The role of catalase in Pseudomonas aeruginosa biofilm resistance to hydrogen peroxide
by James Garrett Elkins

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Land Resources and Environmental Sciences
Montana State University
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Abstract:
Microbial biofilm formation on man-made surfaces can profoundly impact human health and welfare. Biofilms readily develop on medical implants such as catheters and artificial joints causing chronic infections. Industrial processing systems are also plagued with the accumulation of biofilm in pipes, heat exchangers, cooling towers, and other equipment resulting in the loss of system efficiency. Biofilm bacteria are tremendously more difficult to kill with antimicrobial agents than freely suspended organisms. The basis for biofilm resistance to antimicrobial agents has been investigated but, the primary mechanisms responsible for this phenomena are still poorly understood. It has been hypothesized that biofilm bacteria are able to rapidly adapt to an antimicrobial agent and neutralize it with protective proteins/enzymes. This hypothesis was addressed in this study by investigating the adaptive response of Pseudomonas aeruginosa biofilms to the oxidizing biocide, hydrogen peroxide (H_2O_2). P. aeruginosa expresses two catalase enzymes in defense against H_2O_2 known as KatA and KatB. These enzymes catalyticaly degrade H_2O_2 into oxygen and water. P. aeruginosa mutants that are unable to synthesize either KatA or KatB were tested for their susceptibility to H_2O_2 when grown as planktonic cells and biofilms. Biofilms lacking KatA activity were more susceptible to H_2O_2 than the wild-type or KatB- strain but remained much more resistant to the biocide than planktonic cultures. However, the absence of catalase activity in KatA- biofilms allowed efficient biofilm removal with H_2O_2. Biofilms unable to synthesize the inducible KatB catalase were nearly equal to the wild-type strain with respect to H_2O_2 resistance. Using spectrophotometric assays, activity gel staining techniques, and reporter enzyme measurements, catalase expression was monitored in biofilms. Specific catalase levels were essentially equal for biofilms and planktonic cells. Biofilms exposed to relatively high concentrations of H_2O_2 induced the KatB catalase but induction patterns did not differ from planktonic cells exposed to lower H_2O_2 concentrations. In conclusion, constitutive catalase expression is necessary for optimal biofilm resistance to H_2O_2 but other mechanisms of resistance must exist. Biofilms are capable of adapting to exogenous H_2O_2 by inducing KatB synthesis but this enzyme is relatively insignificant to overall biofilm resistance to short-term H_2O_2 exposure.

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BIOFILM RESISTANCE TO HYDROGEN PEROXIDE

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of

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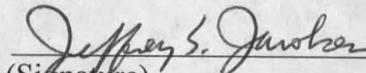
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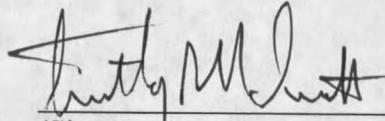
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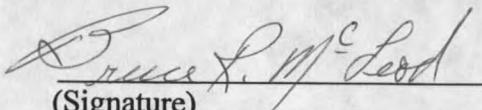Approved for the Department of Land Resources and Environmental Sciences

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ABSTRACT

Microbial biofilm formation on man-made surfaces can profoundly impact human health and welfare. Biofilms readily develop on medical implants such as catheters and artificial joints causing chronic infections. Industrial processing systems are also plagued with the accumulation of biofilm in pipes, heat exchangers, cooling towers, and other equipment resulting in the loss of system efficiency. Biofilm bacteria are tremendously more difficult to kill with antimicrobial agents than freely suspended organisms. The basis for biofilm resistance to antimicrobial agents has been investigated but, the primary mechanisms responsible for this phenomena are still poorly understood. It has been hypothesized that biofilm bacteria are able to rapidly adapt to an antimicrobial agent and neutralize it with protective proteins/enzymes. This hypothesis was addressed in this study by investigating the adaptive response of *Pseudomonas aeruginosa* biofilms to the oxidizing biocide, hydrogen peroxide (H_2O_2). *P. aeruginosa* expresses two catalase enzymes in defense against H_2O_2 known as KatA and KatB. These enzymes catalyticaly degrade H_2O_2 into oxygen and water. *P. aeruginosa* mutants that are unable to synthesize either KatA or KatB were tested for their susceptibility to H_2O_2 when grown as planktonic cells and biofilms. Biofilms lacking KatA activity were more susceptible to H_2O_2 than the wild-type or KatB⁻ strain but remained much more resistant to the biocide than planktonic cultures. However, the absence of catalase activity in KatA⁻ biofilms allowed efficient biofilm removal with H_2O_2. Biofilms unable to synthesize the inducible KatB catalase were nearly equal to the wild-type strain with respect to H_2O_2 resistance. Using spectrophotometric assays, activity gel staining techniques, and reporter enzyme measurements, catalase expression was monitored in biofilms. Specific catalase levels were essentially equal for biofilms and planktonic cells. Biofilms exposed to relatively high concentrations of H_2O_2 induced the KatB catalase but induction patterns did not differ from planktonic cells exposed to lower H_2O_2 concentrations. In conclusion, constitutive catalase expression is necessary for optimal biofilm resistance to H_2O_2 but other mechanisms of resistance must exist. Biofilms are capable of adapting to exogenous H_2O_2 by inducing KatB synthesis but this enzyme is relatively insignificant to overall biofilm resistance to short-term H_2O_2 exposure.

CHAPTER 1

INTRODUCTION

Prokaryotic success in many different environments can likely be attributed to the tendency of bacteria to form biofilms. Biofilms arise when planktonic cells adhere to a surface and undergo phenotypic changes including the expression of genes involved in exopolysaccharide (EPS) synthesis (Davies et al., 1993, Davies and Geesey, 1995). Through binary fission, single cells become microcolonies and eventually, a highly complex microbial community is established. Biofilms consist of microorganisms, a polymer matrix surrounding each cell, a system of voids and flow channels allowing fluid convection throughout the biofilm, and other abiotic materials that become trapped by the EPS matrix (Costerton et al., 1995). As a common soil/water microorganism and opportunistic pathogen, *Pseudomonas aeruginosa* occupies many diverse niches in nature. Since *P. aeruginosa* is often isolated from natural biofilms, this species serves as an appropriate model organism for biofilm research.

Biofilm formation is problematic in many different medical and industrial situations. For instance, the colonization of catheters and prosthetic devices by microorganisms results in chronic systemic infections, especially in immunocompromised patients. *P. aeruginosa* colonization and biofilm formation is often the cause of morbidity and mortality in patients suffering from cystic fibrosis. Regrowth

of coliform biofilms in drinking water distribution systems can also impact human health. In industrial systems, heat exchangers are less efficient when fouled with biofilms. Biofilm accumulation in pipe systems results in pressure loss and poor flow characteristics. The warm, oxygenated waters found in cooling towers are especially conducive to biofilm formation, resulting in loss of cooling efficiency and structural integrity. The corrosion of metal surfaces is also enhanced when colonized with bacteria (Costerton et al., 1988).

Antimicrobial agents are widely used to control unwanted microbial growth in medical and industrial situations. Antimicrobial agents include all classes of antibiotics (i. e. β-lactams) as well as low molecular weight, broad spectrum biocides such as hypochlorous acid and hydrogen peroxide. The use of these compounds to kill or remove biofilms often proves difficult since sessile bacteria are much more resistant to killing than freely suspended organisms (Ashby et al., 1994; Brown and Gilbert, 1993; LeChevallier et al., 1988). Currently, the basis for the reduced efficacy of antimicrobial agents against biofilm organisms is poorly understood. Knowing the underlying mechanisms responsible for biofilm resistance will allow for the development of improved antimicrobial compounds as well as better application protocols for compounds already in use.

Several biofilm structural and physiological characteristics have been assessed for their role in biofilm resistance to antimicrobial agents. The EPS matrix may provide protection by establishing a reaction/diffusional barrier that prevents penetration of the antimicrobial compound into the biofilm (Nichols et al., 1989; Tresse and Junter, 1995). Poor biocide penetration has been shown to enhance the survival of biofilm bacteria

although some investigators argue that slow penetration alone is insufficient to account for the resistance biofilms display to many bactericidal compounds (Brown and Gilbert, 1994; Stewart, 1996). Low physiological activity has been demonstrated in *P. aeruginosa* biofilms due to oxygen limitation (Xu et al., 1998). The mode of action for many antimicrobial agents such as β-lactams is growth dependent; therefore, growth limiting conditions within a biofilm may account for reduced susceptibility.

Another mechanism that may contribute to the reduced efficacy of antimicrobial agents against biofilm organisms is physiological adaptation to the compound itself. Upon exposure to a particular agent, bacteria may possess the genetic and physiological capability of synthesizing proteins/enzymes that are able neutralize or catalyticaly degrade the compound in use. Physiological adaptation to antimicrobial agents has been observed in biofilm disinfection experiments but the actual mechanisms responsible for adaptation are not understood (Sanderson and Stewart, 1997).

Adaptive stress responses to oxidizing agents have been well characterized in enteric bacteria. One of the best described adaptive responses to an oxidizing agent includes the expression of catalase in defense against the oxidizing biocide, hydrogen peroxide (H_2O_2). Catalase enzymatically degrades H_2O_2 into oxygen and water. *E. coli* increases total cellular catalase activity when exposed to sub-lethal doses of H_2O_2, enabling the organism to become resistant to much higher concentrations of the oxidant (Christman et al., 1985). Catalase expression and its role in scavenging H_2O_2 has been studied in organisms such as *E. coli* for nearly 30 years and a comprehensive review of this work is provided in this thesis. Since past research has been conducted almost exclusively with planktonic organisms, little is known about the importance of catalase

expression in biofilm resistance to H_2O_2.

Hassett and co-workers (Brown et al., 1995; Hassett et al., 1992) have studied catalase expression in planktonic *P. aeruginosa* cells under both oxidative and non-oxidative conditions. Two genes, termed *katA* and *katB*, encoding two distinct catalases have been identified in *P. aeruginosa*. The *katA* gene is constitutively expressed throughout the aerobic growth cycle while *katB* transcription occurs only when cells are under oxidative attack by H_2O_2. Since adaptation to H_2O_2 has been described for planktonic cells of *P. aeruginosa*, relatively simple yet fundamentally important questions could be addressed in this thesis regarding the importance of adaptive responses in biofilm resistance to H_2O_2.

By assessing the susceptibility of mutant biofilms that are unable to synthesize either KatA or KatB to H_2O_2, the following hypotheses were tested:

1. *P. aeruginosa* biofilms that cannot constitutively express catalase (KatA⁻) will be hypersusceptible to H_2O_2.

   o KatA mutants have essentially no catalase activity. What fraction of overall biofilm resistance to H_2O_2 can be attributed to constitutive catalase expression?

2. Catalase (KatB) induction in response to H_2O_2 is necessary for biofilm resistance to H_2O_2.

   o If *P. aeruginosa* biofilms are unable to initiate an adaptive response to peroxide by inducing KatB, will they be more susceptible to H_2O_2 relative to wild-type biofilms.

By studying the expression patterns of both enzymes under oxidative and non-oxidative stress conditions, these hypotheses were tested:

3. Even in the absence of H_2O_2, catalase genes (*katA*, *katB*, or both) are upregulated

in biofilms resulting in a high specific catalase activity relative to planktonic organisms.

- o Perhaps biofilms are resistant to H_2O_2 because of differential gene regulation that results in relatively high biofilm specific catalase activity.

4. *P. aeruginosa* biofilms initiate an oxidative stress response upon exposure to H_2O_2.

- o Although this has been demonstrated in planktonic cells (Brown et al., 1995), catalase expression, specifically *katB* induction, may be differentially regulated in biofilms.

This thesis begins with a comprehensive literature review (Chapter 2) covering pertinent topics such as possible mechanisms of biofilm resistance to antimicrobial agents, evidence for physiological adaptation to antimicrobials, the use of oxidizing biocides to control problematic biofilms, oxygen radical chemistry and biology, and oxidative stress genetics and physiology in enteric bacteria and *P. aeruginosa*. Chapter 3 features the main scientific study completed for this thesis in a professional publication format. In addition to assessing the importance of adaptive responses in biofilm resistance to oxidizing agents, this research represents a novel approach toward understanding basic differences in oxidative stress physiology between planktonic organisms and biofilms. Also resulting from this work, we have described an excellent model system for examining resistance mechanisms in biofilms (Hassett et al., 1999a) as well as uncover other aspects of biofilm physiology that affect biofilm resistance to oxidizing agents (Hassett et al., 1999b). These contributions should provide further insight regarding differences in biofilm and planktonic cell physiology and will hopefully assist in the development of improved antimicrobial agents and biofilm control strategies.

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CHAPTER 2

LITERATURE REVIEW

Introduction

One of the primary reasons behind the problematic nature of biofilms is their ability to resist killing by antimicrobial agents. Biofilm resistance to numerous antimicrobial compounds has been demonstrated repeatedly (Brown and Gilbert, 1993; LeChevallier et al., 1988; Sanderson and Stewart, 1997) and, to date, no antimicrobial agent has been shown to eliminate biofilms as effectively as it can suspended organisms.

Biofilm resistance appears to be universal for all types of compounds including antibiotics such as β-lactams and aminoglycosides (Anwar et al., 1990) as well as low molecular weight biocides like hypochlorous acid and monochloramine (Berman et al., 1988; Sanderson and Stewart, 1997). Biofilm resistance to such a wide range of antimicrobial agents is the basis for the problematic nature of biofilms in so many different situations. This is especially true in the prevention of nosocomial infections where conventional antibiotics are proving to be poorly effective against many types of pathogenic organisms. Biofilm formation by inherently antibiotic resistant strains of bacteria greatly increases the potential for disease causing organisms to evade disinfection.

Control of biofilms in industrial settings generally relies on the use of broad

spectrum, low molecular weight biocides that can be purchased in bulk quantities. Of

the many types of biocides employed in industry, members of the oxidizing class of

biocides remain the most widely used (Kuo and Smith, 1996). Oxidizing biocides

include halogenated compounds such as hypochlorous acid (HOCl), monochloramine

(NH_2Cl), chlorine dioxide (ClO_2), and hypobromous acid (HOBr), or oxygen derivatives

such as ozone (O_3) and hydrogen peroxide (H_2O_2). Biofilms composed of single

organisms or consortia of bacterial species have demonstrated resistance to oxidizing

biocides. LeChevallier et al. (1988) challenged planktonic and biofilm cultures of

heterotrophic bacteria isolated from drinking water and *Klebsiella pneumoniae* isolates

with varying doses of hypochlorous acid and monochloramine. Planktonic cells required

only 0.08 mg L^{-1} of hypochlorous acid or 94 mg L^{-1} of monochloramine to achieve a 99%

reduction in viability within 1 minute of exposure. Biofilms grown on the surfaces of

granular activated carbon particles, metal coupons, and glass microscope slides were 150-

to 3000 fold more resistant to equal doses of hypochlorous acid and 2- to 100 fold more

resistant to equal doses of monochloramine. The authors speculated that the decreased

reactivity of the monochloramine allowed the agent to penetrate deeper into the biofilm

thus improving its efficacy relative to hypochlorous acid.

Currently, the mechanisms responsible for biofilm resistance to antimicrobial

agents are poorly understood. Several unifying characteristics of biofilms and their

structure have been implicated in resistance. These hallmarks include cell encapsulation

within an extra-cellular polysaccharide matrix; decreased physiological activity due to

limitation of electron donors/acceptors or other essential nutrients; and the physiological

production of cell constituents capable of neutralizing biocides. These processes and their role in biofilm resistance to antimicrobial agents will now be reviewed.

Mechanisms of Biofilm Resistance to Antimicrobial Agents

Role of Extracellular Polysaccharide Matrix

One of the prominent features of any biofilm is the presence of an exopolysaccharide (EPS) matrix surrounding each cell. Biofilm formation is initiated when bacteria originating from the external milieu attach to a surface. The ability of a single cell to remain attached to a surface and initiate biofilm formation has been shown to occur through the expression of genes responsible for polysaccharide biosynthesis (Davies et al., 1993; Davies and Geesey, 1995). Cell division and the accretion of additional planktonic cells results in the development of a mature biofilm which includes an EPS matrix. Biofilm polymer matrices are highly hydrated containing up to 95% water and are responsible for the heterogeneous three-dimensional structure of biofilms which include clusters of cells (mushrooms) and flow channels (Costerton et al., 1995).

The EPS matrix of surface attached bacteria has been implicated in biofilm resistance to antimicrobial agents (Nichols et al., 1989). Primarily, the EPS matrix is thought to either slow or prevent the transport of antimicrobial agents from the bulk phase to the encapsulated biofilm cells. This could occur with the matrix serving as a simple physical barrier to convective/diffusive mass transport. Also, the antimicrobial agent could physically adsorb to the matrix thus immobilizing it. Alternatively, reactive biocides such as hypochlorous acid may act upon the EPS matrix, being stoichiometrically consumed. The role of the EPS matrix in protecting biofilm bacteria

from various antimicrobial agents has been determined experimentally. Using microelectrodes, DeBeer et al. (1994) directly measured the penetration of chlorine (added as hypochlorous acid) into *P. aeruginosa* and *K. pneumoniae* biofilms cultured on stainless steel slides. Sharp concentration gradients were established throughout the depth of the biofilm within a few minutes following the addition of chlorine. The ability of chlorine to penetrate these biofilms varied widely with concentration and exposure times, although this variation was thought to be mainly due to heterogeneity in the biofilm structure. Nevertheless, the EPS matrix of these two-species biofilms did establish a reaction-diffusion barrier, enhancing resistance to chlorine.

Artificial biofilms constructed of bacteria entrapped in agarose gel slabs (Chen and Stewart, 1996) or alginate and agarose beads (Xu et al., 1996) have been used as models to study chlorine transport into polymer matrices. Such artificial biofilms posses homogeneous matrices and consistent geometries which minimizes the variablility otherwise encountered with real biofilms. In these studies, chlorine reacted slowly with agarose and penetrated ca. 500 μm thick slab comparatively rapidly (15 min.) when cells of *P. aeruginosa* were absent. However, the entrapment of bacteria in the agarose matrix greatly retarded chlorine transport with penetration equivalent to cell free slabs occurring after 1 hour of exposure. Unlike agarose, alginate presents a significant reaction-diffusion barrier for chlorine. After 45 hours of exposure to 20 mg L^{-1} chlorine, concentrations did not reach 50% of the bulk phase at the center of 3 mm agarose beads containing 2% (w/w) alginate. Agarose/alginate beads containing *P. aeruginosa* at a density 2.5 x 10^9 cfu/cm^3 further retarded chlorine penetration. Entrapment of cells in polymer beads resulted in remarkable resistance to chlorine with 6 hours of exposure

killing only the cells near the surface of the bead.

Others have argued that the biofilm EPS matrix provides only limited or no antimicrobial resistance. Using povidone-iodine as a disinfectant against *P. aeruginosa*, Brown et al. (1995) showed that the iodine demands imparted by biofilm and planktonic samples of equal biomass were similar. Consequently, iodine reacting with the matrix could not explain the difference in resistance observed between planktonic and biofilm cells. Similarly, Nichols et al. (1989) measured the penetration of tobramycin and cefsulodin through mucoid and non-mucoid aggregates of *P. aeruginosa* and concluded that the rates of penetration were not significantly different between the two cell types. Gristina et al. (1989) tested the susceptibility of mucoid and non-mucoid strains of *Staphylococcus epidermis* to a number of antibiotics and concluded that polymer encapsulation provided no protection.

The specific role of the EPS matrix in the protection of natural biofilms from antimicrobial agents remains unclear. In general, the establishment of a diffusion/convection barrier by the EPS layer is insufficient to explain the recalcitrance of biofilms to disinfection (Quesnel et al., 1993; Stewart et al., 1996). This is supported by the fact that biofilm cells remain resistant to biocides even after system equilibrium is reached and solute concentrations within the biofilm equal those in the bulk fluid. This suggests that other mechanisms are involved in biofilm resistance to disinfection. It is likely that the nature of the biocide, primarily its reactivity with EPS, that will be important in determining the degree to which the polysaccharide matrix will have possible protective affects. Specifically, the level of protection afforded by the matrix probably depends on biofilm species composition, age, hydraulic flow conditions, and the

chemical nature of the antimicrobial agent in question.

Resistance Due to Low Physiological Activity

Gradients of metabolic activity have been demonstrated within biofilms (Huang et al., 1998; Huang et al., 1995; Xu et al., 1998). Due to reaction/diffusion limitations on the penetration of electron donors/acceptors, organisms residing near the substratum or deep within microcolonies of a biofilm are likely to be experiencing a nutrient limited physiology. Microelectrodes have been employed to demonstrate that in aerobic, heterotrophic biofilms, oxygen concentrations sharply decline with biofilm depth (Costerton et al., 1995). Xu et al. (1998) recently concluded that oxygen was the primary determinant of physiological activity in *P. aeruginosa* biofilms. Using the ability to induce alkaline phosphatase under phosphate starvation conditions as an indicator of protein synthesis, it was found that only the oxygenated fraction of the biofilm (ca. upper 30 μm) was capable of *de novo* protein synthesis. Kinniment and Wimpenny (1992) used a different approach to demonstrate physiological heterogeneity in *P. aeruginosa* biofilms. Frozen sections of biofilm were sliced horizontally and then assayed for adenylated nucleotides. Based on ratios of ATP, ADP, and AMP, an adenylate energy charge (EC_A) index could be established to predict cellular energy status. Interestingly, AMP was the dominant species of adenine nucleotide in either 55 or 105 hour old biofilms except near the biofilm surface where the ATP/ADP ratio increased. These results suggested that a large percentage of the biofilm was in a metabolically quiescent.

Antibiotic efficacy is often dependant on the target organisms being metabolically active. This is especially true for antibiotics that target growth related phenomena such

as peptidoglycan, nucleic acid, and protein synthesis. Gristina et al. (1989) determined nafcillin, vancomycin, gentamicin, and daptomycin efficacy against suspended and adherent *Staphylococcus epidermidis* and concluded that the increased resistance *S. epidermidis* biofilms displayed to all of the antibiotics was likely due to slow growth. Other studies have shown, however, that slow microbial growth rates were not responsible for increased resistance to several antibiotics applied to differing experimental conditions. Slow growth rates may not lead to *Staphylococcus aureus* biofilm resistance to high molecular weight antibiotics such as benzylpenicillin, tetracycline, and vancomycin (Williams et al., 1997). The efficacies of halogenated biocides such as iodine (Brown and Gauthier, 1993) and fluoride (Embleton et al., 1998) against biofilms have also been shown to be independent of specific growth rate.

Other phenomena related to growth are often implicated in biofilm resistance to antimicrobial agents. Profound changes occur in cells when nutrients such as carbon, nitrogen, phosphate, and/or iron become limiting (Kjelleberg, 1993). Nutrient limitation is also known to induce significant changes in cell permeability to many antimicrobial agents (Brown et al., 1990), therefore decreasing susceptibility to disinfection. Phenotypic changes elicited by nutrient limitation vary widely and many authors speculate that some aspect of a nutrient limited physiology is responsible for the increased resistance of biofilms to antimicrobial agents. Increased EPS production at the onset of stationary phase has often been attributed to antibiotic resistance (Evans et al., 1994). Although, as in many cases noted above, the presence of the EPS envelope alone cannot account for decreased susceptibility. Also, the production of an EPS matrix has been shown to result from just the initial attachment of *P. aeruginosa* to a surface (Davies

et al., 1995) which implies that formation of a polymer matrix is independent of starvation; however, extracellular polymer formation likely plays some role in the long term survival of biofilm organisms (Costerton et al., 1981).

Biofilm Physiological Adaptation to Antimicrobial Agents

Bacteria residing in a biofilm may physiologically adapt to antimicrobial agents, therefore increasing their resistance. Adaptation, in this sense, occurs when the biofilm cell expresses a phenotype that renders the bacterium less susceptible to a particular agent. Since microorganisms have always been confronted with naturally occurring antimicrobial agents, resistance mechanisms have evolved to overcome them. Adaptation strategies include the expression of genes/operons that provide nonspecific protection to various agents while other mechanisms act by irreversible enzymatic degradation. Examples include the induction of membrane bound permeases to pump antibiotics such as tetracycline out of the cell; the generation of intracellular reducing agents like glutathione which can neutralize oxidizing biocides (Chapman et al. 1993; Chesney et al. 1996); and the induction of enzymes that possess high substrate specificity against a particular agent such as β-lactamases for β-lactam antibiotics or catalase for hydrogen peroxide. The onset of physiological resistance to antimicrobials in biofilms can be elicited by exposure to the agent itself, or perhaps by the induction/repression of genes associated with biofilm formation which co-incidentally affect antimicrobial susceptibility.

Physiological adaptation to antimicrobial agents has been observed in biofilms. Giwercman et al. (1991) recorded increases in β-lactamase production in *P. aeruginosa*
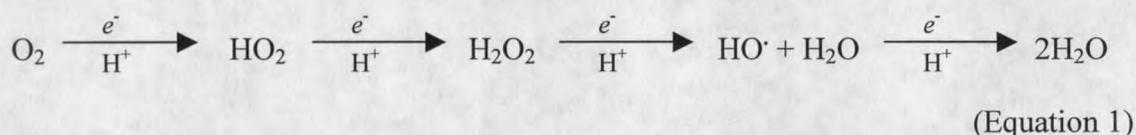
biofilms exposed to imipenem and peperacillin; the ability of the biofilms to induce

β-lactamase upon exposure to the antibiotics afforded the cells increased protection. This

report demonstrated that exposure to the antimicrobial agent was the cause of increased

β-lactamase activity rather than biofilm formation itself. When testing a mathematical

model designed to predict the kinetics of *P. aeruginosa* biofilm disinfection with

monochloramine, Sanderson and Stewart (1997) reported that biofilm cells became

increasingly resistant to subsequent doses of monochloramine at increasing

concentrations. They concluded that the adaptation to the oxidizing agent,

monochloramine, was perhaps due to an oxidative stress response being elicited by the

biofilm organisms.

Oxidizing biocides serve as the primary tools used to combat unwanted biofilm

accumulation and biofouling in industrial settings. Oxidants in the form of reactive

oxygen intermediates (ROIs) (see below) are also employed to kill invading

microorganisms *in vivo* via phagocytosis and the respiratory burst (Babior, 1987). Over

the last several decades, an impressive scientific effort has been focused on

characterizing the genetic, physiological, and biochemical mechanisms responsible for

the bacterial evasion of oxidizing agents. However, a weakness in our knowledge

regarding how bacteria adapt to and evade external oxidants exists since, in the past, only

planktonic organisms cultured in highly artificial environments have been studied. The

information obtained in this manner has unquestionably been crucial to our current

understanding of microbial genetics and physiology; nevertheless, bacteria *in situ* prefer

to associate with surfaces. Oxidative stress responses evoked by biofilm organisms are

yet to be explored and understood. The remainder of this review will cover the known

chemical species that induce oxidative stress responses in microorganisms as well as the genetics and physiology of oxidative stress as it is known to occur in *Eschericia coli* and *Pseudomonas aeruginosa.*

Reactive Oxygen Intermediates

In aerobic organisms, the dependency on molecular oxygen (O_2) as a terminal electron acceptor is not without consequence. Toxic, reactive oxygen intermediates (ROIs) are readily formed during the reduction of O_2 to H_2O through aerobic metabolism. This is due to the paramagnetic nature of O_2 which possesses unpaired electrons in separate orbitals of parallel spin. In order to avoid formation of ROIs, simultaneous reduction of each unpaired electron must occur by another electron of complementary spin. Univalent, single electron reductions frequently occur through aberrant electron flow in membrane associated electron transport systems, thus forming both oxygen radicals and nonradical, but unstable, compounds (Fridovich, 1978; Demple, 1991). ROIs are known to cause damage to nucleic acids (Imlay and Linn 1988), proteins (Tamarit et al., 1998), and lipids (Farr and Kogoma, 1991). As shown in Equation 1, three distinct oxygen species may be created as O_2 is reduced to water:

$$O_2 \xrightarrow[H^+]{e^-} HO_2 \xrightarrow[H^+]{e^-} H_2O_2 \xrightarrow[H^+]{e^-} HO^{\cdot} + H_2O \xrightarrow[H^+]{e^-} 2H_2O$$

(Equation 1)

Superoxide (O_2^-)

The superoxide anion radical is generated via a single electron reduction of molecular oxygen and is the most readily formed of the ROIs (Fridovich, 1978). In