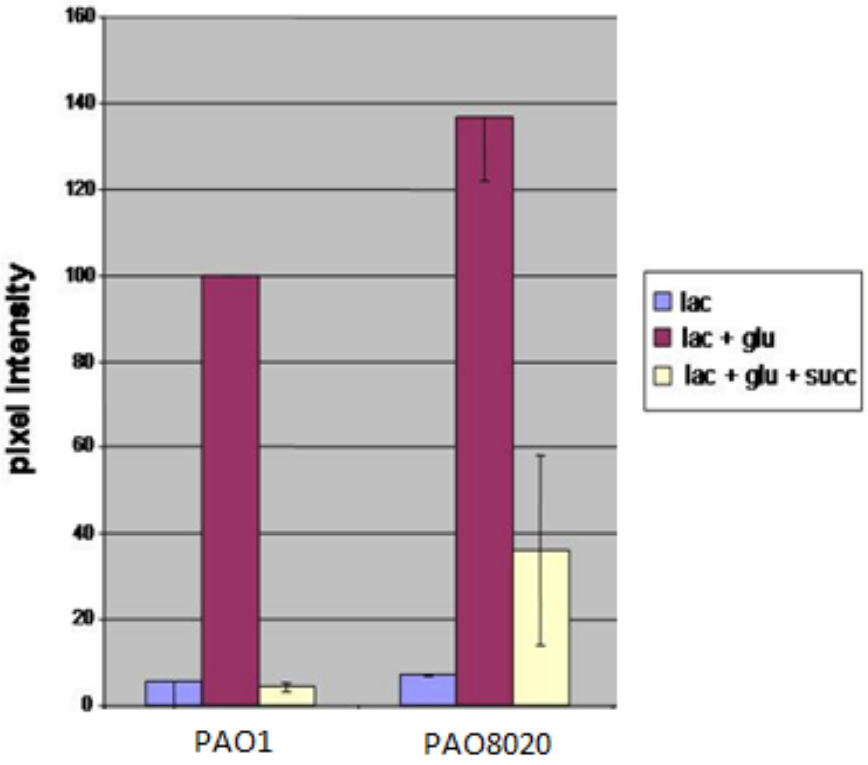

Figure 5. PAO1 and PAO8020 were each cultured in BSM with either 40 mM lactate, 40 mM lactate with 20 mM glucose, or 40 mM lactate with 20 mm glucose and 30 mM succinate. RNA was isolated and primer extensions were performed to target *zwf* mRNA. Bar graph shows levels of *zwf* mRNA for each strain in each growth condition. Data points are an average of multiple data sets.

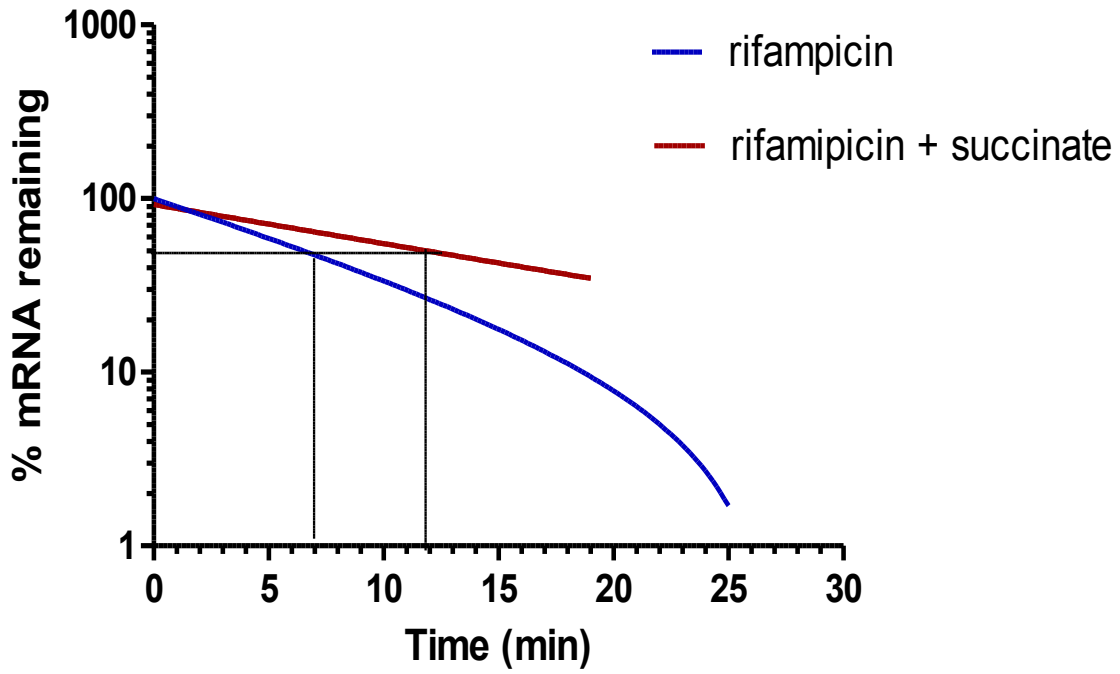

Figure 6. Wild type PAO1 was cultured with 20 mM glucose. Rifampicin was added alone or in combination with 30 mM succinate and aliquots removed over time for primer extension assays and quantitation.

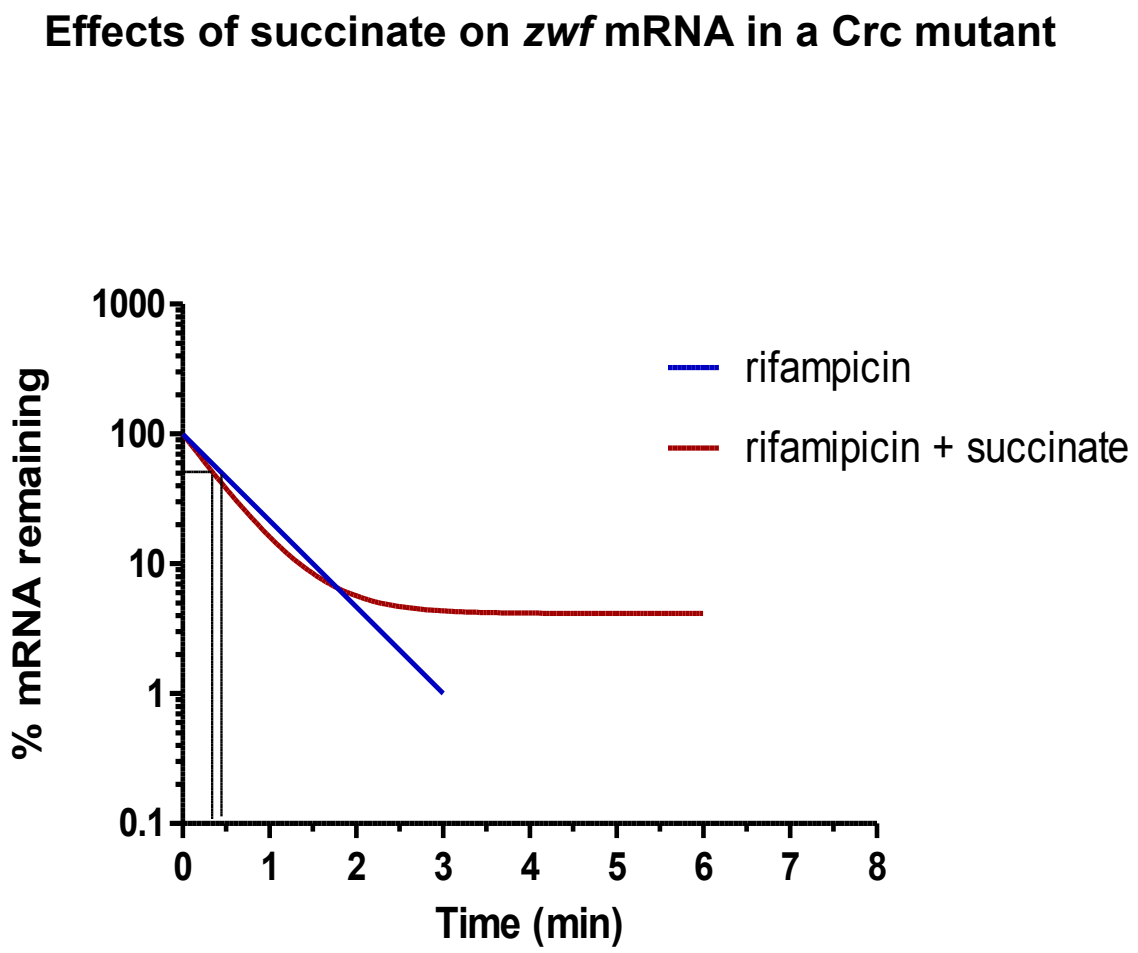

Figure 7. The PAO8020 Crc mutant was cultured in 20 mM glucose. Rifampicin was added alone or in combination with 30mM succinate and aliquots removed over time for primer extension assays and quantitation.

Strains, Plasmids, Primers

		Features	Reference
Strains			
P. aeruginosa			
PAO1		wild type	Holloway 1969
PAO8026		Δ*hexR::aac1*, Gm^r	Proctor 1998
PAO8020		Δ*crc*::Tc^r	Macgregor 1996
Plasmids			
pPZ121		*edd+, glk+*	This study
Primers			
glk	PH68	5'-AATGGTGCCGCCGATA-3'	This study
zwf	PH64	5'-ATCAGGCATTTGTTGTTACC-3'	This study

Figure 8. Strains, plasmids and primers used in this study

DISCUSSION

Identification of the *zwf* transcription start site

The *zwf* gene has been used for many years to look at the effects of carbon source on the *hex* regulon and the mechanisms of catabolite repression. This promoter region is widely used to look at how HexR and Crc affect the genes that are involved in carbohydrate metabolism. It has already been determined that HexR binds upstream of *zwf*. However, the start site for transcription has not been published to date. The promoter identified in this study contains a -10 and -35 sequence that is 90% identical to the consensus sequence for most pseudomonas rpoD promoters. The starting base is centrally located between the two regions of sequence identified by DNase footprinting as a HexR binding domain (Proctor 1998). The upstream region of *zwf* also contains a Catabolite Activity motif (CA motif). The CA motif is the common sequence AACAACAA that overlaps the Shine-Dalgarno sequence. This sequence is identical in *zwf* and now known to be an important interaction site for Crc. These data suggest that the regulaton by Crc is acting directly on zwf, but the next question is whether it is transcriptional or post-transcriptional. This region between zwf and the divergently transcribed *hexr* is an important area for the analysis of the entire *hex* regulon.

Identification of the *glk* transcription start site

The glk gene lies downstream of *edd*. Previous studies mapped a start site for *edd*, which is located upstream of *glk*, but the transcriptional start site for *glk* is not published yet. DNase footprint assays showed a HexR binding region upstream of *edd* but there

is also no analysis of *glk* activity in relation to the HexR protein or catabolite repression. Does it have its own promoter? Is it transcribed through the *edd* promoter? Or is it transcribed alone as well as through the *edd* promoter? If this promoter exists, the question is whether HexR control exists to repress transcription. Promoter prediction software gives a predicted start site of -41 with a 0.88 confidence level (Fruitfly.org). The center of a predicted HexR binding site is located at -54 (Proctor 1998). A promoter was identified in this study for *glk* alone with primer extension assays but the start site does not have the -10 or -35 sequences common to the other promoters identified within the *hex* regulon. This is possibly a weaker sigma 70 promoter or a promoter with a less common sigma factor.

For promoters under HexR regulation, expression is expected in glucose but not in lactate, as lactate does not induce the *hex* regulon. Expression would be expected for both glucose and lactate in HexR mutants due to constitutive expression. Previous studies reported that *glk* is expressed at low levels and this could be why the initial primer extensions did not reveal a start site for transcription. In this *glk* assay, a plasmid containing *glk* was introduced into the wild type and *hexR* mutant cells before extracting RNA for primer extension assays to ensure a high number of transcripts. Due to the typical effects of a high-copy plasmid, a signal is now expected in wild-type PAO1 with lactate, but not as high as those same cells in glucose. The same is also true for HexR mutants: some expression is expected with lactate due to the plasmid insertion but not as high as when the cells are grown in glucose. Interestingly, HexR mutants in lactate had a much greater signal than those mutants grown in glucose. One

explanation could be the presence of TCA after glucose metabolism. A second explanation would be the presence of an independently regulated promoter for *glk*, in addition to the HexR regulated promoter upstream of *edd* that would also transcribe through *glk*. If *glk* was capable of being expressed and regulated independently of HexR, it may explain the higher levels of *glk* expression in PAO8026 cells grown in lactate. The plasmid, pPZ121, is a high copy plasmid containing *edd*, *glk*, and the HexR binding region for *edd*. Normally, a higher amount of *glk* after growth in lactate would not be expected, due to the glucose-induced and HexR regulated *edd* transcript present. However, this plasmid may be causing more repression of the HexR controlled promoters due to the high copy plasmid containing two HexR binding sites.

The high copy plasmid was not used for the S1 Nuclease assays. This could explain why this experiment did not show an independent *glk* promoter, because this promoter could be a weak promoter for glk. However, the proposed *edd-glk* transcript had the same effect for the HexR mutant in the S1 nuclease assays; the signal from cells grown in lactate was higher than the signal from those grown in glucose. More analysis is necessary to further characterize this operon in the *hex* regulon.

Crc acts at a post-transcriptional level and affects mRNA stability

Previous published work showed the effects of catabolite repression on the enzyme activities of hex regulon genes but this study looks at the effects on levels of *zwf* mRNA. The direct comparison of *zwf* in different carbon sources showed that Crc does affect levels of mRNA. To show whether this effect is transcriptional or posttranscriptional, succinate was added after induction with glucose. The increase in wild type mRNA half

life indicates an increase in mRNA stability with the addition of succinate. The lack of this same result with the Crc mutant gives the possibility that the presence of Crc results in an increased stability of *zwf* mRNA. However, as seen in the first direct comparison in different carbon sources, the wild type has less total *zwf* mRNA than the Crc mutant after a full growth cycle. These preliminary results show that Crc does not control zwf at the level of transcription. However, it is evident from this study that changes in expression of carbohydrate metabolism genes are taking place and there is some effect on the stability of mRNA.

This hypothesis that Crc is not acting transcriptionally is supported by a recent publication that attributes Crc control to post-transcriptional small RNA binding (Sonnleitner 2009). Sonnleitner et al determined that Crc control is post-transcriptional using the *amiE* gene. Although the question of Crc control appears to be answered, it is important to note that this group did not look at Crc control over *zwf* or any other member of the *hex* regulon. This work shows that the result seen for other operons is likely the same for the *zwf* operon because Crc is a global regulator of catabolite repression and previous work indicates that Crc regulates different systems with the same mechanisms. The author mentioned that *zwf, glk*, and *edd* all contain a consensus sequence for the CA motif so this contributes to the idea that Crc regulation is the same for the *hex* regulon as it is for the *amiE* operon. This sequence is identical in *zwf* and *edd* and 75% matching in *glk* (Sonnleitner 2009). This motif is an important interaction site for Crc and now this study supports the conclusion that the Crc protein is acting on the *zwf* operon and *hex* regulon. The presence of this motif in *zwf* could

explain the increase in stability of the mRNA; the binding of Crc to the *zwf* mRNA could then prevent degradation. The comparisons between growth conditions give an overall view of the differences between strains in the presence of succinate, a well known mediator of catabolite repression due to its position as a preferred carbon source by *P. aeruginosa*. This comparison alone gives an estimate of the overall transcriptional changes and does not point toward a trend of transcriptional control. These studies independently contribute to the recently published conclusion that Crc is acting post-transcriptionally but also add another layer to the work, by using a different set of genes in *P. aeruginosa.*

Conclusions and Future Directions

This thesis answers a few questions that are beneficial to the overall analysis of the *hex* regulon in *P. aeruginosa.* The transcriptional start sites were identified for the last two remaining genes having evidence of a promoter, and the results from the *hexR* mutant introduced the possibility of an independently controlled promoter for *glk* in addition to the HexR controlled *edd* transcript. Catabolite Repression Control exists at a level that controls steady state levels of *zwf* mRNA and it is evident when cells are grown in the presence of succinate. The trends established by using succinate enriched media on wild type and *crc* mutant cells show that the Crc protein is most likely acting post-transcriptionally, which adds transcriptional evidence for the *hex* regulon that correlates with the published assertion that Crc acts post-transcriptionally in *P. aeruginosa*.

To fully characterize the entire regulon, it will be necessary to gain a definitive answer on the regulation of *glk*; most likely using anaerobic conditions. Although Crc is not

considered a transcriptional protein, it will be interesting to continue looking at mRNA to know the role of catabolite repression in stability. An important step will be the relationship between Crc and HexR and any other regulatory proteins that control metabolism.

Given the complexities involved in Catabolite Repression, it is likely that HexR and Crc are part of an even broader level of regulation. These insights into their function will help to further characterize the pathways used for glucose metabolism, and gain more understanding of the requirements for biofilm production. In addition to the most important goal of eradicating the mucosal infections, these findings will only increase the knowledge of bacterial defenses, and possibly find more uses for their unique metabolic activities.

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