Gene expression in Pseudomonas aeruginosa biofilms : evidence for biofilm specific regulation and iron override effects on quorum sensing
by Nikki Bollinger

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology
Montana State University
© Copyright by Nikki Bollinger (2001)

Abstract:
Prior studies established that the Pseudomonas aeruginosa oxidative stress response is influenced by iron availability, whereas more recent evidence demonstrated that it was also controlled by quorum sensing (QS) regulatory circuitry. In the present study, sodA (encoding manganese-cofactored superoxide dismutase, Mn-SOD) and Mn-SOD were used as a reporter gene and endogenous reporter enzyme, respectively, to reexamine control mechanisms that govern the oxidative stress response, and to better understand how QS and a nutrient stress response interact or overlap in this bacterium. In cells grown in trypticase soy broth (TSB), Mn-SOD was expressed in wild-type stationary phase planktonic cells, but not in a Iasl or IasR mutant. However, Mn-SOD activity was completely suppressed in the wild-type strain when the TSB medium was supplemented with iron. Reporter gene studies indicated that sodA transcription could be variably induced in iron-starved cells of all three strains, depending on growth stage. Iron starvation induction of sodA was greatest in the wild-type strain and least in the IasR mutant, and was maximal in stationary phase cells. Reporter experiments in the wild-type strain showed increased lasI::lacZ transcription in response to iron limitation, whereas the expression level in the las mutants was minimal and iron starvation induction of lasI::lacZ did not occur. Studies comparing sodA expression in P. aeruginosa biofilms and planktonic cultures were also initiated. In wild-type biofilms, Mn-SOD was not detected until after 6 d, although it could be rapidly detected in iron-limited biofilms. Unlike planktonic bacteria, Mn-SOD was constitutive in the lasI and IasR mutant biofilms, but could be suppressed if the growth medium was amended with 25 |iM ferric chloride. This study demonstrated that: i) the nutritional status of the cell

GENE EXPRESSION IN *PSEUDOMONAS AERUGINOSA* BIOFILMS: EVIDENCE
FOR BIOFILM SPECIFIC REGULATION AND IRON OVERRIDE EFFECTS ON
QUORUM SENSING

by

Nikki Bollinger

A thesis submitted in partial fulfillment of the requirements for the degree

of

Master of Science

in

Microbiology

MONTANA STATE UNIVERSITY
Bozeman, Montana

March 2001

APPROVAL

of a thesis submitted by

Nikki Bollinger

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

Timothy R. McDermott, Ph.D. _____ _Nov. 29, 2000_
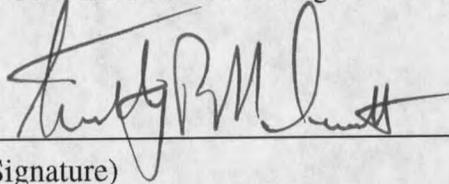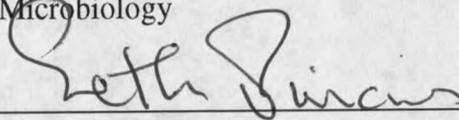
(Signature)   Date

Approved for the Department of Microbiology

Seth Pincus, Ph.D. _____ Nov. 30, 2000

(Signature)   Date
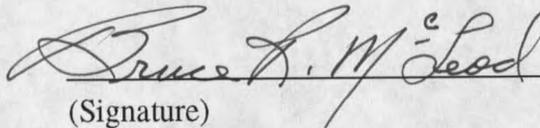
Approved for the College of Graduate Studies

Bruce R. McLeod, Ph.D. _____ 2-28-01

(Signature)   Date

iii.

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a master's degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library.

If I have indicated my intention to copyright this thesis by including a copyright notice page, copying is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for permission for extended quotation from or reproduction of this thesis in whole or in parts may be granted only by the copyright holder.

Signature _____

Date _____11/22/00_____

ACKNOWLEDGEMENTS

I would like to give special thanks to my major advisor, Dr. Tim McDermott, for his constant support, encouragement, and direction during this research. Dr. McDermott was open to my suggestions during this research but at the same time ensured that my research stayed on a steady and focused path toward completion in a timely manner. Appreciation is also extended to Dr. Michael Franklin and Dr. Anne Camper for their review of this manuscript and their suggestions throughout this research.

I would also like to give my appreciation to several people that worked in the lab while I conducted my research. Lina Botero has given me technical assistance while using equipment in the lab as well as suggestions with specific protocols. Also a thank you is extended to Jim Elkins and Jesse Fredericks who helped me perfect specific protocols such as native gels and protein assays.

This thesis is dedicated to my family because without their support I would have not been able to complete this research successfully. I extend my deepest gratitude to my parents, Wayne and Pamela Bollinger, my sister, Kelly, and my brother, Trey, for their encouragement and friendship.

TABLE OF CONTENTS

vii.

LIST OF FIGURES

ABSTRACT

Prior studies established that the *Pseudomonas aeruginosa* oxidative stress response is influenced by iron availability, whereas more recent evidence demonstrated that it was also controlled by quorum sensing (QS) regulatory circuitry. In the present study, *sodA* (encoding manganese-cofactored superoxide dismutase, Mn-SOD) and Mn-SOD were used as a reporter gene and endogenous reporter enzyme, respectively, to re-examine control mechanisms that govern the oxidative stress response, and to better understand how QS and a nutrient stress response interact or overlap in this bacterium. In cells grown in trypticase soy broth (TSB), Mn-SOD was expressed in wild-type stationary phase planktonic cells, but not in a *lasI* or *lasR* mutant. However, Mn-SOD activity was completely suppressed in the wild-type strain when the TSB medium was supplemented with iron. Reporter gene studies indicated that *sodA* transcription could be variably induced in iron-starved cells of all three strains, depending on growth stage. Iron starvation induction of *sodA* was greatest in the wild-type strain and least in the *lasR* mutant, and was maximal in stationary phase cells. Reporter experiments in the wild-type strain showed increased *lasI::lacZ* transcription in response to iron limitation, whereas the expression level in the *las* mutants was minimal and iron starvation induction of *lasI::lacZ* did not occur. Studies comparing *sodA* expression in *P. aeruginosa* biofilms and planktonic cultures were also initiated. In wild-type biofilms, Mn-SOD was not detected until after 6 d, although it could be rapidly detected in iron-limited biofilms. Unlike planktonic bacteria, Mn-SOD was constitutive in the *lasI* and *lasR* mutant biofilms, but could be suppressed if the growth medium was amended with 25 µM ferric chloride. This study demonstrated that: i) the nutritional status of the cell

CHAPTER 1

INTRODUCTION

Historically, *Pseudomonas aeruginosa* has been one of the most extensively studied gram-negative bacteria. Recently, it has been one of the key organisms utilized in the study of quorum sensing (QS), a mechanism that enables bacteria to regulate the expression of numerous genes based, in part, on population density. Although every case of QS studied thus far has been connected to some aspect of cell-density and the accumulation of autoinducer molecules, one must ask if other parameters are involved in QS or indeed the control of QS. Further, QS-based gene expression in *P. aeruginosa* must be carefully compared between biofilms and planktonic cultures.

To date, the effects of nutrient availability or cell nutritional status have not been studied in the context of QS. In this thesis, the iron nutritional status of *P. aeruginosa* planktonic and biofilm cultures was manipulated to examine the effect of nutrient starvation on QS-based gene regulation. Manganese superoxide dismutase (MnSOD), an enzyme previously found to be affected by iron limitation and QS, was used as a reporter enzyme. During the course of this thesis research, iron availability was found to override QS control of MnSOD. Further, an example dynamic gene expression in biofilms was also revealed.

The thesis is composed of two components; a literature review and the research conducted for this thesis. The literature review briefly addresses the medical and environmental significance of this bacterium, and then summarizes aspects of quorum sensing, biofilms and iron regulation.

CHAPTER 2

LITERATURE REVIEW

Characteristics of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram negative rod that is an opportunistic pathogen (92) and found in numerous medical settings. This bacterium infects immunocompromised individuals including cancer patients (6), human immunodeficiency virus infected patients (34), and is particularly problematic for patients with severe burn wounds (59) or cystic fibrosis (CF) (28).

Numerous factors are critical to the virulence of *P. aeruginosa*. Alginate, lipopolysaccharide, toxins (exotoxin A), proteases (elastase and protease), and hemolysins (phospholipase and rhamnolipid) are all virulence factors that are synthesized by *P. aeruginosa* (80). They have been shown to contribute to the virulence of the bacterium in animal models (74), in *in vitro* experiments (7), and in clinical studies (110). The production of various *P. aeruginosa* virulence factors relies on a specific environmental stimulus such as nutrient availability and temperature (7). However, in general, the expression of virulence factors is dependent upon the density of cells in a bacterial population. The mechanism by which *P. aeruginosa* controls virulence expression in a cell-density dependent manner is termed quorum sensing (38). One of the first examples in this bacterium was the observation that *apr* (codes for alkaline protease) transcription requires LasR (40), which was also found to be a critical component of the QS system in *P. aeruginosa* (40). Synthesis of other virulence factors, such as the LasB elastase and the LasA protease, were also found to be under the control of LasR (39, 101).

Quorum Sensing

Background

Microorganisms have numerous signal transduction mechanisms that enable them to sense and transduce environmental signals into discrete cellular responses. In addition, it is thought that bacteria are capable of conducting a census of their population and regulating the expression of genes, operons, and regulons through QS-based circuitry. One of the best-known QS-controlled activities is bioluminescence in the symbiotic marine bacterium, *Photobacterium (Vibrio) fischeri* (38). When cell density is low, *P. fischeri* cultures appear dark but once a critical cell density is reached a blue-green light is emitted.

The bioluminescence in *P. fischeri* involves the secretion and accumulation of low molecular weight N-acyl-L-homoserine lactones (AHL). Synthesis of these AHL signals is controlled by products of the regulatory genes *luxI* (the signal generator) and *luxR* (the response regulator). LuxR, LuxI, and the diffusible autoinducer (3-oxohexanoyl homoserine lactone), also known as VAI-1, were among the first components of QS discovered in *Vibrio fischeri* (38). LuxR acts as a VAI-1 receptor in addition to a transcriptional activator, while LuxI directs the synthesis of VAI-1. Initially, the autoinducer passively diffuses out of cells down a concentration gradient and intracellular concentrations of the autoinducer gather near and within the bacteria. The accumulation of AHLs enables *P. fischeri* to sense the surrounding population and manage the expression of bioluminescence genes (*lux*) if needed.

Quorum Sensing in *Pseudomonas aeruginosa*

P. aeruginosa contains two separate quorum sensing systems known as *las* and *rhl*. The *las* system involves an AI synthase, LasI (a homologue to LuxI) regulates the production of N-(3-oxododecanoyl) homoserine lactone (PAI-1) (87). This diffusible extracellular signal activates the *lasR*-encoded transcriptional activator, LasR (homologue to LuxR), to induce virulence genes such as *lasB, lasA* (101), *apr* (40), and *toxA* (86). LasI production is positively regulated by activated LasR and PAI-1 at the level of transcription, which leads to the synthesis of more PAI-1 (94). The QS system has been determined to be critical in *P. aeruginosa* virulence *in vivo*, based on observations that the Δ*lasR* mutant was avirulent in a mouse model (100).

The *rhl* system also regulates the expression of specific virulence factors (108, 70). The *rhl* system is composed of RhlI, which is the *rhlI*-encoded AI synthase, and RhlR, which is the *rhlR*-encoded transcriptional activator (81, 70). The autoinducer, N-butyrl homoserine lactone, also known as PAI-2, regulates rhamnolipid synthesis (108), expression of alginate, and pyocyanin production. The stationary phase sigma factor RpoS was first reported to also be controlled by the rhl system (71), but more recent observations argue that this is not the case (106). A model that describes known QS-regulated gene expression in *P. aeruginosa* is shown in Figure 1.
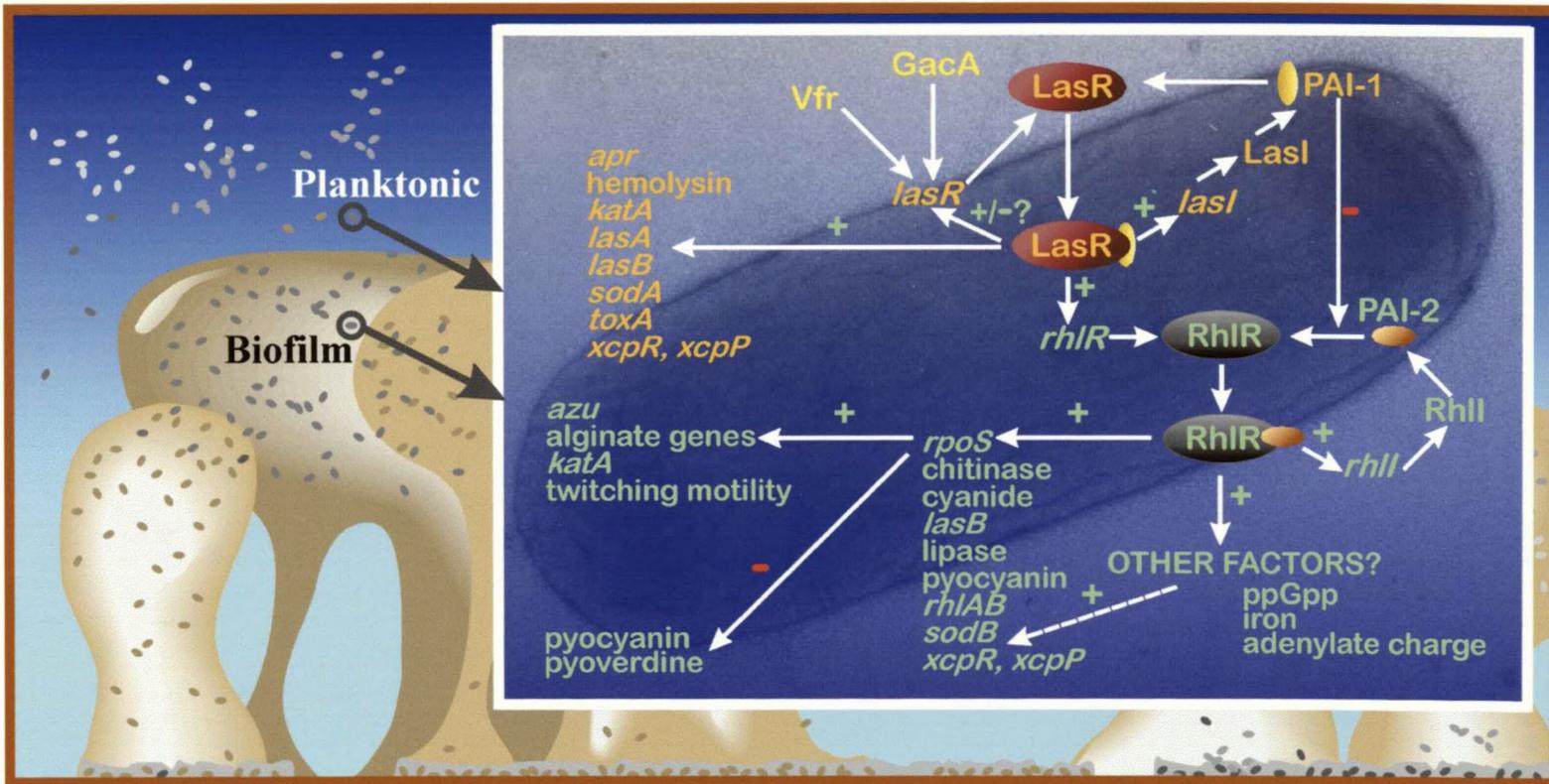
Fig. 1. Model of QS control in *P. aeruginosa*. This figure is based upon many research contributions within the past 6 years concerning genes and gene products under quorum sensing control in *P. aeruginosa*. It provides an update of a previous tier of control proposed by Pearson et al., (1997).

BIOFILMS

Biofilm Formation

In the open environment, bacterial biofilms are very common, where virtually every surface is coated with varying amounts of microbial biomass. Biofilm formation is often initiated when a conditioning film forms from the adsorption of organic molecules and/or ions as a substratum is submerged in an aquatic environment (15). As cells attach to the substratum, the microorganisms start to undergo cell division and ultimately may form mushroom-shaped microcolonies and stalk-like structures (19) surrounded by an organic polymer matrix (69). The microcolonies are separated by water channels, which allow nutrients to diffuse into the biofilm and waste products to flow out (25). The shape of biofilms is generally not identical because they may be arranged as "patches" or they may be found as a uniform coverage across a surface. The surface to which biofilm cells adhere may be either inert or living (20).

Bacterial biofilms are also found in the medical setting, most often when contaminated surfaces come into contact with natural fluids such as blood or urine. Human health problems that are viewed as biofilm associated include cystic fibrosis and apparently irreversible colonization of prosthetic devises (e.g. catheters), medical implants (e.g. heart valves), or on dental surfaces (plaque) of some individuals (98).

Biofilm structural features are dependent on species composition, the flow rate of the over-lying fluid, pH, temperature, and nutrient supply (15). Much of biofilm architecture is determined by exopolyssacharides (EPS) synthesis and the environmental factors that influence EPS levels (1, 17, 19). The EPS matrix synthesized by biofilm cells is negatively charged and functions as an ion-exchange resin. The EPS is also thought to protect the biofilm cells from environmental challenges such as desiccation (17), nutrient

limitation (84), and antimicrobial agents (1, 13). Decreased susceptibility of biofilm bacteria to antimicrobial agents is a critical issue in the treatment of medical implant and cystic fibrosis infections. It has been proposed that biofilm matrix properties retard antibiotic transport, rendering the antimicrobial agents incapable of completely penetrating the biofilm, and thus resulting in either no or underexposure of some bacteria to the antibiotic (18). Another hypothesis suggests that the physiology of metabolically quiescent biofilm bacteria decreases their susceptibility to antibiotics that require growth in order to effect killing (11).

Biofilm Dynamics

Evidence suggests that cells grown on solid surfaces or agar have different physiological characteristics than those grown in liquid media. A well-known example of this difference is swarmer cell differentiation on agar surfaces exhibited by numerous microorganisms such as *Vibrio, Proteus, Clostridium, Bacillus,* and *Serratia* species (57). When *Vibrio parahaemolyticus* grows in a liquid environment, each bacterial cell generates one polar flagellum that allows the cells to swim through the liquid media. On the other hand, cells that grow on a solid surface are characterized by suspended septation and intitiated elongation. These bacteria also produce many peritrichous lateral flagella in addition to their polar flagellum which allow them to move over surfaces, termed swarming (5, 107). Other studies reveal differences in growth rate, exopolymer production, fimbriae and flagellar synthesis, and susceptibility to antibiotics between biofilm and planktonic cultures (42).

It is necessary to understand the mechanisms of biofilm formation when trying to prevent harmful biofilms such as those found in cystic fibrosis patients. In addition to basic differences between biofilm and planktonic cells for any given bacterium, species-to-

species variations in such differences will also occur. During the initial phases of biofilm formation, all cells are metabolically active but following the primary colonization and formation of small microcolonies, a change in the cells' activity occurs. Usually cells proximal to the periphery of the biofilm or adjacent to water channels have higher metabolic activity than cells established deeply within the polysaccharide matrix (1). The down regulation of cell metabolic activity within a biofilm is likely not an abrupt event, but instead would probably occur gradually with increasing distance from the biofilm or microcolony surface. Cells in the centers of the largest microcolonies would exhibit decreased growth activity, whereas cells in smaller colonies continue to stay very active, but would begin to show reduced growth activity after a while (98). Microcolony size is not the only parameter that would determine growth activity. A widespread depletion of the nutrient supply due to the high cell density of bacteria and species-species nutrient competition could aid in causing the decrease in metabolic activity. This implies that even microcolonies in a thin biofilm cannot be considered independent units since the activity of the single microorganisms inside one microcolony is dependent on factors caused by surrounding microcolonies such as the exhaustion of nutrients.

Perturbations of growth conditions can lead to changes in biofilm cell gene expression. In 1999, Sternberg, *et al.* (98) showed that an addition of a more easily metabolizable carbon source did in fact, activate cells in microcolonies. Even the cells in the center of the microcolonies, which were the first to decrease their growth activity, reacted to the better nutrient supply. This study indicates that biofilms are dynamic and that every cell in a maturing biofilm is prepared to respond quickly to refreshed nutrient supplies.

Pseudomonas aeruginosa Biofilms and Quorum Sensing

Davies, *et al.* (24) determined that the *las* quorum sensing system in *P. aeruginosa* biofilms controls the normal development of biofilms. Biofilm structure of a *lasI* mutant was significantly altered relative to the wild type strain, with the most significant difference being SDS sensitivity (24). SDS resistance in the *lasI* mutant could be restored by the addition of 10µM 3-oxododecanoyl-homoserine lactone, the autoinducer controlled by the *lasI* gene product. It is not clear which QS-regulated genes are necessary for normal biofilm formation. Potentially important biofilm cell behavior is inferred from reporter gene and adhesion studies. The expression of *algC,* a gene encoding phosphomannomutase (an extracellular polysaccharide), which is an important regulation point in the alginate biosynthetic pathway, is increased when *P. aeruginosa* is attached to and grows on an abiotic surface (23). This would imply that alginate synthesis is up-regulated upon attachment. Another physiological difference of biofilms is the induction of type IV pili during the formation of these structured communities (85). In addition, type IV pili have been demonstrated to be crucial for bacterial adhesion to eukaryotic cell surfaces and for pathogenesis (109). This finding is an important indication of the overlapping mechanism of biofilm formation on inert surfaces and the factors required for bacterial attachment to living surfaces and pathogenesis *in vivo.*

Iron Regulation

Background

Iron is a micronutrient that bacteria utilize to carry out various cellular processes critical to their survival. This element is fundamental for cellular metabolism because it is

required as a cofactor for many enzymes (103). Iron is required for the transport and storage of oxygen, reduction of ribonucleotides and dinitrogen, decomposition of peroxides, and electron transport via different carriers (2). Aerobic microorganisms are not always able to uptake this metal in its elemental ionic form from the extracellular medium. They generate and secrete siderophores, which are low-molecular-weight iron carriers (78). Siderophores bind Fe^{3+} with high specificity and high affinity (79). Following the chelation of Fe^{3+}, the bacterial cell recovers the ferri-siderophore complexes through distinct outer membrane receptors (29). At physiological pH, iron is rendered unavailable in aerobic environments due to the small value for the solubility product constant of the hydroxide. However, an excess of iron could be toxic because it catalyzes Fenton reactions and forms active species of oxygen. Therefore, the accumulation of iron must be regulated in order to maintain the intracellular concentration of the metal within a specific range. Since no known mechanisms for excreting iron out of the bacterial cell are known, these microorganisms must control their internal iron concentration by controlling its transport through the cellular membrane (3, 22).

Iron Transport Regulation

A gene named *fur* (for ferric uptake regulation) in *E. coli* encodes a 17-kDa polypeptide (4) that behaves as a repressor of transcription of iron-regulated promoters due to its Fe^{2+}-dependent DNA binding activity (26, 32). When bacteria grow in iron-rich conditions, Fur chelates the divalent ion, resulting in a dimer protein configuration that is capable of binding target DNA sequences known as Fur boxes or iron boxes (21). The C-terminus is involved in the dimerization while the N-terminus contains DNA binding domains (99). In *Vibrio*, Fur is an abundant protein, being measured at about 2500

molecules per cell during logarithmic phase, while increasing to 7500 molecules during stationary phase (104). The Fur- Fe^{2+} complex prevents the transcription of genes and operons preceded by the iron box sequence (3). However, when the iron concentration is low, the equilibrium favors unbound Fur, allowing the RNA polymerase access to the promoters region. Typically, genes coding for the biosynthesis of siderophores and other iron-related functions are under iron control and are up-regulated by iron starvation (45, 66). In *P. aeruginosa,* Fur may regulate expression of a sigma factor, known as PvdS, which then controls the expression of a specific set of genes (73). Homologues of the *fur* gene have been identified in *P. aeruginosa* (91) which are capable of complementing an *E. coli fur* mutant. This complementation indicates that the molecular mechanisms that direct transcriptional regulation by iron are common among gram negative bacteria.

Superoxide Dismutase

Background

The consequences of living in an aerobic environment could be devastating due to the high reactivity of oxygen. When oxygen is univalently reduced, reactive oxygen species (ROS) form within bacterial cells (37, 47). Superoxide, O_2^-, is a by-product of aerobic metabolism and may accumulate inside the cell if not scavenged by an enzyme known as superoxide dismutase (SOD) (75). Superoxide dismutase reduces toxic O_2^- within the cell by converting it to hydrogen peroxide (H_2O_2), which eventually is broken down to water and O_2 by the enzyme catalase (36, 37). *P. aeruginosa* possess two types of superoxide dismutases (49), which are classified according to their metal cofactors. Mn-SOD and FeSOD are located in the cytoplasm and protect DNA and proteins from

oxidative damage. FeSOD is encoded by *sodB*, which is constitutively expressed and viewed as a housekeeping gene (61). MnSOD is encoded by *sodA*, which is regulated by increases in internal O_2^- concentration and cell density (27). Superoxide dismutase expression in *P. aeruginosa* is, in part, controlled by the availability of iron in the bacterial cell. When iron is abundant, only FeSOD is expressed, whereas under iron-limiting conditions, which may occur in stationary phase, Mn-SOD is induced (50). If superoxide were allowed to accumulate, it would react with and damage numerous molecules. Mutants that lack SOD demonstrate aerobic hypermutagenesis (33).

Role of Superoxide in Oxidative Damage

One model often used to explain how oxidative damage occurs is referred to as the Haber-Weiss Scheme (9, 35). This hypothesis suggests oxygen damages DNA indirectly through the Fenton reaction as follows:

$$O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+}$$

$$H_2O_2 + Fe^{2+} \rightarrow OH^{\circ} + OH^- + Fe^{3+} \text{ (Fenton Reaction)}$$
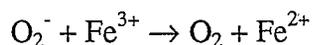
Superoxide acts as a reductant for iron, which produces a hydroxyl radical by transferring the electron to H_2O_2. The hydroxyl radical then attacks DNA. Evidence that suggests O_2^- produces DNA damage *in vivo* was gathered by conducting experiments with *E. coli* strains lacking cytosolic SOD (33). These mutants are hypermutagenic if placed in air-saturated media, which implies that elevated concentrations of O_2^- result in enhanced DNA damage. Data that supports this hypothesis revealed that SOD⁻ mutants were 10-fold more susceptible to DNA oxidation by H_2O_2 than were wild type cells (14). The amount of DNA damage caused by oxidants was so immense that SOD mutants weren't able to grow

Conclusion

While bacterial gene regulation is complex, interesting patterns are beginning to emerge. Available data in the literature suggests that quorum sensing, iron regulation and superoxide dismutase production are all related. My thesis research took advantage of the fact that endogenous reporter enzyme Mn-SOD is controlled by both QS and iron. My research initiate studies that were designed to tease apart regulatory relationships between the QS and Fur regulatory systems. To what extent do they overlap? Does QS control iron responses or vice versa? Are their regulatory effects additive? I found that: i) the nutritional status of the cell must be taken into account when evaluating QS-based gene expression; ii) QS may also have negative regulatory functions; iii) QS-based gene regulation models based on studies with planktonic cells must be modified in order to explain biofilm gene expression behavior; and iv) gene expression in biofilms is dynamic.

CHAPTER 3

GENE EXPRESSION IN *Pseudomonas aeruginosa*: EVIDENCE OF IRON OVERRIDE EFFECTS ON QUORUMS SENSING AND BIOFILM-SPECIFIC GENE REGULATION

Introduction

Pseudomonas aeruginosa is ubiquitous, being found in diverse environments such as soil, freshwater, and marine environments. It is also an opportunistic pathogen of the airways of cystic fibrosis patients, and in immunocompromised hosts including cancer, AIDS, and burn patients (43). Similar to other pathogens and gram negative bacteria, *P. aeruginosa* has a global regulatory system known as quorum sensing (QS) that controls expression of numerous genes, many of which are associated with virulence (31, 76). Bacterial QS, or cell-to-cell communication, is a process in gram-negative and some gram-positive bacteria where low molecular weight diffusible molecules synthesized by one cell trigger gene activation in other cells (44). In gram-negative bacteria, the signaling molecules are either homoserine lactone/acyl side chain-based (HSL, autoinducers), diketopiperazine (DKPs) (58), or via 2-heptyl-3-hydroxy-4-quinolone (90), while gram-positive bacteria use small peptides. Because of its aforementioned ubiquity in nature and its importance in disease, *P. aeruginosa* is a model organism for QS study.

QS is viewed as a cell-density dependent phenomenon that allows bacteria to communicate, sense population density, and ultimately coordinate transcription of many genes. Bacteria monitor their population by sensing the level of autoinducer signal molecules (46). HSL-based QS in *P. aeruginosa* is a multi-tiered process governed by two gene tandems, *lasRlasI* and *rhlRrhlI* (86, 87, 88). The *las* system is composed of LasR, a transcriptional regulator protein, and LasI, an autoinducer synthase that produces

one of the three known *Pseudomonas* HSL's, PAI-1 [*N*-(3-oxododecanoyl)-L-homoserine lactone]. The second tier consists of RhlR, which, like LasR, is a transcriptional regulator, and RhlI, an autoinducer synthase that catalyzes the synthesis of a second HSL, PAI-2 (*N*-buturyl-L-homoserine lactone). PAI-1 interacts with the regulator LasR to activate transcription of target genes (87). The LasR-PAI-1 complex will activate the transcription of *lasI* and several genes important in defense against oxidative stress such as those coding for the major catalase, KatA, and the manganese superoxide dismutase (Mn-SOD) (56). Further, recent work by Greenberg and colleagues has identified many other QS-regulated genes that were previously unrecognized (105).

Whether considered in either disease or environmental settings, an important aspect of *P. aeruginosa* ecology is its propensity to form biofilms. *P. aeruginosa* biofilms have high cell densities and an architecture that typically consists of highly ordered "mushroom-" and pillar-like structures (19). This important aspect of *P. aeruginosa* biology has also been shown to be influenced by QS (24). QS-deficient mutants form a thin, tightly packed biofilm, differing markedly from wild-type biofilm architecture, suggesting that particular aspects of biofilm cell physiology are under control of QS and are important for normal biofilm formation. The physiology of bacterial biofilms is viewed to be different from that of planktonic cultures (19), but the true extent of such potential differences is still poorly understood.

I examined *P. aeruginosa* biofilm responses to environmental stimuli as a means of studying gene expression and physiology of biofilm bacteria. We have elected to focus on the oxidative stress response because our knowledge of the antioxidant responses in this organism is firmly grounded genetically and physiologically (49-53), and because anti-oxidant enzymes are of central importance to the pathogenecity of this organism (55). Further, it has recently been found that key components of the oxidative stress response are regulated by QS (56). Curiously, earlier studies had also implicated iron availability as

a significant controlling factor in expression levels of antioxidant enzymes in *P. aeruginosa* (49-56). As QS has thus far been found to exert its effects when cell densities are high, a condition which is found in biofilms and which can lead to localized areas of high nutrient demand, we have hypothesized that nutrient limitation may also be an important factor to consider in studies aimed at understanding QS and biofilm biology (D.J. Hassett, U. A. Ochsner, T. de Kievit, B.H. Iglewski, L. Passador, T.S. Livinghouse, J.A. Whitsett, and T.R. McDermott, submitted for publication). The availability of well-defined QS mutants offers an excellent opportunity to examine and compare gene expression in biofilms and planktonic cells under conditions where the availability of a specific nutrient can be conveniently and reliably manipulated.

Materials and Methods

Bacterial Strains, Plasmids and Growth Conditions

P. aeruginosa wild-type strain PAO1 (60), the *lasI*::Tn*10* mutant PAO-JP1 (89), and the *lasR* mutant PAO-R1 (87) were used in this study. Plasmids pDJH201, [contains *sodA::lacZ* (56)], and pPCS223 [contains *lasI::lacZ* (102)], were used in reporter gene experiments. These plasmids were introduced into the different strains by electroporation and maintained with carbenicillin (300 mg · L^{-1}). In each case, plasmid transformation was verified by restriction enzyme analysis of plasmid preparations (93) of the different transformants. In some experiments, iron bioavailability in the medium was manipulated by the addition of iron (25 μM $FeCl_3$) or the iron-specific chelator 2,2-dipyridyl (500 μM for trypticase soy broth (TSB) medium or 50 μM for 1/10 TSB).

Planktonic cultures were grown in TSB at 37°C in a rotary shaker at 300 rpm. Culture volumes did not exceed 10% of the flask volume to ensure maximum aeration. Biofilms were cultured using a drip-flow reactor system previously described by Huang et al. (62) and included 316L stainless steel slides (1.3 x 7.6 cm) as the substratum. Briefly, 10 ml of 1/10 strength TSB medium were added to each chamber (four chambers per reactor), followed by inoculation with 1 ml of stationary phase culture of the test strain grown in TSB medium. The reactor was then incubated horizontally at 37°C for 24 h to allow bacterial attachment to the substratum. Following the attachment period, the reactor was inclined 10° and a constant drip of 1/10 strength TSB was allowed to flow over the slides at a rate of 50 ml h^{-1}. Biofilms were cultured in a 37° C incubator. To achieve this, the sterile 1/10 TSB media contained in the external carboy was pre-equilibrated to 37° C prior to entry into the drip flow reactor. To accomplish this, the media was first preheated to 42° C by pumping through masterflex silicone tubing coiled in a 42° C water bath fixed atop the incubator chamber (Figure 2). The feed tubing leaving the 42° C water bath was foam insulated (to reduce thermal loss) and channeled through the heat vent hole of the culture incubator. A mercury thermometer was attached to the media flow tubing via aluminum tape, providing for isothermic association with the tubing and verified the medium was 37°C as it entered the incubator chamber. A final temperature equilibration step designed to guarantee appropriate temperature involved additional coiling (3 m flow length) of the feed tubing in distilled water (2 liter beaker) that was equilibrated at chamber temperature. This ensured a final medium temperature of 37° C prior to entry into the drip flow reactor. Silicone tubing exiting each reactor chamber was used to pump the waste out of the chamber and into external waste carboys.