



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
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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 *IasI* 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 *IasI* and *IasR* mutant biofilms, but could be suppressed if the growth medium was amended with 25 μ M ferric chloride. This study demonstrated that: i) the nutritional status of the cell

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FOR BIOFILM SPECIFIC REGULATION AND IRON OVERRIDE EFFECTS ON
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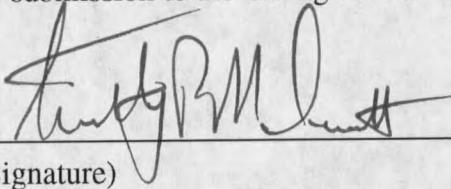
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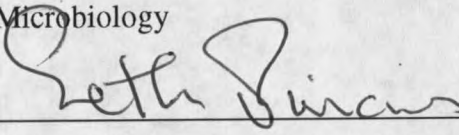
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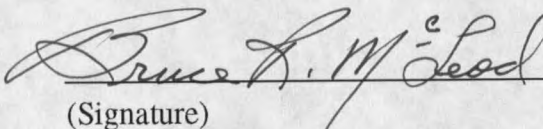
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TABLE OF CONTENTS

	page
LIST OF FIGURES	vii
ABSTRACT	viii
1. INTRODUCTION	1
2. LITERATURE REVIEW	2
CHARACTERISTICS OF <i>PSEUDOMONAS AERUGINOSA</i>	2
QUORUM SENSING	3
Background	3
Quorum Sensing in <i>Pseudomonas aeruginosa</i>	4
BIOFILMS	6
Biofilm Formation	6
Biofilm Dynamics	7
<i>Pseudomonas aeruginosa</i> biofilms and quorum sensing	9
IRON REGULATION	9
Background	9
Iron Transport Regulation	10
SUPEROXIDE DISMUTASE	11
Background	11
Role of Superoxide in Oxidative Damage	12
CONCLUSION	14
3. GENE EXPRESSION IN <i>Pseudomonas aeruginosa</i>	
BIOFILMS: EVIDENCE OF IRON OVERRIDE EFFECTS ON QUORUM	
SENSING REGULATION AND BIOFILM-SPECIFIC GENE	
REGULATION	15

INTRODUCTION.....	15
MATERIALS AND METHODS.....	17
Bacterial Strains, Plasmids, and Growth Conditions.....	17
Cell-free Extract Preparation, Nondenaturing Gel Electrophoresis, and Reporter Enzyme Activity.....	20
RESULTS.....	20
Mn-SOD regulation.....	20
Effects of Iron Concentration on <i>lasI</i> Expression.....	25
Production of Mn-SOD in Biofilms.....	25
DISCUSSION.....	28
Nutritional Override of Quorum Sensing.....	28
Biofilm Gene Expression.....	33
4. THESIS SUMMARY AND CONCLUSIONS.....	35
REFERENCES CITED.....	37

LIST OF FIGURES

Figure	page
1. Model of QS control in <i>P. aeruginosa</i>	5
2. Biofilm Set-up and assembly for growing biofilms at specific temperatures.	19
3. Growth curve of <i>Pseudomonas aeruginosa</i> in planktonic cultures.....	21
4. Superoxide dismutase activity in planktonic cells cultured in TSB.....	22
5. Transcription of <i>sodA</i> in mid log phase and stationary phase cells of PAO1, PAO-JP1, and PAO-R1 as affected by iron limitation	24
6. Expression of <i>lasI</i> in response to iron deprivation	26
7. Mn-SOD expression in biofilms	27
8. Mn-SOD expression in PAO1 biofilms as affected by iron starvation	29
9. A potential mechanism for dual control of <i>sodA</i> by iron-sensitive and quorum sensing circuitry	32

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 μ M 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-butyryl 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.

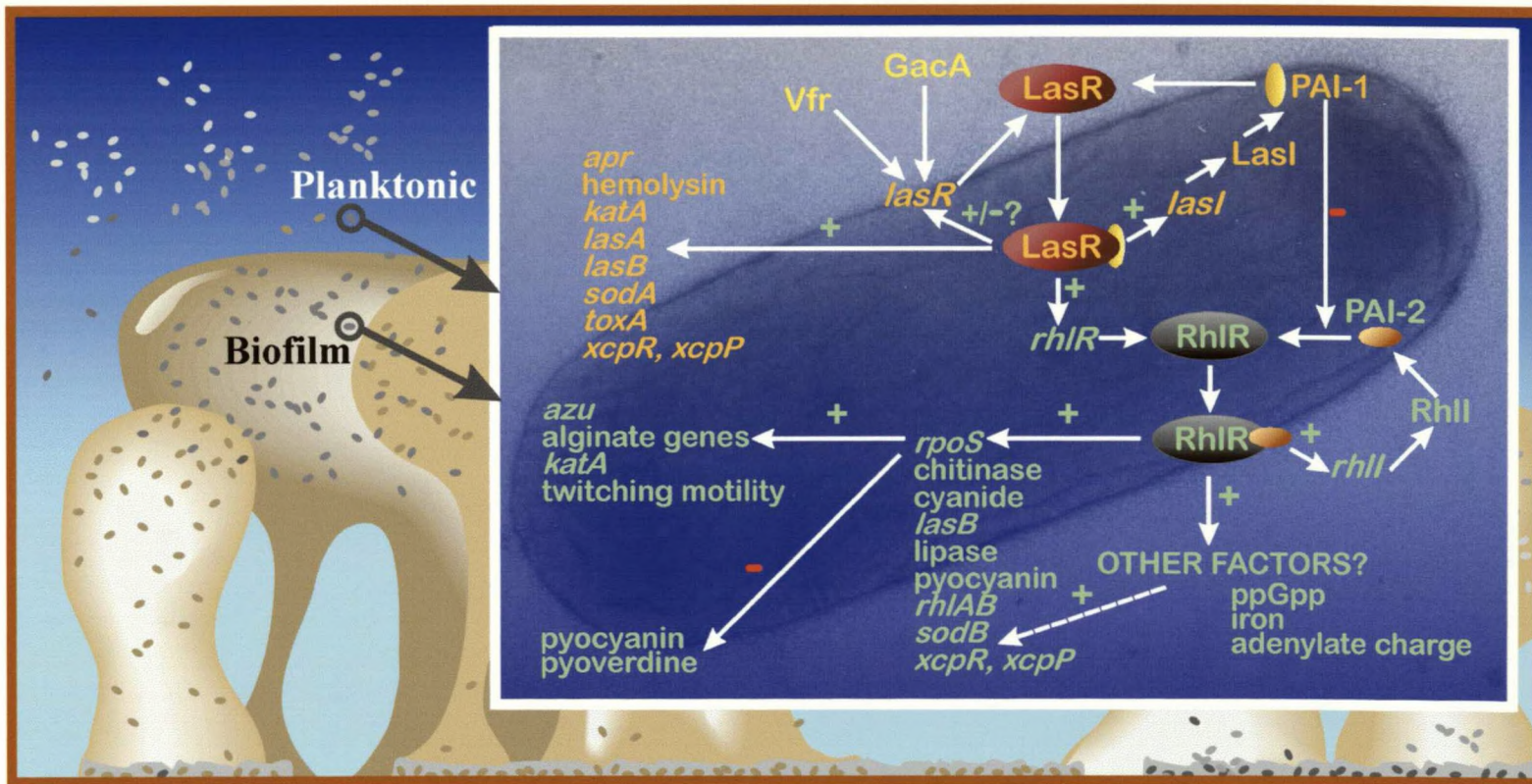


Fig. 1. Model of QS control in *P. aeruginosa*. This figure is based upon many research contributions within the past 6 years concerning genes and gene products under quorum sensing control in *P. aeruginosa*. It provides an update of a previous tier of control proposed by Pearson et al., (1997).

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 devices (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 exopolysaccharides (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 initiated 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 10 μ M 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

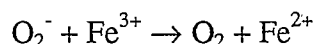
Background

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:



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
REGULATIONIntroduction

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*-butyryl-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 antioxidant enzymes are of central importance to the pathogenicity 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*::Tn10 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 ($300 \text{ mg} \cdot \text{L}^{-1}$). 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 ($25 \text{ } \mu\text{M}$ FeCl_3) or the iron-specific chelator 2,2-dipyridyl ($500 \text{ } \mu\text{M}$ for trypticase soy broth (TSB) medium or $50 \text{ } \mu\text{M}$ 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.

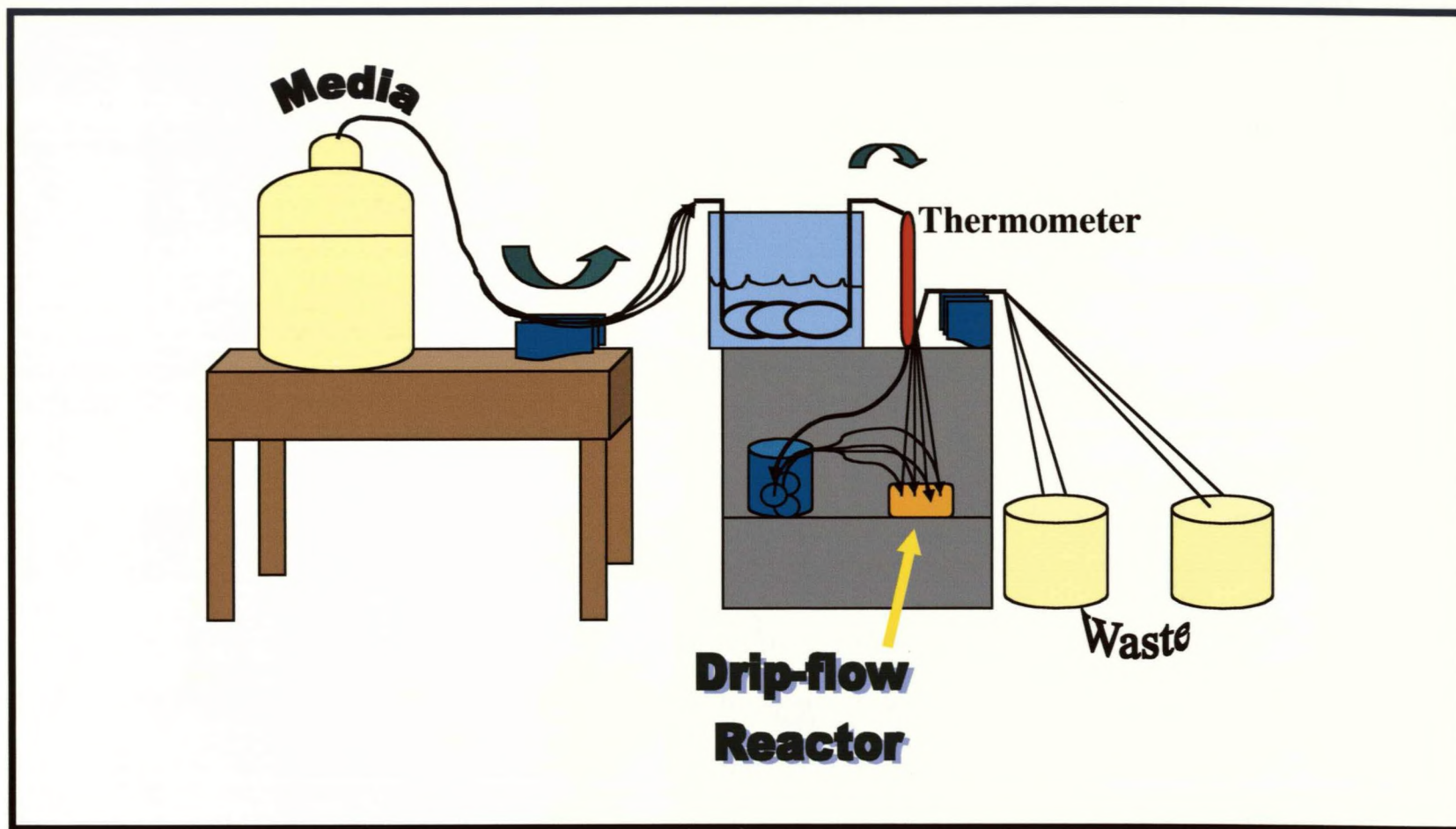


Fig. 2. Biofilm set-up and assembly for growing biofilms at specific temperatures.

Cell-free Extract Preparation, Nondenaturing Gel Electrophoresis, and Enzyme and Reporter Activity

Nondenaturing gel electrophoresis methods were as described previously by Hassett et al. (55). Briefly, cell extracts were prepared from cultures harvested by centrifugation at 10,000 x g for 10 min at 4°C. Bacteria were washed twice in ice-cold 50 mM potassium phosphate buffer, pH 7.0, and sonicated in an ice water bath for 30 s. The sonicate was clarified by centrifugation at 13,000 x g for 10 min at 4°C. Extract protein content was estimated using the Bradford assay (8). Superoxide dismutase activity stains in nondenaturing gels were used to semi-quantitatively follow induction of Mn-SOD and to assess its activity separately from the constitutively expressed Fe-SOD (coded by *sodB*) (55). Gel images were computer scanned and stored as Powerpoint image files. LacZ reporter enzyme expression was measured as β -galactosidase activity as previously described by Miller (77).

Results

Mn-SOD Regulation

Initial experiments compared Mn-SOD levels in the *P. aeruginosa* wild-type strain PAO1 and the *lasI* mutant PAO-JP1 at specific growth phases (Fig. 3). In PAO1, Mn-SOD activity was detectable in stationary phase cells ($t = 12$ h, Fig. 4A), which correlates with the production and accumulation of the quorum sensing autoinducer molecule PAI-1 (56). Mn-SOD was absent from stationary phase TSB cultures of the *lasI* mutant JP1, which cannot synthesize PAI-1 (89), and the *lasR* mutant PAO-R1, which lacks the regulatory protein essential to QS (39, 40) (results not shown). All of these observations

were consistent with previous findings which showed that Mn-SOD production is controlled by QS in this organism (56).

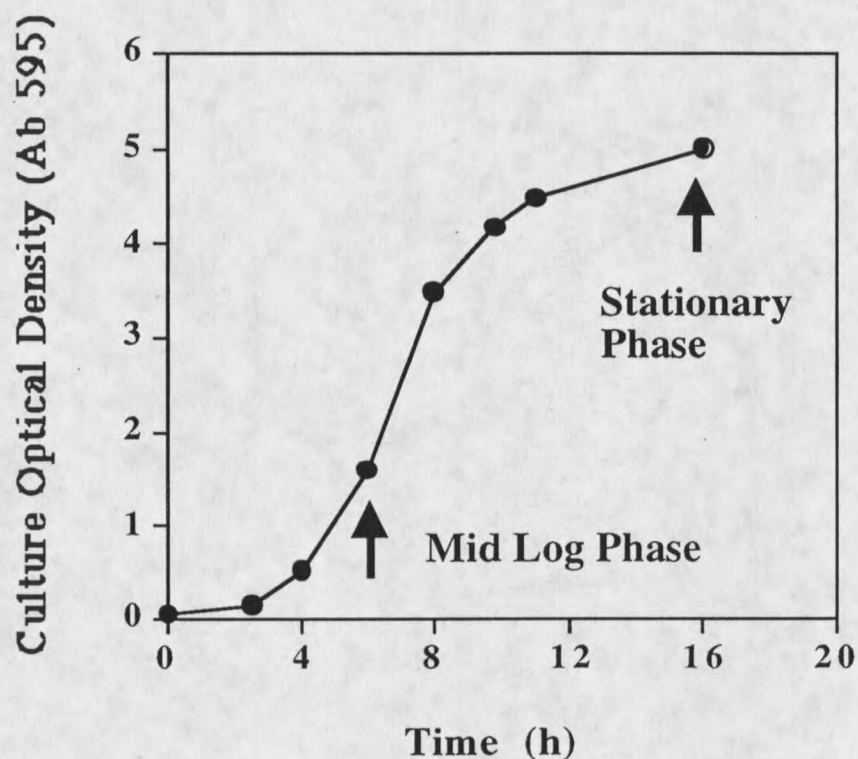


Fig. 3. Growth curve of *Pseudomonas aeruginosa* in planktonic culture.

Assessment of iron effects on QS-controlled gene expression was initiated in experiments where either the TSB medium was supplemented with 25 μM FeCl_3 , or 500 μM 2,2-dipyridyl was added to render the iron unavailable. In wild-type cells cultured to

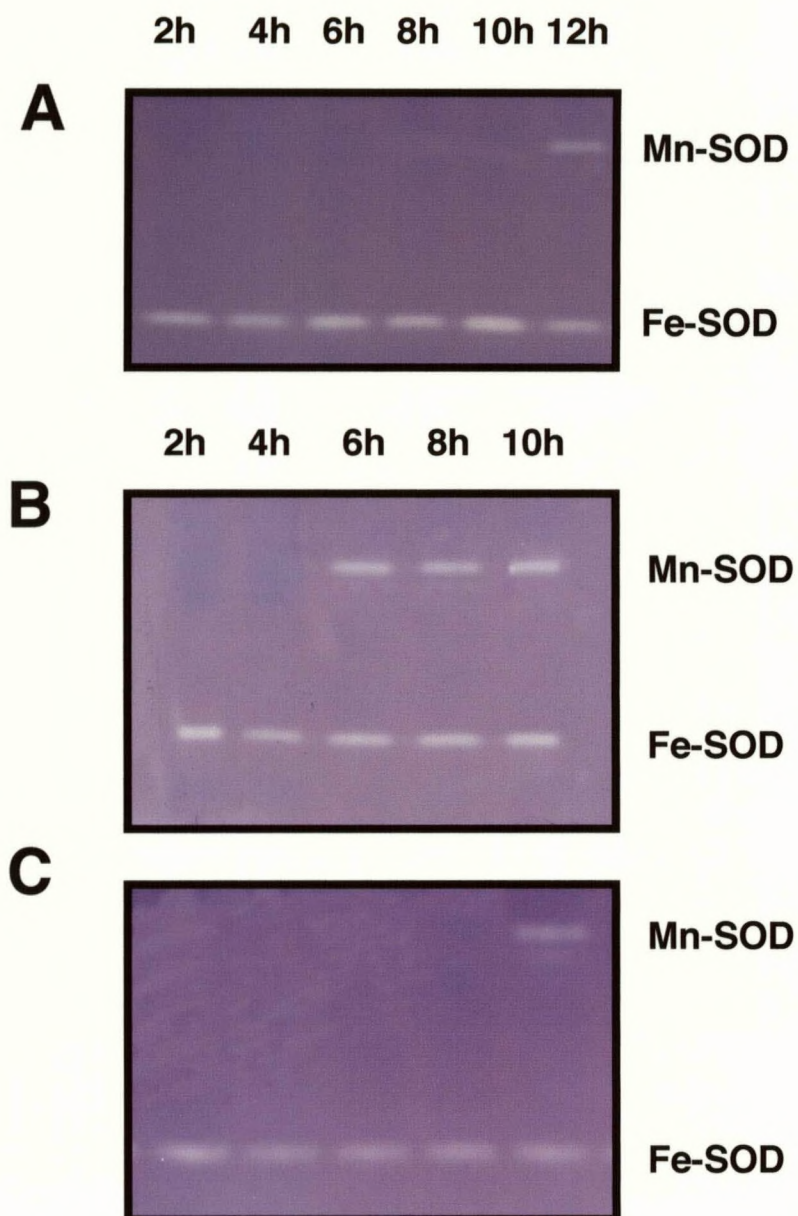


Fig. 4. Superoxide dismutase activity in planktonic cells cultured in TSB. Cell extracts (50 μ g protein per lane) of each strain were prepared at different time points corresponding to different growth stages; 6 h = mid log phase, 10 h = early stationary phase. (A) SOD activity stains of wild type strain PAO1 cultured in TSB, (B) PAO1 in TSB amended with the iron-specific chelator 2,2-dipyridyl, (C) and the *lasI* mutant PAO-JP1 in TSB amended with the iron-specific chelator 2,2-dipyridyl were prepared as described in the Materials and Methods. Results are of one of three independent experiments demonstrating this response.

stationary phase in iron-supplemented TSB medium, no Mn-SOD activity was observed in native gels (results not shown). When 2,2-dipyridyl was included in the culture media, Mn-SOD expression occurred in mid log phase and stationary phase PAO1 cells (Fig. 4B) but only in stationary phase in the *lasI* mutant PAO-JP1 (Fig. 4C) and the *lasR* mutant PAO-R1 (results not shown). The iron sensitive expression of *sodA* in PAO1 is consistent with prior work (52, 53, 54) and demonstrated that either excess or limiting iron can override QS control of *sodA* expression in *P. aeruginosa*.

To establish whether the iron effects on Mn-SOD expression occurs at the transcriptional level, a plasmid containing a *sodA::lacZ* transcriptional fusion was transformed into PAO1, PAO-JP1, and PAO-R1, and the above experiments were repeated. As shown in Fig. 5, iron limitation increased *sodA* expression in PAO1 mid-log phase cells. Iron limitation also caused equivalent levels of *sodA* up-regulation in mid-log phase cells of PAO-JP1 (Fig. 5), even though the isozyme was not apparent in activity stains of JP1 cell extracts (Fig. 4C). Reporter gene activity was also increased in iron-starved mid log phase cells of PAO-R1, but the increase was smaller than in strains PAO1 or PAO-JP1 (Fig. 5). In stationary phase cells, up-regulation of *sodA* in response to iron-starvation also occurred. β -galactosidase reporter enzyme levels measured in PAO1, JP1, and PAO-R1 were approximately 4-, 11-, and 7-fold greater, respectively, under iron limiting growth conditions as compared to cells with ample iron. The reporter gene results with both mid log and stationary phase cells again demonstrated that QS control of *sodA* could be overridden by the iron starvation response, but also showed that the autoinducer

PAI-1 and the regulatory protein LasR were still required for maximal *sodA* expression, regardless of iron availability. Further, these studies suggested that Mn-SOD activity in the *lasI* mutant was attenuated by abnormal post-translational activity because iron starvation-based induction of *sodA* in mid log phase cells did not result in increased Mn-SOD levels in native gel SOD activity stains.

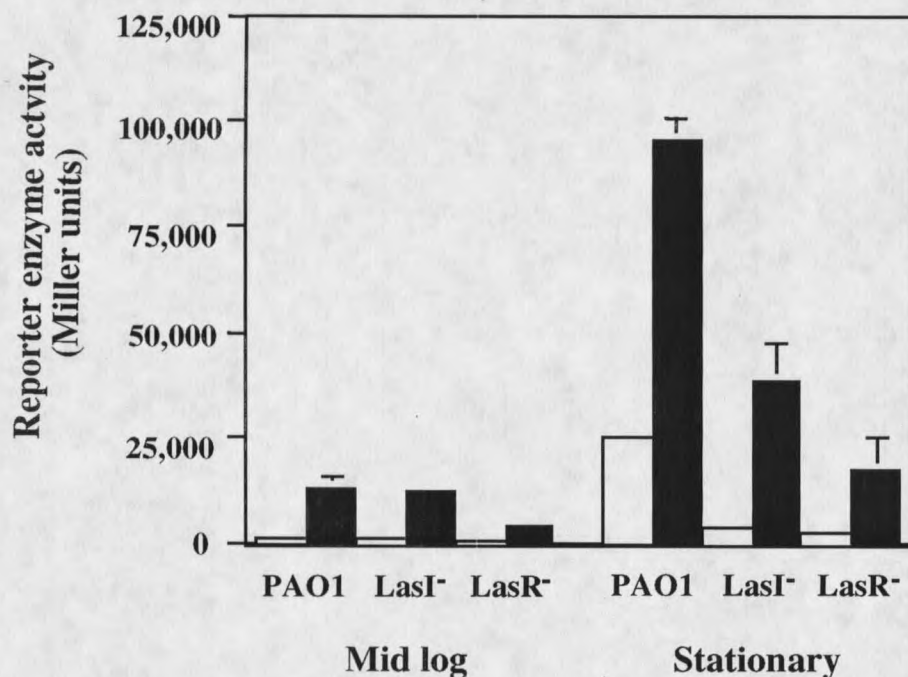


Fig.5. Transcription of *sodA* in mid log phase and stationary phase cells of PAO1, PAO-JP1, and PAO-R1 as affected by iron limitation. Relative transcription levels were determined by measuring the reporter enzyme β -galactosidase as described by Miller (77) in planktonic cells harvested at mid log (6h) and late stationary (16h) phase. Open bars, cells grown in TSB; filled bars, cells grown in TSB containing the iron-specific chelator 2,2-dipyridyl. The data represent the mean of three independent experiments (2-3 cultures per experiment). Where visible, error bars denote one standard error.

Effects of Iron Concentration on *lasI* Expression

Additional experiments were conducted to determine if the iron starvation effect on *sodA* expression could be linked to *las* gene expression. Over three independent experiments, iron limitation increased *lasI* expression in the wild-type strain by approximately 30-35% (Fig. 6). The increase, while relatively small, was highly reproducible and statistically significant. However, no increase in *lasI::lacZ* reporter activity (range; 90-150 Miller units) could be measured under the same culture conditions for the *lasI* mutant and the *lasR* mutant (results not shown), implying again that the iron-based response appeared to require LasR and PAI-1 for the maximal effect to be observed. The lack of an iron effect on *lasI* induction in the LasI⁻ mutant also demonstrated that potential alternative autoinducers were most likely not participating in the iron-stress response under the conditions used for cell culture in these experiments.

Production of Mn-SOD in Biofilms

An additional important motivation for this study was to also investigate potential differences in gene expression/regulation between biofilm cells and planktonic cells. The combination of mutants and the use of Mn-SOD as a native reporter enzyme provided an opportunity to assess the same regulatory issues when the cells were grown as biofilms and to determine if gene expression patterns were different. Surprisingly different Mn-SOD expression patterns were encountered between wild-type and QS mutant biofilms. Mn-SOD was only observed in mature (6 d) PAO1 biofilms (Fig. 7A), whereas it was expressed within the first 24 h of *lasI* mutant biofilm formation (Fig. 7B). In identical biofilm experiments with the *lasR* mutant, Mn-SOD levels were the same as observed with the *lasI* mutant (results not shown). Additional biofilm experiments were then conducted

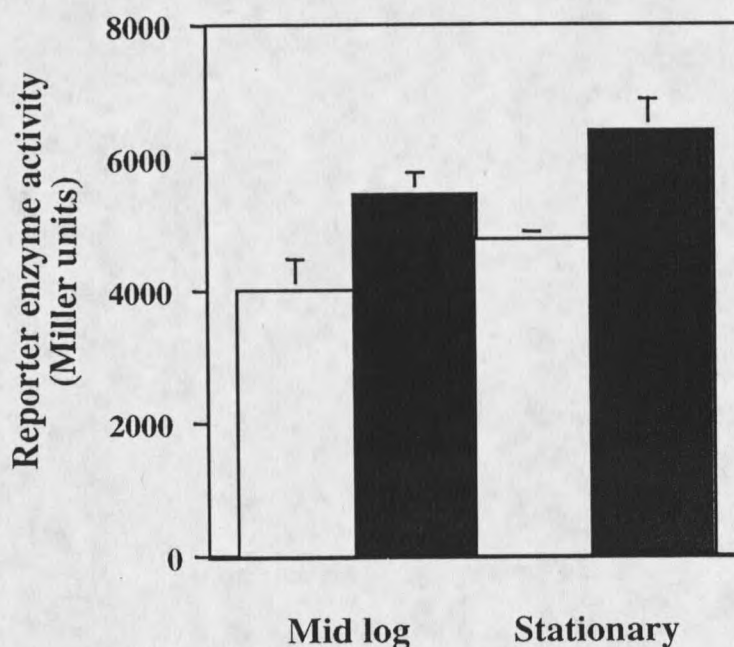


Fig. 6. Expression of *lasI* in response to iron deprivation. Wild-type strain PAO1 was transformed with pPCS223 that contains the *lasI::lacZ* reporter fusion (105), and then cultured to mid log phase or stationary phase in TSB broth (open bars) or TSB broth amended with the iron-specific chelator 2,2-dipyridyl (filled bars). The data represent the average of the means of three independent experiments (2 cultures per experiment). Error bars, where visible, represent one standard error of the three experimental means. Differences between iron treatments for each culture stage are statistically significantly different ($p = 0.01$).

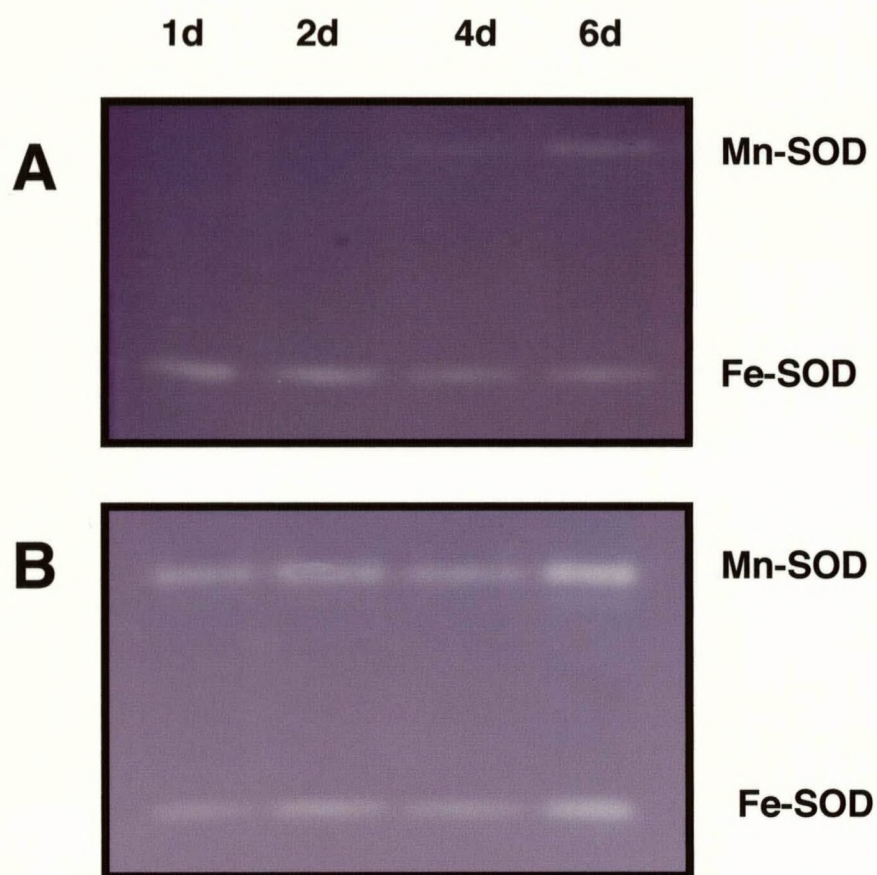


Fig. 7. Mn-SOD expression in biofilms. SOD activity stains of cell extracts (50 μ g protein per lane) of the wild-type strain PAO1 (A) and the *lasI* mutant PAO-JP1 (B) obtained from biofilms cultured with 1/10 TSB for up to 6 days. The data is representative of duplicate experiments, and the location of the Mn-SOD and Fe-SOD bands are as shown.

to assess whether biofilm cells would respond to iron manipulation as was observed with planktonic cultures. When 2,2-dipyridyl was included in the biofilm flow media, Mn-SOD was evident in PAO1 biofilms; Mn-SOD activity stains obtained from 24 h biofilm cell extracts appeared weak, but by day 2, they were roughly equivalent to the *lasI* and *lasR* mutant biofilms (Fig. 8) in the other experiments. Given the apparent constitutive *sodA* expression in *lasI* and *lasR* biofilms, further biofilm experiments sought to establish whether *sodA* expression in JP1 and PAO-R1 biofilms was still sensitive to environmental iron concentrations. This was confirmed by supplementing the biofilm medium with iron (25 μ M FeCl_3). When iron was provided at such ample levels, Mn-SOD was absent in biofilms of wild-type and both mutant strains for the duration of the experiments (up to 6 d, results not shown). During the course of all biofilm experiments, no apparent changes in the Fe-SOD activity band occurred (results not shown).

Discussion

Nutritional override of quorum sensing

Prior to the discovery of QS in this organism, the production of several gene products now known to be QS-regulated were initially found to be up-regulated by nutritional factors. An example of overlap between a nutrient limitation response and QS-regulated activity in *P. aeruginosa* is pyocyanin production. This exoproduct is typically observed in stationary phase cells and is controlled by QS (10), but can also be induced by phosphate starvation (49). The affects of other nutrients such as iron have been observed more frequently. Elastase synthesis has been shown to be QS- controlled (86, 108), however its production occurs maximally when *P. aeruginosa* is iron-limited but is

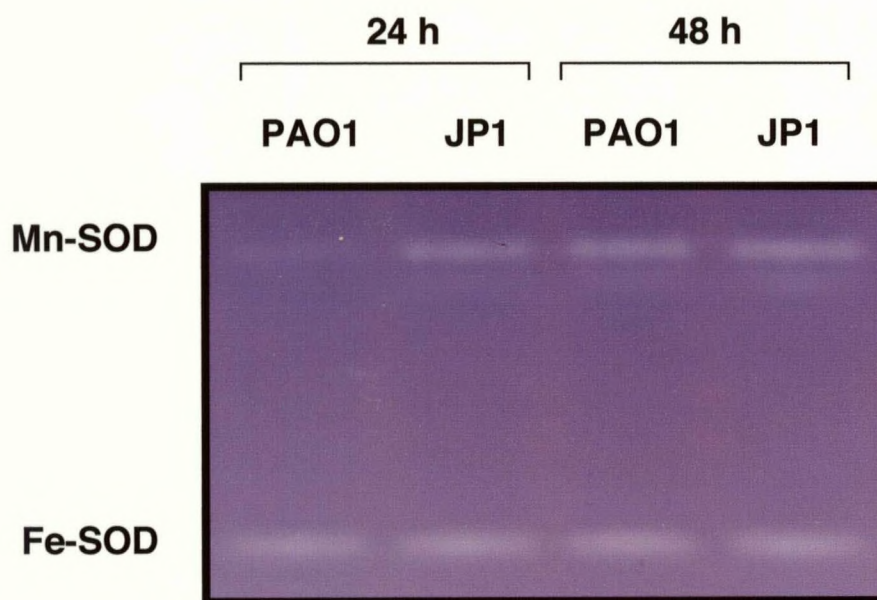


Fig. 8. Mn-SOD expression in PAO1 biofilms as affected by iron starvation. SOD activity was detected using activity stains as described in Materials and Methods. Cell extracts (50 μ g per lane) were obtained from PAO1 biofilms grown for 1d or 2d in 1/10 TSB containing the iron-specific chelator 2,2-dipyridyl, and from 1d and 2d PAO-JP1 biofilms grown in 1/10 TSB. The data is representative of duplicate experiments, and the location of the Mn-SOD and Fe-SOD bands are as shown.

restricted in the presence of iron (7, 63, 96). Expression of *sodA* has also been previously shown to be iron-regulated (52, 53, 54), and then later found to be controlled by QS (58). Reporter experiments in the current study established that *sodA* can be induced by iron limitation in either log phase or stationary phase cells of the wild-type strain (Fig. 4A, 4B, Fig. 5) and QS mutants (Fig. 5). However, relative to the wild-type strain, maximal iron stress-dependent *sodA* induction was much lower in mid log phase *lasR* cells and in stationary phase cells of both *las* mutants (Fig. 5). This implies that the autoinducer PAI-1 and the LasR regulatory protein were still required for maximum iron-starved *sodA* induction under these experimental conditions. This was particularly apparent in stationary phase cells, where QS is thought to be most active in gene regulation.

In addition, I note that apparent wild type *sodA* transcription occurred in mid log phase cells of the *LasI* mutant (Fig. 5), but it did not translate into mature Mn-SOD enzyme as measured by activity stains in native gels (Fig. 4C). While activity stains in native gels are only semi-quantitative, cell extract sample loading in these experiments was adequate to detect meaningful SOD enzyme levels, provided for reasonable strain and growth condition comparisons, and allowed for sensitive detection of this isozyme in a wild-type background. This basic observation implies that post-transcriptional processing of the *sodA* mRNA was altered in the *las* mutants. Further, it suggests that *LasI*, or the autoinducer it synthesizes (PAI-1), is involved in some as yet unknown, but essential, cellular activity at low cell densities.

The overall iron effect on *lasI* expression (Fig. 6) was small relative to increases in *lasI* transcription previously observed (71), but the increase was highly reproducible, and thus directly links the iron stress response with QS circuitry. Integration of QS with nutrient availability should not be unexpected, and it is perhaps no coincidence that QS-

based regulation is most often observed in stationary phase cultures when cell densities approach levels that represent a significant nutrient sink and indeed growth rates are declining due to limitation of some nutrient(s). Nutrients such as iron are subject to biotic and abiotic redox reactions, typically yielding insoluble precipitates under aerobic conditions. Thus, the bioavailability of such nutrients can be low regardless of local biological demand. In biofilms where cell densities can approach 10^{10} - 10^{12} cells per cm^3 (16), low nutrient bioavailability coupled with high localized nutrient demand could result in a nutrient stress response. Lazazzera (72) has recently reviewed a similar concept for *Bacillus subtilis*, and there are other reports that suggest QS and nutrient sensing in gram negative bacteria are integrated. Kjelleberg et al. have connected carbon starvation, QS, and the stringent response in a marine *Vibrio* isolate (41, 43, 82, 83, 97). Other similar interactions integrating nutrient limitation, cell communication, and the stringent response have also been reported for *Myxococcus xanthus* (48, 67, 68, 95), and thus serve to further demonstrate the complexity of cell-to-cell signaling systems.

When considering possible strategies for manipulating QS for controlling bacterial infections, the effects of multiple control mechanisms or nutritionally-based regulatory override systems must be recognized. The onset of QS-regulated gene expression relative to the accumulation of autoinducers or to other metabolites, and the timing of their accumulation relative to changes in the cell nutritional state, represents a critical area of research. To account for the observations made in this work, I examined the promoter region of the operon that contains *sodA* (Fig. 9). Iron boxes [Fur- Fe^{2+} binding site (53)] and putative LasR-PAI-1 binding sites (designated as Lux boxes) were found and are located such that under high iron conditions, the Fur repressor protein would bind as a dimer to the iron boxes, inhibiting binding and thereby reducing transcriptional activation by the LasR-PAI-1 complex. The occurrence of dual regulatory elements in the promoter

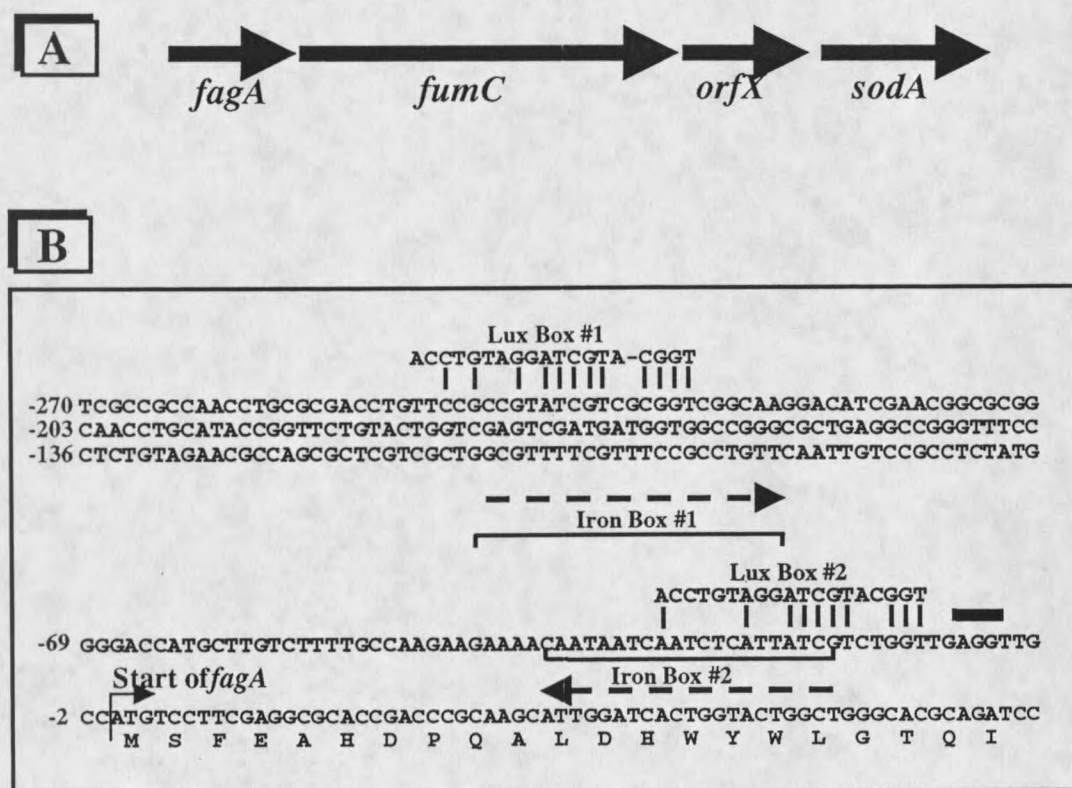


Fig. 9. A potential mechanism for dual control of *sodA* by iron-sensitive and quorum sensing circuitry. (A) Gene arrangement of the *fagA fumC orfX sodA* operon. (B) DNA sequence directly upstream of *fagA*, which is promoter proximal in the depicted operon (53, 54). The Fur regulatory protein (Encoded by *fur*, which is itself regulated by iron availability) utilizes Fe^{2+} as a corepressor (52). The Fe(II) -Fur complex directly binds to the promoter region of genes that contain a specific regulatory sequence known as the "iron box" (26). Sequence analysis suggests the presence of "iron boxes" (orientation of each shown as dashed line), which is the binding site for Fe(II) -Fur. Also, Mn-SOD production is elevated in *fur* mutants (55). Iron boxes are located at nucleotide positions -18 to -37 and at -21 to -42. Positions of putative "Lux boxes", the binding site for the PAI-1-LasR complex, are also shown at -11 to -29 and at -228 to -247. Nucleotide positions that are homologous with the consensus *Vibrio* "Lux box" are indicated by the connecting lines. Under high iron conditions, binding of the iron box by the Fe(II) -Fur complex would inhibit the LasR-PAI-1 complex from binding to the Lux box #2 region and would also inhibit transcription possibly originating upstream due to potential activation from binding at Lux box #1. Upon iron starvation, the Fe(II) -Fur complex would not be present and thus the LasR-PAI-1 complex would be free to activate transcription. Bold thick line indicates potential ribosome binding site.

region of other QS-regulated genes has not been studied, but represents an important issue for understanding and manipulating QS in bacteria.

Biofilm Gene Expression

Another important issue addressed in this study included *P. aeruginosa* biofilm cell physiology. The use of defined regulatory mutants and an endogenous reporter enzyme that had been shown to be controlled by QS and nutritional effects presented an opportunity to assess basic gene expression in biofilms and make relevant comparisons to planktonic cells. *P. aeruginosa* has been used as a model organism for studying biofilm behavior and for the development of biofilm control strategies (19), and though recent progress has been significant, *P. aeruginosa* biofilm cell physiology is still only poorly understood. Particularly lacking is information regarding gene expression/regulation. Gradients in metabolic activity have been shown to exist in *P. aeruginosa* biofilms, and some information regarding adaptive gene regulation has also been recently published (62). These studies examined *P. aeruginosa* gene regulation in response to environmental stress, showing, for example, that induction of *phoA* in response to phosphate limitation only occurred in the upper region (ca. 20%) of the biofilm (62).

In the present study, differences in *sodA* expression between wild-type and QS mutant biofilms were significant. Whereas Mn-SOD activity was not detected in wild type biofilms until after 6 d, it was detectable within the first 24 h of biofilm formation by both *las* mutants. The notable lack of Mn-SOD in wild-type biofilms implies the cells were not limited for iron and is consistent with an analysis of iron availability under these growth conditions. Chemical analysis of TSB medium showed an iron concentration of 15 μM . The iron content in the 1/10 TSB used for biofilm experiments would be proportionately lower, but the constant flow would continuously deliver fresh medium over the

suggests that at least in the case of *sodA* control, basic iron sensor-response circuitry is unchanged for cells growing in either setting. The basis for the apparent up-regulation of *sodA* in the QS mutant biofilms under conditions that are not obviously iron-limiting is not currently understood, but is the focus of continuing experiments.

In summary, this study demonstrated that: i) the nutritional status of the cell must be taken into account when evaluating QS-based gene expression; ii) QS-based gene regulation models based on studies with planktonic cells must be modified in order to explain biofilm gene expression behavior; and iii) gene expression in biofilms is dynamic. In addition, the results from the *las* mutant biofilm studies implied that QS regulation can exert negative regulatory control. Determining physiological differences between biofilms and planktonic cultures is critical to the understanding and eventual treatment of *P. aeruginosa* infections such as that found in the cystic fibrosis lung, or for removing problematic biofilms from industrial or environmental settings. Previous studies showed evidence that bacteria in lung tissue grow under iron limited conditions (12, 30, 46). Further experiments aimed at understanding *in situ* conditions and gene expression, particularly as related to *P. aeruginosa* infections, should offer significant opportunity to improve our understanding of disease and its control.

CHAPTER 4

THESIS CONCLUSION AND SUMMARY

Although extensive research has been conducted on *Pseudomonas aeruginosa* and its quorum sensing circuitry, it is evident from the research conducted in this

thesis that there is a lot that is unknown. This thesis provides an initial foundation that explains how factors other than cell density can control the expression of quorum sensing regulated genes. Since iron concentration has been determined to up- or down-regulate the quorum sensing regulated gene, Mn-SOD, it is not inconceivable that globally regulated genes can be controlled by more than one factor. Even though this conclusion is novel, it is not surprising.

Biofilm physiology has also been studied in this thesis. The research confirms previous hypotheses that biofilms are dynamic and their physiology differs from planktonic cultures containing the same organism(s). This conclusion verifies the theory that in order to determine how bacteria evade antibiotics in human infections, it is crucial to study these microorganisms in biofilm cultures since this is how they usually occur in the body.

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