ENVIRONMENTAL AND GENETIC FACTORS LEADING TO *MYCOBACTERIUM AVIUM* BIOFILM FORMATION

by

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This dissertation has been read by each member of the dissertation committee and has been found to be satisfactory regarding content, English usage, format, citation, bibliographic style, and consistency, and is ready for submission to the Division of Graduate Education.

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Henriette Geier

April 2008

DEDICATION

Es ist nicht genug zu wissen, man muß auch anwenden.
Es ist nicht genug zu wollen, man muß auch tun.

[Knowing is not enough; we must apply.
Willing is not enough; we must do.]

Johann Wolfgang v. Goethe

Dieses Werk ist meinen Eltern gewidmet, die immer an mich geglaubt haben und mich in allen meinen Projekten unterstützt haben, auch wenn es manchmal schwer für sie war. Danke, dass Ihr mir Flügel gegeben habt … und Wurzeln.

I would like to dedicate this dissertation to my parents who always believed in me and supported all my ideas and dreams, even though it was hard for them at times.
Thanks for giving me wings … and roots.

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ABSTRACT

This dissertation investigated the role of environmental and genetic factors that lead to biofilm formation by the environmental and waterborne pathogen *Mycobacterium avium.* The bacterium causes respiratory and also cutaneous disease predominantly in immunocompromised patients, but infection also occurs in otherwise healthy individuals. *M. avium* has been frequently isolated from drinking water and it has been shown that the bacterium forms biofilms in drinking water systems. Biofilms show enhanced resistance to antibiotics and disinfectants and therefore are difficult to combat.

Biofilm formation is a complex multistep process, and the bacteria change their physiology quite significantly upon entering the attached life style. The process is initiated by a number of triggers that are of both environmental and microbial origin. In this dissertation, biofilm formation was investigated using three different approaches. Firstly, the effect of the universal quorum-sensing signal autoinducer-2 (AI-2) was investigated. The addition of AI-2 to *M. avium* cultures results in enhanced biofilm formation and transcriptional studies revealed that the bacterium reacts to the compound with upregulation of the oxidative stress response. Oxidative stress was then directly investigated by addition of hydrogen peroxide, also resulting in enhanced biofilm formation. Therefore, it was concluded that oxidative stress leads to *M. avium* biofilm formation. Furthermore, the importance of the mycobacterial cell wall was studied by comparing biofilm formation of mutants in cell wall-related proteins with the parent strain. These studies revealed a role in biofilm formation that is dependent on the material properties of the surface.

Finally, the effect of other bacterial strains and their metabolic by-products on *M. avium* biofilm formation was studied, as well as the influence of the medium. Although the presence of other bacteria influenced biofilm formation, concentration of the important co-factor iron was shown to be particularly significant in enhancing the process.

Collectively, these data suggest that *M. avium* biofilm formation is enhanced when the bacterium encounters oxidative stress or other bacteria that may represent potential competitors for nutrients or space. It was also shown that the mycobacterial cell wall plays an important part in the attachment process.

CHAPTER 1

Jedem Anfang wohnt ein Zauber inne.

[An enchantment is inherent in every beginning]

Herrmann Hesse

THE SCOPE OF THE THESIS

The goal of this thesis was to investigate the environmental and genetic factors that contribute to *Mycobacterium avium* biofilm formation under conditions similar to drinking water systems. Chapter 2 introduces *M. avium* as a waterborne and opportunistic pathogen with many exceptional features, such as inherent drug resistance, which is in part due to its extraordinary cell wall. The concept of biofilms as matrix-enclosed microbial communities and their impact on health and the environment will also be discussed. Furthermore, the chapter introduces quorum sensing, the cell-to-cell communication in bacteria, and its universal signaling molecule, Autoinducer-2 (AI-2). Factors that are believed to lead to biofilm formation, such as oxidative stress and iron availability, are considered.

The objective of the work presented in Chapter 3 was to investigate the role of the universal quorum-sensing molecule AI-2 in biofilm formation. Addition of AI-2 at different concentrations to *M. avium* cultures indicated a role of AI-2 in the process of biofilm formation. Whole-genome microarray analysis in the absence and presence of AI-2 revealed a set of five genes that were significantly upregulated in response to the

molecule. Analysis of those five genes indicated a role in the oxidative stress response, similar to that obtained by addition of hydrogen peroxide. In turn, hydrogen peroxide added to *M. avium* cultures also induced biofilm formation. Therefore, it was concluded that AI-2 acts as an oxidative stressor rather than a quorum-sensing signal in *M. avium* cultures. It is intriguing that AI-2, a molecule produced a by a wide variety of bacterial species triggers a response in *M. avium*, a bacterium that is unable to produce the molecule itself. Therefore, the word parainducer as opposed to autoinducer, was introduced.

The work presented in Chapter 4 aimed to investigate the role of the bacterial cell wall in biofilm formation. It was hypothesized that serovar-specific glycopeptidolipids (ssGPLs) are involved in mycobacterial biofilm formation. *M. avium* strains with mutations in the synthase of these cell envelope-related proteins were tested for their ability to form biofilms on a number of different materials under different environmental conditions. The adherence of the cells to polyvinyl chloride microtiter plates, plastic and glass chamber slides and stainless steel coupons in a recirculation system were tested. The study revealed that the mutants did not adhere to the polyvinylchloride, they attached very well to the chamber slides, but showed different morphologies than the parent strain. In the recirculation system, the cells attached in equal numbers to the stainless steel coupons; however, the parent strain accumulated in greater numbers in the planktonic phase.

These experiments show that the cell wall properties of *M. avium* are very important for the bacterium's ability to attach to surfaces. In addition, this study showed that the characteristics of the surface directly influence the bacterial adherence.

Chapter 5 describes how the presence of other bacterial species influences biofilm formation in *M. avium.* Experiments conducted with cell-free supernatants from a number of both gram-positive and gram-negative bacteria showed that some of the supernatants had either an enhancing or inhibitory effect on *M. avium* biofilm formation. Time-course experiments revealed that depending on the growth stage of the tested bacteria, the influence of their supernatants on *M. avium* cultures was dependent at least in part on the iron metabolism of the cells. qRT-PCR experiments carried out with *M. avium* cultures grown in the presence and absence of iron revealed that iron induces oxidative stress in bacteria and therefore indirectly promotes biofilm formation.

In summary, this dissertation highlights the importance of some of the environmental and biological factors leading to increased biofilm formation in the opportunistic environmental pathogen *M. avium*. Chapter 3 of this dissertation is reproduced with permission from Geier, H., S. Mostowy, G. A. Cangelosi, M. A. Behr, and T. E. Ford. 2008. Autoinducer-2 triggers the oxidative stress response in Mycobacterium avium, leading to biofilm formation. Appl Environ Microbiol 74:1798-804. Chapter 4 was part of a study carried out in collaboration with Dr. Gerard Cangelosi and published in Freeman, R., H. Geier, K. M. Weigel, J. Do, T. E. Ford, and G. A. Cangelosi. 2006. Roles for cell wall glycopeptidolipid in surface adherence and planktonic dispersal of Mycobacterium avium. Appl Environ Microbiol 72:7554-8.

CHAPTER 2

INTRODUCTION

Biology and Characteristics of Mycobacteria

Overview

Mycobacteria are a unique and large group of microorganisms that inhabit a wide variety of environments. Over 70 *Mycobacterium* species have been defined, 30 of which cause disease in humans and animals. They can be divided into two groups: the strictly pathogenic mycobacteria, such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*, and the environmental mycobacteria, represented among others by the *Mycobacterium avium* complex (MAC), which is comprised of *M. avium* subspecies *avium* (MAA), *M. avium* subspecies *paratuberculosis* (MAP), *M. avium* subspecies *silvaticum* and *M. intracellulare* (14). In order to distinguish between those *M. avium* strains that were isolated from birds (*M. avium* subspecies *avium*) and those that have their origin in mammalian (including human) hosts, a new designation was proposed: *M. avium* subspecies *hominissuis* (72), which is also a member of the MAC.

M. tuberculosis, the causative agent of tuberculosis, and *M. leprae*, the causative agent of leprosy, have probably been present in humans since antiquity, with references to both diseases in the Old Testment.

The pathogenicity of *M. avium* was first recognized in AIDS patients, and is a frequent cause of infection in humans and animals. Members of the MAC are the main source for non-tuberculous mycobacterial infections in developed countries. They infect

mainly the immuno-compromised, however, lymphadenitis in children, respiratory infections, skin lesions, intestinal and disseminated disease have been diagnosed in otherwise healthy individuals (10, 34). The cells can invade and multiply within host cells and cause fever, night sweats and weight loss (50). An increasingly recognized source of infection is water; drinking water, swimming pools and hot tubs are niches for mycobacterial colonization due to its inherent resistance to disinfectants and its tolerance to high temperatures. The hydrophobic nature of the cells allows them to aerosolize, which can lead to granulomatous pneumonitis in people who are frequently exposed to these sources, such as lifeguards (90). Other sources include food or contact with animals.

Epidemiology studies employing DNA finger printing techniques, serology studies and other molecular tools have established that person-to-person transmission is unlikely to occur (70, 84, 110).

Physiology and Genetics

A number of mycobacterial strains have been sequenced so far, showing that the genome sizes differ between the strictly pathogenic and the environmental mycobacteria. The environmental mycobacteria have larger genomes than *M. tuberculosis* and *M. leprae*, reflecting their need to adapt to a wide variety of habitats. The genome sizes range from 3.3 mp in *M. leprae* and 4.4 mp in *M. tuberculosis* to 5.5 mp in *M. avium* and 6.5 mp in *M. marinum.*

Genetic manipulation has proven to be difficult in mycobacteria and site-specific mutations are hard to accomplish (78). Transposon mutagenesis generates stable, single

insertions that can be afterwards linked to the gene. These mutations allow for insertions in every gene of the bacterial chromosome, allowing the generation of efficient transposon systems.

Mycobacteria are equipped with an exceptional cell envelope. The presence of mycolic acids is unique to the genus *Mycobacterium* (52). The cell wall consists of an arabinogalactan-peptidoglycan-mycolic acid layer, which makes it thick and waxy, resulting in the hydrophobic nature of the cell (5, 6). This hydrophobicity enables the cells to adhere to many surfaces as well as to be readily aerosolized (34).

Significant ecological niches (and therefore sources of infection) are water and soils. Due to their physiologic ecology, environmental mycobacteria are able to withstand harsh conditions. The bacteria have an acidic pH optimum and are able to grow under reduced oxygen concentrations, which favor soils and natural waters as habitats (9, 41, 61, 86). In addition to their acid and low-oxygen tolerance, the bacteria can also tolerate high salt concentrations, which enables them to thrive in brackish water, seawater and wastewater, which are often sources for drinking water (42).

Most of the environmental mycobacteria, including MAC, are slow growers with generation times of about one day. The slow growth results from the fact that *M. avium* only has one rRNA cluster at its disposal (7), and, in addition, the cells have high energy requirements for the synthesis of long chain fatty acids (C_{60}-C_{80}), lipids and waxes (8). The slow growth can be advantageous in that the cells can survive starvation and antimicrobial and disinfectant exposure. The cells are resistant to antibiotics, ozone and chlorine-based disinfectants and heavy metals (32, 87, 106). Mycobacteria are considered oligotrophs, which means that they can not only persist but also grow in natural and

drinking water. This has been demonstrated by Falkinham et al. (33), by comparing MAC numbers recovered from treated and untreated drinking-water systems and raw water sources. The fact that the mycobacterial numbers were higher in the distribution system samples (about 25 000 fold) than in those collected just after treatment suggests that the bacteria proliferate in the distribution system, potentially attached to the drinking water pipes in biofilms (33). This also suggests that the biofilm serves as a reservoir for *M. avium* within the distribution system. Due to their tolerance to high temperatures, some mycobacterial species are more often found in the hot water than in the cold water supplies (109).

The presence of mycobacteria in drinking water and irrigation systems results in their occurrence in recreational waters, such as swimming pools, hot tubs and spas (31, 46, 56, 59).

Biofilms

Overview

Biofilms are complex microbial communities that live attached to surfaces. Once the cells are attached, their physiology changes significantly and they start producing a protective layer consisting of extracellular polymeric substances (EPS) that quite colloquially can be described as “slime”. Due to that slimy protective layer and other changes in their physiology, many biofilm cells are much more resistant to antibiotics and disinfectants than their planktonic counterparts (100). They form on ship hulls, where biofouling increases the roughness leading to an increase in hydrodynamic drag of the vessel. Bacteria colonize all sorts of industrial filters and in water distribution pipes

where they can cause serious economic consequences (20). In medical settings, biofilms can form on medical devices, such as catheters, bronchoscopes, medical implants, contact lenses and many more (22, 37, 108). Biofilms are also common on biotic surfaces; such as teeth (dental plaque) or lung epithelia (in patients with cystic fibrosis infections) or open wounds causing infections that are very hard to combat (1, 54, 67). Due to their increased resistance to antibiotic treatment, infections are very persistent and often the biofilm has to be removed mechanically.

Biofilms are the predominant bacterial mode of growth in the environment, since the attached life style provides protection under harsh conditions, such as high shear stress or nutrient-limitation (82, 96).

Biofilm Formation

Biofilm formation is a complex process that can be divided into several stages and is dependent on a number of environmental and genetic factors. The initial microbial adhesion events are characterized by the physico-chemical properties of the surface and the bulk fluid. The process starts with the adsorption of macromolecules on the surface, thus forming a conditioning film that then enables the attachment of the cells. The components of the conditioning film are mostly small organic molecules. In aquatic and terrestrial environments, the conditioning layer consists mostly of polysaccharides, glycoproteins and humic acids. (16).

Both the macromolecules and the cells are transported to the surface by means of mass transport. The most important component is the convective mass transfer, which is dependent on the hydrodynamics at the site of attachment. Within pipes transporting

potable water for example, two main flow conditions occur: laminar and turbulent flow. Laminar flow shows parallel smooth flow patterns with little or no lateral mixing, whereas the turbulent flow is random and chaotic allowing for bacteria and nutrients to be mixed and transported nearer to the surface. A combination of convection, diffusion and sedimentation enables the close approach between surface and cells needed for adhesion to occur (12). It is very important to take microbial mass transport into account when biofilm experiments are to be compared. The attachment behavior of cells in otherwise identical experimental settings can be different under different hydrodynamic settings.

The initial adhesion can be mediated by non-specific attractive Lifshitz-van der Waals Forces, whereas electrostatic forces are mostly repulsive since both the bacterial cell envelope and the majority of surfaces are negatively charged. These repulsive forces become operative at distances of 10-20 nm and are overcome at a distance of approximately 5 nm (12). To overcome this distance, specific adhesion receptors are needed to facilitate strong adhesion. Fimbriae, the proteinacious, non-flagellar appendages as well as flagella are involved in the attachment process by overcoming the initial electrostatic repulsion barrier between cell and substratum (21).

Biofilm formation is dependent on a large number of environmental cues and signals, which will be discussed more in detail in the following chapter. The physico-chemical models help to explain the initial stages of biofilm formation, but the biological factors are very important for the attachment and maturation of a biofilm. It has been shown that proteinaceous cell surface structures, such as pili, fimbrae, flagella and curli are crucial for the early attachment processes (3, 80, 83). These are structural components that serve as sensory systems for the environmental cues leading to biofilm formation.

Flagella and type IV pili mediate motility, which has been proven to be essential for initial biofilm formation (80). The flagella-mediated motility is important for the early contact of the cells with the surface, whereas the type IV-pili-mediated twitching motility is important for the formation of microcolonies and the stabilization of the biofilm (80).

Following the first irreversible step the cells establish a permanent foothold on the surface. At this stage, EPS production sets in. The regulation of this transition has been demonstrated by Garrett and co-workers, who showed that the expression of the sigma factor AlgT (AlgU) required for alginate synthesis resulted in downregulation of flagellar biosynthesis genes in *Pseudomonas aeruginosa* (39). Davies and co-workers were the first to show contact-induced gene expression in *Pseudomonas aeruginosa* when they demonstrated a 3-5 fold upregulation in alginate expression in recently attached cells compared to their planktonic counterparts (24). Irreversible attachment and EPS production represent the onset of biofilm maturation. As the cells are growing, the biofilm develops into elaborate three-dimensional structures, a process which is among other factors regulated by quorum sensing (25). The cells start to differentiate within the biofilm community and take on specialized functions, comparable with multicellular organisms (103).

After the full development of a biofilm is achieved, cells begin to senesce and detach. Individual cells as well as bigger parts can leave the biofilm due to nutrient depletion, quorum sensing or shear forces (79, 85, 102). Detachment can be due to physical factors, such as erosion, shear, sloughing or abrasion; or due to physiological factors, for example proteases produced by biofilm cells (111). Depending on the internal cohesivity and adhesivity, the whole biofilm or parts of it can detach (12, 89).

Conclusion

Biofilms are highly structured microbial communities; and within these communities the cells differentiate biochemically and morphologically. Biofilms seem to be the predominant mode of growth in the environment and can give valuable insight into how pathogens spread in the environment.

Most laboratory studies are performed with monospecies biofilms, which have the advantage that the growth conditions can be kept constant in order to obtain reproducibility for the laboratory experiments. However, most environmental biofilms consist of many different bacterial species, algae, protozoa and other microorganisms . These communities are challenging to study, but give valuable insight into the interactions between species in the environment.

Quorum Sensing

Introduction

Quorum sensing is a term that describes the communication between bacteria. This communication occurs via small signaling molecules, the so-called autoinducers (AI). The cells produce these AI in order to convey information within the population, and in response, the population reacts by changing its behavior. Therefore, the population acts as a multicellular organism (112). By definition, quorum sensing is cell density dependent; once a threshold concentration of cells (and therefore AI produced by the cells) is reached, the cells switch their behavior in a concerted action (73). Three different classes of AI have been identified so far: Acylated homoserine lactones (AHLs), that are predominantly employed by gram-negative bacteria, autoinducing peptides (AIP) that are produced by gram-positive bacteria and autoinducer-2 (AI-2), a family of AI molecules that is considered a universal quorum sensing signal (73). AHLs and AIP are species-specific, whereas AI-2 is produced and recognized by at least one hundred bacterial species.

Once an AI binds to its cognate receptor located on the outer cell membrane, it triggers a signaling cascade within the cell resulting in a change of its transcriptional profile and therefore the population's behavior (73). Processes regulated by quorum sensing mostly require high cell densities, such as bioluminescence, biofilm formation, sporulation or virulence. These are processes that would be inefficient if not executed by the whole population. The precise regulation makes costly effects such as bioluminescence more efficient for the cells.

Quorum Sensing in Gram-negative Bacteria

Quorum sensing was discovered in the marine microorganism *Vibrio fischeri* (91), which colonizes the light organs of squid and other marine animals which benefit from the light for the attraction of prey, for example.

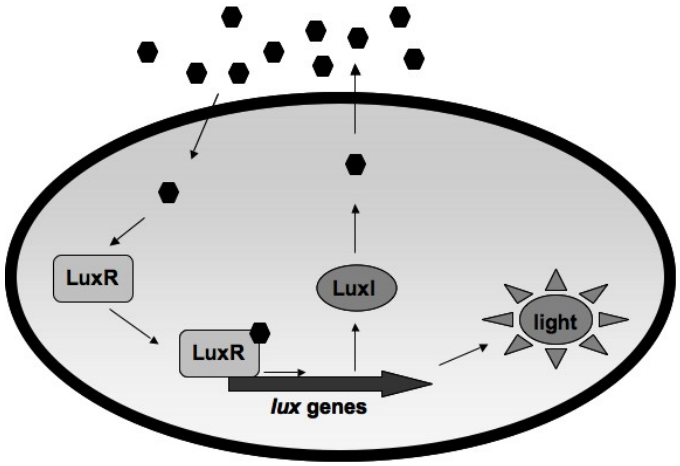

Figure 2.1. Quorum sensing circuit in *Vibrio fischeri*. Autoinducer-1 is produced by the LuxI synthase. Once the threshold concentration is reached, AI-1 binds to the transcriptional regulator LuxR which then binds to the LuxR operon and starts gene transcription (adapted from 112).

The question why the cells only produced light under certain circumstances (i.e. in a dense culture) led to the discovery of AHLs. The QS circuit of *V. fischeri* (Figure 2.1) serves as a model for the pathways in gram-negative bacteria. Although most of the QS circuits are more elaborate, the principle is very similar for AHL-signaling. A LuxS-type synthase is responsible for the production of the AI and a LuxR-type transcription regulator binds to the target genes in order to initiate or inhibit their transcription (38). The LuxI-synthase is among the target genes, resulting in a positive feedback loop; therefore the term autoinducer. AHLs have the same homoserine lactone core, however the length and structure of the acyl side chain determines their species-specificity (38).

Quorum Sensing in Gram-positive Bacteria

In gram-positive bacteria, the signal is a small peptide that binds to the sensor histidine

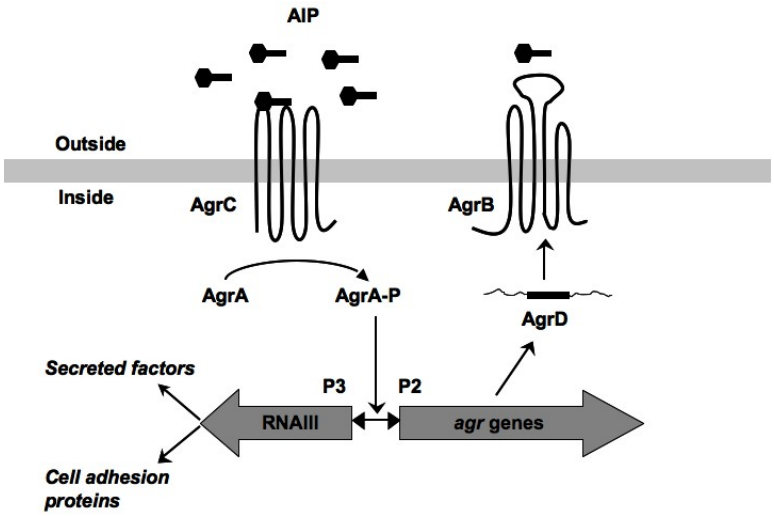

Figure 2.2. Quorum sensing circuit in the gram-positive bacterium *Staphylococcus aureus.* The AIP is produced and posttranslationally modified. After secretion, it binds to a histidine protein kinase of a two-component system and the signal is then transferred to a response regulator by phosphorylation (adapted from 112).

kinase of a two-component system (Figure 2.2). The signal is conveyed by a phosphorylation cascade that activates a response regulator, which in turn activates gene transcription (63). The AIP is produced and posttranslationally modified before secretion (63). The sequence and structure of the AIPs are highly specific and thus confer intraspecies communication. One prominent example for a gram-positive quorum sensing circuit is *Staphylococcus aureus* (Figure 2.2) (112), a pathogen that regulates the expression of virulence factors via quorum sensing. At low cell densities, the bacteria

show attachment and colonization behavior, whereas at high cell density, the bacteria become virulent and overcome the host immune system (112).

Since virulence is often regulated by QS (15, 43), the interruption of the signaling process has become a target for therapeutic approaches. In the case of *S. aureus*, an antibody-based approach has been demonstrated to interrupt QS by scavenging the AIP. This approach has been shown to be sufficient to suppress the expression of virulence factors and infection in mice (81).

Autoinducer-2: The Bacterial Esperanto

Beyond the species-specific signaling via AHL and AIP, interspecies signaling is accomplished through the autoinducer-2 family of universal signals (92). AI-2 is produced by a wide variety of gram-positive and gram-negative bacteria. It is produced by the LuxS synthase, which is a part of the *S*-adenosyl-L-methionine (SAM) pathway. The toxic byproduct *S*-adenosylhomocysteine (SAH) is converted to adenine, homocysteine and the AI-2 precursor 4,5-dihydroxy-2,3-pentonedione (DPD), in a concerted action of the Pfs and LuxS enzymes (92). Two of the structures have been identified as active molecules bound to their cognate receptors, (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (*S*-THMF borate) in *V. harveyi* (18) and (2*R*,4*S*)-2-methyl-2,3,3,4-tdtrahydroxytetrahydrofuran (*R*-THMF) in *Salmonella typhimurium* (74) (Figure 2.3).

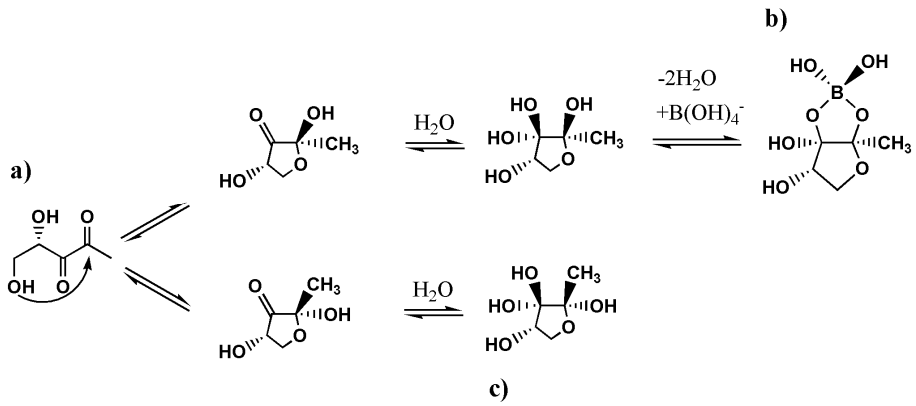

Figure 2.3. Autoinducer-2 is an equilibrium of interconverting molecules derived from the same precursor, DPD (74). a): DPD, b) *S*-THMF borate, c) *R*-THMF

The presence of boron as the central atom in the form recognized by the marine microorganism *V. harveyi* was surprising at first, but makes sense considering that there are elevated levels of boron in seawater. There is no boron in the soil, and therefore, the non-borated form of AI-2 is active in *S. typhimurium* (112).

LuxS is the main component in AI-2 signaling; however the enzyme is not solely dedicated to producing AI-2. It plays an important role in the activated methyl cycle, which may in fact be its main role and AI-2 may be a metabolic by-product in some species (26, 116). Much discussion has evolved as to whether AI-2 is a true signaling molecule. From an evolutionary standpoint (28, 58), true signaling only occurs if the signal evolved for a specific purpose and if the receiver's response evolved in parallel (68). That means, if cell A emits a compound X and cell B reacts to it, X is called a signal only if cell A produced the compound with the purpose of eliciting a response in cell B and cell B benefits from receiving X. If X is a metabolic byproduct released by cell A, and cell B benefits from receiving it, X is called a cue (28, 58). LuxS is an important enzyme in a detoxifying metabolic pathway and therefore, AI-2 is a metabolic byproduct

that is secreted by the cell. For AI-2 to be a true signal, it must be (i) secreted by the cell, (ii) taken up by the receiving cell, (iii) elicit a response from that cell because the receiver's response has evolved and (iv) benefit both the producer and the receiver (28). To date, only two processes have been shown to be regulated by AI-2 signaling: bioluminescence in *V. harveyi* (104) and an ABC transporter in *S. typhimurium* (105). There are many other bacteria that show an effect in response to AI-2; however, criterium (iv) is rarely the case and therefore, the cells rather respond to an environmental cue that is less specific than a AHL signal. Nevertheless, sensing of AI-2 enables the cells to notice the presence of other bacteria even though their identity is not entirely clear. The cells are able to monitor their environment by sensing AI-2. Therefore it makes sense that bacteria that are unable to produce AI-2, can actually sense and respond to it. So far, two cases have been shown in the literature where AI-2 elicits a response in a non-AI-2 producing strain. First, *P. aeruginosa* increased its virulence upon exposure to AI-2 (30), and second, Geier et al. (40) revealed that *M. avium* enhanced biofilm formation upon addition of the molecule (Chapter 3).

Quorum Sensing and Biofilm Formation

Quorum sensing is an example of the social behavior of microorganisms. In a review, Bernhard Crespi put well-known behavioral traits of higher organisms in context with microorganisms and pointed out similarities between biofilms and ant nests or beehives (23). The cooperative nature of biofilms is now becoming clear as cellular division of labor can be shown for single cells within the community (for a review see 101).

Biofilms are very dense populations, and in some cases can harbor up to 700 bacterial species, as shown for biofilms colonizing human teeth (64). Therefore, interspecies communication would seem a sensible means to coordinate the cohabitation of the population via interspecies quorum sensing. Indeed, a role of AI-2 in multispecies biofilm formation has been shown (69, 88, 117). Several studies have demonstrated a role for AI-2 in monospecies biofilm formation. The effect of the molecule can be inhibitory on biofilm formation, as shown for *Bacillus cereus* (2), *V. cholerae* (45) and *Eikenella corrodens* (4). AI-2 promotes biofilm formation in *Escherichia coli* (44, 47), *Streptococcus mutans* (39, 65, 71, 114, 117) and *Aggregatibacter actinomycetemcomitans* (36, 95). The influence of AHL signals on biofilm formation has also been shown for several species. Davies et al. were among the first to show that AHL signaling is involved in *P. aeruginosa* biofilm formation (25). By comparing the biofilm structure of QS mutants and wild type biofilms they showed that the architecture was not as elaborate and the biofilms more susceptible to SDS treatment when the QS circuit is knocked out (25).

The regulatory mechanisms, i.e. how QS regulates biofilm formation, are not entirely clear. Biofilm-related characteristics such as motility and EPS production are often involved. However, if these studies were performed by using gene knockouts for global QS regulators, many metabolic pathways in the cell could be affected leading to altered phenotypes.

Conclusion

Much effort has been made to reveal the mechanisms behind QS. What initially seemed to be a very straightforward concept turned out to be a very complex network of pathways and regulators. Some quorum sensing circuits act in concert, or are hierarchical, and there seems to be a complex interaction between signals produced by the cell and environmental cues. One example is the complex QS regulon in *V. cholerae.* It has been shown that the interaction between the QS circuit and the second messenger cyclic di-GMP (c-di-GMP) regulates biofilm formation in this bacterium. The QS system inhibits biofilm formation, whereas c-di-GMP, which contains information about the local environmental conditions, promotes it (113).

The initial paradigm stated that the cells grow until a critical population density is reached and binding of the AI to a receptor triggers a change in gene expression. Newer findings however suggest that it is not just a minimum concentration that needs to be reached, but an optimum concentration is necessary to trigger the effect. In their paper about AI-2 in mixed species biofilms, Rickard et al. pointed out that the concentration optimum is crucial for biofilm formation to occur (88). The study described in Chapter 3 of this thesis describes how small concentrations of AI-2 promote biofilm formation, whereas higher concentrations inhibit it (40).

In conclusion it can be said that QS is an important regulatory mechanism for a number of processes. However, QS is often tightly linked to metabolic and environmental conditions that cannot be neglected.

Environmental Factors Leading to Biofilm Development

Introduction

As discussed in the previous section, social engagements are important in maintaining biofilms as a microbial population. However, environmental cues are essential for the cells to monitor their environments. Changes in the surroundings of the cells must be sensed and processed, which enables the population to react appropriately.

Some environmental cues are sensed similar to the QS principle: The cue binds to a sensor kinase situated on the cell surface. The kinase is part of a two-component system, and the signal is transferred by phosphorylation to a response regulator, which then initiates gene transcription. Similar to the quorum sensing mechanism, environmental cues can trigger co-ordinate regulation of genes, change the population's behavior or metabolic pathways.

These cues are important for both the initiation and maintenance of biofilms. A sudden change in the environmental conditions often requires that the cells take action in order to adapt to the changing environment. This adaptation includes the expression of motility-related genes, transport mechanisms or EPS production, which are often under the control of the same global regulator (49, 60).

Therefore, the cues can lead to biofilm formation, but also act as metabolic cues.

Nutrient Availability

Nutrient availability influences biofilm formation in different ways for different bacterial strains. Some species, such as *Myxococcus xanthus* and *Escherichia coli* start

forming biofilms in response to nutrient limitation (79), since many macromolecules and nutrients accumulate on the surface. *P. aeruginosa* on the other hand forms biofilms in nutrient-rich environments. For some species, starvation is a signal for the cells to detach from a biofilm and spread out in search for more nutritious niches (79, 51).

The nutrient availability is to some extent involved in the maturation of the biofilm; it determines the thickness of the biofilm in a way that a maximal number of cells has optimal access to the nutrients in the bulk fluid (99). In laboratory biofilms, the composition of the growth medium has been shown to be an important factor in biofilm formation. De Kievit and co-workers showed that in M9 medium, *P. aeruginosa* forms a thick, multilayer biofilm; however, when grown in FAB medium, the bacteria only formed a sparse monolayer, resembling those biofilms formed by quorum sensing deficient mutants (27). The authors inferred from these findings that citrate as a carbon source prevented quorum sensing. They further investigated the impact of the two media on motility and found that the twitching motility was markedly decreased in FAB medium. Interestingly, these findings were only valid in a static system. When the motility was assayed in a flow system, the medium had no influence whatsoever. Similar findings were described by Klausen et al., who demonstrated that biofilms grown on citrate as a carbon source were densely packed and flat whereas those grown on glucose showed the typical mushroom-like structures (62).

Catabolite repression has also been shown for *E. coli* which does not form biofilms when grown in the presence of glucose (53).

Temperature

Temperature is an important factor in the physiology of a microbe. However, the optimal growth temperature does not always reflect the optimal temperature for biofilm formation. Extreme temperatures trigger a variety of bacterial stress responses, which are often coupled with biofilm formation. Bacteria seem to form more biofilm at temperatures lower than their optimal growth temperature. *Legionella pneumophila*'s growth optimum is at 36°C, whereas it forms biofilms at an optimum temperature of 20°C (48). The same optimum biofilm temperature of 20°C was determined for *Listeria monocytogenes* compared to 37°C, and this process was also dependent on the surface (17, 76). In a very elegant experiment, Kaplan and Fine created a temperature gradient in petri-dishes to demonstrate that higher temperatures are a cue for biofilm dispersal in a number of bacterial strains (57). *Neisseria subflava, Aggregatibacter actinomycetemcomitans, Haemophilus aphrophilus* and *Streptococcus mitis* dispersed along the gradient from 32°C to 36°C. Unfortunately, temperatures lower than 32°C were not tested. In *E. coli,* low temperature was also identified as a cue for biofilm formation. Furthermore, a microarray analysis comparing 37°C and 23°C growth temperature revealed that the low temperature induces a number of stress-related genes under the control of the general regulator of the stress response, RpoS (115). The opposite effect, the induction of RpoS and biofilm formation under high temperatures, was demonstrated for *Pseudomonas putida* (98). The list of temperature-related studies in the biofilm field is long, and its importance as a cue in biofilm formation and detachment is evident.

Oxidative Stress

Every pathogen that enters a mammalian host is exposed to oxidative stress as a result of the host's immune response. Oxidative stress causes damage to lipids, DNA and proteins, which can be deleterious to the cell, leading to a loss of function of membranes and proteins, blocking of DNA replication or directly causing mutations (13). Numerous studies on oxidative stress and its effect on biofilm formation have been reported for a wide range of bacterial species. In many cases, oxidative stress is linked to other processes in the cell, such as virulence traits, biofilm formation, motility and others. It is often one global regulatory protein that controls many functions in the cell that are therefore coupled. In *Campylobacter jejuni,* the global regulator, CsrA, regulates oxidative stress, biofilm formation, motility and attachment, and it represses host cell invasion (35). In *Serratia marcescens* and *Neisseria gonorrhoeae* an OxyR homologue mediates surface attachment and biofilm formation (93, 94). OxyR is a LysR-family transcription factor that is conserved in a number of gram-negative and –positive bacteria, where it acts as the global regulator for the oxidative stress response (19, 66, 75, 29).

Iron

Iron is an important cofactor and is required for the growth of almost all living cells. However, cytotoxic effects of reactive oxygen species are largely mediated by iron. Iron in its bivalent state (Fe^{2+}) is reduced, cytoxic and soluble. In the Fenton reaction, it generates reactive oxygen species (ROS) by consuming oxygen (107). Therefore, the

more bivalent iron is taken up, the more ROS are generated, which activates the oxidative stress response in the cell, which in turn leads to biofilm formation.

Iron is essential for biofilm formation in *Mycobacterium smegmatis* (77). It plays a regulatory role in the transition from the planktonic to the biofilm mode of growth. The fatty acid metabolism as well as surface motility are dependent on iron availability in this bacterium. It has been shown that supplemental iron above 1 μM is necessary for proper biofilm development, whereas iron is not needed for planktonic growth (77).

Iron is also a cue involved in *P. aeruginosa* biofilm formation. In an iron-chelated environment, biofilm formation was inhibited and twitching motility was stimulated (97). The opposite effect was observed with *Staphylococcus aureus,* where low iron content favors biofilm formation, and addition of iron leads to biofilm dispersal (55).

Iron can be found in many different environments, from drinking water pipes to mammalian hosts. This abundance in the environment and its dual role as an important cofactor and potential inhibitory compound make strict and elaborate iron regulation necessary, with iron being an important environmental cue.

Conclusion

The environmental cues above only represent a small fraction of those involved in biofilm formation. Bacteria are constantly exposed to changes in their milieu and need to adapt quickly. Many of the environmental cues indicate stress for the bacteria, and biofilm formation might act as a shield against these stressors. However, much research needs to be done in order to better understand the interactions of these factors rather than studying the effect of one factor at a time. This will make it possible to study possible synergistic or antagonistic effects of environmental factors on biofilm formation.

References

1. Anderson, G. G., S. Moreau-Marquis, B. A. Stanton, and G. A. O'Toole. 2008. In vitro analysis of tobramycin-treated Pseudomonas aeruginosa biofilms on cystic fibrosis-derived airway epithelial cells. Infect Immun 76:1423-33.

2. Auger, S., E. Krin, S. Aymerich, and M. Gohar. 2006. Autoinducer 2 affects biofilm formation by Bacillus cereus. Appl Environ Microbiol 72:937-41.

3. Austin, J. W., G. Sanders, W. W. Kay, and S. K. Collinson. 1998. Thin aggregative fimbriae enhance Salmonella enteritidis biofilm formation. FEMS Microbiol Lett 162:295-301.

4. Azakami, H., I. Teramura, T. Matsunaga, H. Akimichi, Y. Noiri, S. Ebisu, and A. Kato. 2006. Characterization of autoinducer 2 signal in Eikenella corrodens and its role in biofilm formation. J Biosci Bioeng 102:110-7.

5. Belisle, J. T., M. R. McNeil, D. Chatterjee, J. M. Inamine, and P. J. Brennan. 1993. Expression of the core lipopeptide of the glycopeptidolipid surface antigens in rough mutants of Mycobacterium avium. J Biol Chem 268:10510-6.

6. Belisle, J. T., L. Pascopella, J. M. Inamine, P. J. Brennan, and W. R. Jacobs, Jr. 1991. Isolation and expression of a gene cluster responsible for biosynthesis of the glycopeptidolipid antigens of Mycobacterium avium. J Bacteriol 173:6991-7.

7. Bercovier, H., O. Kafri, and S. Sela. 1986. Mycobacteria possess a surprisingly small number of ribosomal RNA genes in relation to the size of their genome. Biochem Biophys Res Commun 136:1136-41.

8. Brennan, P. J., and H. Nikaido. 1995. The envelope of mycobacteria. Annu Rev Biochem 64:29-63.

9. Brooks, R. W., B. C. Parker, H. Gruft, and J. O. Falkinham, 3rd. 1984. Epidemiology of infection by nontuberculous mycobacteria. V. Numbers in eastern United States soils and correlation with soil characteristics. Am Rev Respir Dis 130:630-3.

10. Bruijnesteijn van Coppenraet, L. E., P. E. de Haas, J. A. Lindeboom, E. J. Kuijper, and D. van Soolingen. 2008. Lymphadenitis in children is caused by Mycobacterium avium hominissuis and not related to 'bird tuberculosis'. Eur J Clin Microbiol Infect Dis 27:293-9.

11. Busscher, H. J., R. Bos, and H. C. van der Mei. 1995. Initial microbial adhesion is a determinant for the strength of biofilm adhesion. FEMS Microbiol Lett 128:229-34.

12. Busscher, H. J., M. M. Cowan, and H. C. van der Mei. 1992. On the relative importance of specific and non-specific approaches to oral microbial adhesion. FEMS Microbiol Rev 8:199-209.

13. Cabiscol, E., J. Tamarit, and J. Ros. 2000. Oxidative stress in bacteria and protein damage by reactive oxygen species. Int Microbiol 3:3-8.

14. Cangelosi, G.A., J.E. Clark-Curtiss, M.A. Behr, T.J. Bull, and T. Stinear. 2004. Biology of waterborne pathogenic mycobacteria. In: Bartram, J., J.A. Cotruvo, A. Dufour, G.Rees, and S. Pedley, (eds). Pathogenic Mycobacteria in Water: a guide to public health consequences, monitoring and management. London: IWA Publishing, pp. 39-54

15. Cegelski, L., G. R. Marshall, G. R. Eldridge, and S. J. Hultgren. 2008. The biology and future prospects of antivirulence therapies. Nat Rev Microbiol 6:17-27.

16. Chamberlain, A.H.L., 1992. The role of adsorbed layers in bacterial adhesion. In: Biofilms – Science and Technology, Melo, L.F., Bott, R., Fletcher M. and Capdeville, B. eds. Alvor, Portugal, May 18-29 Kluwer Academic Publishers, London, p. 59

17. Chavant, P., B. Martinie, T. Meylheuc, M. N. Bellon-Fontaine, and M. Hebraud. 2002. Listeria monocytogenes LO28: surface physicochemical properties and ability to form biofilms at different temperatures and growth phases. Appl Environ Microbiol 68:728-37.

18. Chen, X., S. Schauder, N. Potier, A. Van Dorsselaer, I. Pelczer, B. L. Bassler, and F. M. Hughson. 2002. Structural identification of a bacterial quorum-sensing signal containing boron. Nature 415:545-9.

19. Christman, M. F., R. W. Morgan, F. S. Jacobson, and B. N. Ames. 1985. Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in Salmonella typhimurium. Cell 41:753-62.

20. Coetser, S. E., and T. E. Cloete. 2005. Biofouling and biocorrosion in industrial water systems. Crit Rev Microbiol 31:213-32.

21. Corpe W.A. Microbial surface components involved in adsorption of microorganisms onto surfaces. In: Bitton G, Marshall KC, eds. Adsorption of microorganisms to surfaces. New York: John Wiley & Sons; 1980. pp. 105–44.

22. Costerton, J. W., L. Montanaro, and C. R. Arciola. 2005. Biofilm in implant infections: its production and regulation. Int J Artif Organs 28:1062-8.

23. Crespi, B. J. 2001. The evolution of social behavior in microorganisms. Trends Ecol Evol 16:178-183.

24. Davies, D. G., A. M. Chakrabarty, and G. G. Geesey. 1993. Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by Pseudomonas aeruginosa. Appl Environ Microbiol 59:1181-6.

25. Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280:295-8.

26. De Keersmaecker, S. C., K. Sonck, and J. Vanderleyden. 2006. Let LuxS speak up in AI-2 signaling. Trends Microbiol 14:114-9.

27. De Kievit, T. R., R. Gillis, S. Marx, C. Brown, and B. H. Iglewski. 2001. Quorum-sensing genes in Pseudomonas aeruginosa biofilms: their role and expression patterns. Appl Environ Microbiol 67:1865-73.

28. Diggle, S. P., A. Gardner, S. A. West, and A. S. Griffin. 2007. Evolutionary theory of bacterial quorum sensing: when is a signal not a signal? Philos Trans R Soc Lond B Biol Sci 362:1241-9.

29. Dowds, B. C. 1994. The oxidative stress response in Bacillus subtilis. FEMS Microbiol Lett 124:255-63.

30. Duan, K., C. Dammel, J. Stein, H. Rabin, and M. G. Surette. 2003. Modulation of Pseudomonas aeruginosa gene expression by host microflora through interspecies communication. Mol Microbiol 50:1477-91.

31. Embil, J., P. Warren, M. Yakrus, R. Stark, S. Corne, D. Forrest, and E. Hershfield. 1997. Pulmonary illness associated with exposure to Mycobacterium-avium complex in hot tub water. Hypersensitivity pneumonitis or infection? Chest 111:813-6.

32. Falkinham, J. O., 3rd, K. L. George, B. C. Parker, and H. Gruft. 1984. In vitro susceptibility of human and environmental isolates of Mycobacterium avium, M. intracellulare, and M. scrofulaceum to heavy-metal salts and oxyanions. Antimicrob Agents Chemother 25:137-9.

33. Falkinham, J. O., 3rd, C. D. Norton, and M. W. LeChevallier. 2001. Factors influencing numbers of Mycobacterium avium, Mycobacterium intracellulare, and other Mycobacteria in drinking water distribution systems. Appl Environ Microbiol 67:1225-31.

34. Falkinham, J. O., 3rd. 2003. Mycobacterial aerosols and respiratory disease. Emerg Infect Dis 9:763-7.

35. Fields, J. A., and S. A. Thompson. 2008. Campylobacter jejuni CsrA mediates oxidative stress responses, biofilm formation, and host cell invasion. J Bacteriol.

36. Fong, K. P., W. O. Chung, R. J. Lamont, and D. R. Demuth. 2001. Intra- and interspecies regulation of gene expression by Actinobacillus actinomycetemcomitans LuxS. Infect Immun 69:7625-34.

37. Fraser, V. J., M. Jones, P. R. Murray, G. Medoff, Y. Zhang, and R. J. Wallace, Jr. 1992. Contamination of flexible fiberoptic bronchoscopes with Mycobacterium chelonae linked to an automated bronchoscope disinfection machine. Am Rev Respir Dis 145:853-5.

38. Fuqua, W. C., S. C. Winans, and E. P. Greenberg. 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J Bacteriol 176:269-75.

39. Garrett, E. S., D. Perlegas, and D. J. Wozniak. 1999. Negative control of flagellum synthesis in Pseudomonas aeruginosa is modulated by the alternative sigma factor AlgT (AlgU). J Bacteriol 181:7401-4.

40. Geier, H., S. Mostowy, G. A. Cangelosi, M. A. Behr, and T. E. Ford. 2008. Autoinducer-2 triggers the oxidative stress response in Mycobacterium avium, leading to biofilm formation. Appl Environ Microbiol 74:1798-804.

41. George, K. L., and J. O. Falkinham, 3rd. 1986. Selective medium for the isolation and enumeration of Mycobacterium avium-intracellulare and M. scrofulaceum. Can J Microbiol 32:10-4.

42. George, K. L., B. C. Parker, H. Gruft, and J. O. Falkinham, 3rd. 1980. Epidemiology of infection by nontuberculous mycobacteria. II. Growth and survival in natural waters. Am Rev Respir Dis 122:89-94.

43. Girard, G., and G. V. Bloemberg. 2008. Central role of quorum sensing in regulating the production of pathogenicity factors in Pseudomonas aeruginosa. Future Microbiol 3:97-106.

44. Gonzalez Barrios, A. F., R. Zuo, Y. Hashimoto, L. Yang, W. E. Bentley, and T. K. Wood. 2006. Autoinducer 2 controls biofilm formation in Escherichia coli through a novel motility quorum-sensing regulator (MqsR, B3022). J Bacteriol 188:305-16.

45. Hammer, B. K., and B. L. Bassler. 2003. Quorum sensing controls biofilm formation in Vibrio cholerae. Mol Microbiol 50:101-4.

46. Havelaar, A. H., L. G. Berwald, D. G. Groothuis, and J. G. Baas. 1985. Mycobacteria in semi-public swimming-pools and whirlpools. Zentralbl Bakteriol Mikrobiol Hyg [B] 180:505-14.

47. Herzberg, M., I. K. Kaye, W. Peti, and T. K. Wood. 2006. YdgG (TqsA) controls biofilm formation in Escherichia coli K-12 through autoinducer 2 transport. J Bacteriol 188:587-98.

48. Hindre, T., H. Bruggemann, C. Buchrieser, and Y. Hechard. 2008. Transcriptional profiling of Legionella pneumophila biofilm cells and the influence of iron on biofilm formation. Microbiology 154:30-41.

49. Hoang, H. H., N. Gurich, and J. E. Gonzalez. 2008. Regulation of motility by the ExpR/Sin quorum-sensing system in Sinorhizobium meliloti. J Bacteriol 190:861-71.

50. Horsburgh, C. R., Jr. 1991. Mycobacterium avium complex infection in the acquired immunodeficiency syndrome. N Engl J Med 324:1332-8.

51. Hunt, S. M., E. M. Werner, B. Huang, M. A. Hamilton, and P. S. Stewart. 2004. Hypothesis for the role of nutrient starvation in biofilm detachment. Appl Environ Microbiol 70:7418-25.

52. Inderlied, C. B., C. A. Kemper, and L. E. Bermudez. 1993. The Mycobacterium avium complex. Clin Microbiol Rev 6:266-310.

53. Jackson, D. W., J. W. Simecka, and T. Romeo. 2002. Catabolite repression of Escherichia coli biofilm formation. J Bacteriol 184:3406-10.

54. James, G. A., E. Swogger, R. Wolcott, E. D. Pulcini, P. Secor, J. Sestrich, J. W. Costerton, and P. S. Stewart. 2008. Biofilms in chronic wounds. Wound Repair Regen 16:37-44.

55. Johnson, M., A. Cockayne, P. H. Williams, and J. A. Morrissey. 2005. Iron-responsive regulation of biofilm formation in staphylococcus aureus involves fur-dependent and fur-independent mechanisms. J Bacteriol 187:8211-5.

56. Kahana, L. M., and J. M. Kay. 1997. Pneumonitis due to Mycobacterium avium complex in hot tub water: infection or hypersensitivity? Chest 112:1713-4.

57. Kaplan, J. B., and D. H. Fine. 2002. Biofilm dispersal of Neisseria subflava and other phylogenetically diverse oral bacteria. Appl Environ Microbiol 68:4943-50.

58. Keller, L., and M. G. Surette. 2006. Communication in bacteria: an ecological and evolutionary perspective. Nat Rev Microbiol 4:249-58.

59. Khoor, A., K. O. Leslie, H. D. Tazelaar, R. A. Helmers, and T. V. Colby. 2001. Diffuse pulmonary disease caused by nontuberculous mycobacteria in immunocompetent people (hot tub lung). Am J Clin Pathol 115:755-62.

60. Kim, H. S., M. A. Lee, S. J. Chun, S. J. Park, and K. H. Lee. 2007. Role of NtrC in biofilm formation via controlling expression of the gene encoding an ADP-glycero-manno-heptose-6-epimerase in the pathogenic bacterium, Vibrio vulnificus. Mol Microbiol 63:559-74.

61. Kirschner, R. A., Jr., B. C. Parker, and J. O. Falkinham, 3rd. 1992. Epidemiology of infection by nontuberculous mycobacteria. Mycobacterium avium, Mycobacterium intracellulare, and Mycobacterium scrofulaceum in acid, brown-water swamps of the southeastern United States and their association with environmental variables. Am Rev Respir Dis 145:271-5.

62. Klausen, M., A. Heydorn, P. Ragas, L. Lambertsen, A. Aaes-Jorgensen, S. Molin, and T. Tolker-Nielsen. 2003. Biofilm formation by Pseudomonas aeruginosa wild type, flagella and type IV pili mutants. Mol Microbiol 48:1511-24.

63. Kleerebezem, M., L. E. Quadri, O. P. Kuipers, and W. M. de Vos. 1997. Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. Mol Microbiol 24:895-904.

64. Kolenbrander, P. E., R. J. Palmer, Jr., A. H. Rickard, N. S. Jakubovics, N. I. Chalmers, and P. I. Diaz. 2006. Bacterial interactions and successions during plaque development. Periodontol 2000 42:47-79.

65. Lemos, J. A., T. A. Brown, Jr., and R. A. Burne. 2004. Effects of RelA on key virulence properties of planktonic and biofilm populations of Streptococcus mutans. Infect Immun 72:1431-40.

66. Maciver, I., and E. J. Hansen. 1996. Lack of expression of the global regulator OxyR in Haemophilus influenzae has a profound effect on growth phenotype. Infect Immun 64:4618-29.

67. Marsh, P. D. 2005. Dental plaque: biological significance of a biofilm and community life-style. J Clin Periodontol 32 Suppl 6:7-15.

68. Maynard Smith, J. and Harper, D. 2003. Animal signal. New York, NY, Oxford University Press.

69. McNab, R., and R. J. Lamont. 2003. Microbial dinner-party conversations: the role of LuxS in interspecies communication. J Med Microbiol 52:541-5.

70. Meissner, G., and W. Anz. 1977. Sources of Mycobacterium avium complex infection resulting in human diseases. Am Rev Respir Dis 116:1057-64.

71. Merritt, J., F. Qi, S. D. Goodman, M. H. Anderson, and W. Shi. 2003. Mutation of luxS affects biofilm formation in Streptococcus mutans. Infect Immun 71:1972-9.

72. Mijs, W., P. de Haas, R. Rossau, T. Van der Laan, L. Rigouts, F. Portaels, and D. van Soolingen. 2002. Molecular evidence to support a proposal to reserve the designation Mycobacterium avium subsp. avium for bird-type isolates and 'M. avium subsp. hominissuis' for the human/porcine type of M. avium. Int J Syst Evol Microbiol 52:1505-18.

73. Miller, M. B., and B. L. Bassler. 2001. Quorum sensing in bacteria. Annu Rev Microbiol 55:165-99.

74. Miller, S. T., K. B. Xavier, S. R. Campagna, M. E. Taga, M. F. Semmelhack, B. L. Bassler, and F. M. Hughson. 2004. Salmonella typhimurium recognizes a chemically distinct form of the bacterial quorum-sensing signal AI-2. Mol Cell 15:677-87.

75. Mongkolsuk, S., S. Loprasert, W. Whangsuk, M. Fuangthong, and S. Atichartpongkun. 1997. Characterization of transcription organization and analysis of unique expression patterns of an alkyl hydroperoxide reductase C gene (ahpC) and the peroxide regulator operon ahpF-oxyR-orfX from Xanthomonas campestris pv. phaseoli. J Bacteriol 179:3950-5.

76. Norwood, D. E., and A. Gilmour. 2001. The differential adherence capabilities of two Listeria monocytogenes strains in monoculture and multispecies biofilms as a function of temperature. Lett Appl Microbiol 33:320-4.

77. Ojha, A., and G. F. Hatfull. 2007. The role of iron in Mycobacterium smegmatis biofilm formation: the exochelin siderophore is essential in limiting iron conditions for biofilm formation but not for planktonic growth. Mol Microbiol 66:468-83.

78. Otero, J., W. R. Jacobs, Jr., and M. S. Glickman. 2003. Efficient allelic exchange and transposon mutagenesis in Mycobacterium avium by specialized transduction. Appl Environ Microbiol 69:5039-44.

79. O'Toole, G., H. B. Kaplan, and R. Kolter. 2000. Biofilm formation as microbial development. Annu Rev Microbiol 54:49-79.

80. O'Toole, G. A., and R. Kolter. 1998. Flagellar and twitching motility are necessary for Pseudomonas aeruginosa biofilm development. Mol Microbiol 30:295-304.

81. Park, J., R. Jagasia, G. F. Kaufmann, J. C. Mathison, D. I. Ruiz, J. A. Moss, M. M. Meijler, R. J. Ulevitch, and K. D. Janda. 2007. Infection control by antibody disruption of bacterial quorum sensing signaling. Chem Biol 14:1119-27.

82. Parsek, M. R., and P. K. Singh. 2003. Bacterial biofilms: an emerging link to disease pathogenesis. Annu Rev Microbiol 57:677-701.

83. Pawar, D. M., M. L. Rossman, and J. Chen. 2005. Role of curli fimbriae in mediating the cells of enterohaemorrhagic Escherichia coli to attach to abiotic surfaces. J Appl Microbiol 99:418-25.

84. Penny, M. E., R. B. Cole, and J. Gray. 1982. Two cases of Mycobacterium kansasii infection occurring in the same household. Tubercle 63:129-31.

85. Picioreanu, C., M. C. van Loosdrecht, and J. J. Heijnen. 2001. Two-dimensional model of biofilm detachment caused by internal stress from liquid flow. Biotechnol Bioeng 72:205-18.

86. Portaels, F., and S. R. Pattyn. 1982. Growth of mycobacteria in relation to the pH of the medium. Ann Microbiol (Paris) 133:213-21.

87. Rastogi, N., C. Frehel, A. Ryter, H. Ohayon, M. Lesourd, and H. L. David. 1981. Multiple drug resistance in Mycobacterium avium: is the wall architecture responsible for exclusion of antimicrobial agents? Antimicrob Agents Chemother 20:666-77.

88. Rickard, A. H., R. J. Palmer, Jr., D. S. Blehert, S. R. Campagna, M. F. Semmelhack, P. G. Egland, B. L. Bassler, and P. E. Kolenbrander. 2006. Autoinducer 2: a concentration-dependent signal for mutualistic bacterial biofilm growth. Mol Microbiol 60:1446-56.

89. Rittmann B.E. Detachment from biofilms. 1989. In: Structure and functions of biofilms. Characklis WG, Wilderer PA (eds). New York: John Wiley & Sons Ltd., pp. 49-58.

90. Rose, C. S., J. W. Martyny, L. S. Newman, D. K. Milton, T. E. King, Jr., J. L. Beebe, J. B. McCammon, R. E. Hoffman, and K. Kreiss. 1998. "Lifeguard lung": endemic granulomatous pneumonitis in an indoor swimming pool. Am J Public Health 88:1795-800.

91. Ruby, E. G., and K. H. Nealson. 1976. Symbiotic association of Photobacterium fischeri with the marine luminous fish Monocentris japonica; a model of symbiosis based on bacterial studies. Biol Bull 151:574-86.

92. Schauder, S., K. Shokat, M. G. Surette, and B. L. Bassler. 2001. The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. Mol Microbiol 41:463-76.

93. Seib, K. L., H. J. Wu, Y. N. Srikhanta, J. L. Edwards, M. L. Falsetta, A. J. Hamilton, T. L. Maguire, S. M. Grimmond, M. A. Apicella, A. G. McEwan, and M. P. Jennings. 2007. Characterization of the OxyR regulon of Neisseria gonorrhoeae. Mol Microbiol 63:54-68.

94. Shanks, R. M., N. A. Stella, E. J. Kalivoda, M. R. Doe, D. M. O'Dee, K. L. Lathrop, F. L. Guo, and G. J. Nau. 2007. A Serratia marcescens OxyR homolog mediates surface attachment and biofilm formation. J Bacteriol 189:7262-72.

95. Shao, H., R. J. Lamont, and D. R. Demuth. 2007. Autoinducer 2 Is Required for Biofilm Growth of Aggregatibacter (Actinobacillus) actinomycetemcomitans. Infect Immun 75:4211-8.

96. Shirtliff, M. E., J. T. Mader, and A. K. Camper. 2002. Molecular interactions in biofilms. Chem Biol 9:859-71.

97. Singh, P. K., M. R. Parsek, E. P. Greenberg, and M. J. Welsh. 2002. A component of innate immunity prevents bacterial biofilm development. Nature 417:552-5.

98. Srivastava, S., A. Yadav, K. Seem, S. Mishra, V. Chaudhary, and C. S. Nautiyal. 2008. Effect of High Temperature on Pseudomonas putida NBRI0987 Biofilm Formation and Expression of Stress Sigma Factor RpoS. Curr Microbiol.

99. Stanley, N. R., and B. A. Lazazzera. 2004. Environmental signals and regulatory pathways that influence biofilm formation. Mol Microbiol 52:917-24.

100. Stewart, P. S., and J. W. Costerton. 2001. Antibiotic resistance of bacteria in biofilms. Lancet 358:135-8.

101. Stewart, P. S., and M. J. Franklin. 2008. Physiological heterogeneity in biofilms. Nat Rev Microbiol 6:199-210.

102. Stoodley, P., R. Cargo, C. J. Rupp, S. Wilson, and I. Klapper. 2002. Biofilm material properties as related to shear-induced deformation and detachment phenomena. J Ind Microbiol Biotechnol 29:361-7.

103. Stoodley, P., K. Sauer, D. G. Davies, and J. W. Costerton. 2002. Biofilms as complex differentiated communities. Annu Rev Microbiol 56:187-209.

104. Surette, M. G., M. B. Miller, and B. L. Bassler. 1999. Quorum sensing in Escherichia coli, Salmonella typhimurium, and Vibrio harveyi: a new family of genes responsible for autoinducer production. Proc Natl Acad Sci U S A 96:1639-44.

105. Taga, M. E., J. L. Semmelhack, and B. L. Bassler. 2001. The LuxS-dependent autoinducer AI-2 controls the expression of an ABC transporter that functions in AI-2 uptake in Salmonella typhimurium. Mol Microbiol 42:777-93.

106. Taylor, R. H., J. O. Falkinham, 3rd, C. D. Norton, and M. W. LeChevallier. 2000. Chlorine, chloramine, chlorine dioxide, and ozone susceptibility of Mycobacterium avium. Appl Environ Microbiol 66:1702-5.

107. Touati, D. 2000. Iron and oxidative stress in bacteria. Arch Biochem Biophys 373:1-6.

108. Velraeds, M. M., H. C. van der Mei, G. Reid, and H. J. Busscher. 1997. Inhibition of initial adhesion of uropathogenic Enterococcus faecalis to solid substrata by an adsorbed biosurfactant layer from Lactobacillus acidophilus. Urology 49:790-4.

109. von Reyn, C. F., R. D. Arbeit, C. R. Horsburgh, M. A. Ristola, R. D. Waddell, S. M. Tvaroha, M. Samore, L. R. Hirschhorn, J. Lumio, A. D. Lein, M. R. Grove, and A. N. Tosteson. 2002. Sources of disseminated Mycobacterium avium infection in AIDS. J Infect 44:166-70.

110. von Reyn, C. F., J. N. Maslow, T. W. Barber, J. O. Falkinham, 3rd, and R. D. Arbeit. 1994. Persistent colonisation of potable water as a source of Mycobacterium avium infection in AIDS. Lancet 343:1137-41.

111. Walker, J. T., D. J. Bradshaw, M. Finney, M. R. Fulford, E. Frandsen, O. S. E, J. M. Ten Cate, W. R. Moorer, A. J. Schel, A. Mavridou, J. J. Kamma, G. Mandilara, L. Stosser, S. Kneist, R. Araujo, N. Contreras, P. Goroncy-Bermes, D. O'Mullane, F. Burke, A. Forde, M. O'Sullivan, and P. D. Marsh. 2004. Microbiological evaluation of dental unit water systems in general dental practice in Europe. Eur J Oral Sci 112:412-8.

112. Waters, C. M., and B. L. Bassler. 2005. Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol 21:319-46.

113. Waters, C. M., W. Lu, J. D. Rabinowitz, and B. L. Bassler. 2008. Quorum sensing controls biofilm formation in Vibrio cholerae through modulation of cyclic di-GMP levels and repression of vpsT. J Bacteriol 190:2527-36.

114. Wen, Z. T., P. Suntharaligham, D. G. Cvitkovitch, and R. A. Burne. 2005. Trigger factor in Streptococcus mutans is involved in stress tolerance, competence development, and biofilm formation. Infect Immun 73:219-25.

115. White-Ziegler, C. A., S. Um, N. M. Perez, A. L. Berns, A. J. Malhowski, and S. Young. 2008. Low temperature (23 degrees C) increases expression of biofilm-, cold-shock- and RpoS-dependent genes in Escherichia coli K-12. Microbiology 154:148-66.

116. Winzer, K., K. R. Hardie, and P. Williams. 2003. LuxS and autoinducer-2: their contribution to quorum sensing and metabolism in bacteria. Adv Appl Microbiol 53:291-396.

117. Yoshida, A., T. Ansai, T. Takehara, and H. K. Kuramitsu. 2005. LuxS-based signaling affects Streptococcus mutans biofilm formation. Appl Environ Microbiol 71:2372-80.

CHAPTER 3

AUTOINDUCER-2 TRIGGERS THE OXIDATIVE STRESS RESPONSE IN *MYCOBACTERIUM AVIUM* LEADING TO BIOFILM FORMATION

Reproduced with permission from Geier, H., S. Mostowy, G. A. Cangelosi, M. A. Behr, and T. E. Ford. 2008. Autoinducer-2 triggers the oxidative stress response in Mycobacterium avium, leading to biofilm formation. Appl Environ Microbiol **74:**1798-804.

Abstract

Mycobacterium avium is an environmental organism and opportunistic pathogen with inherent resistance to drugs, environmental stresses and the host immune response. To adapt to these disparate conditions, *M. avium* must control its transcriptional response to environmental cues. *M. avium* forms biofilms in various environmental settings, including drinking water pipes and potable water reservoirs. In this study we investigated the role of the universal signaling molecule autoinducer-2 (AI-2) on biofilm formation by *M. avium*. The addition of the compound to planktonic *M. avium* cultures resulted in increased biofilm formation. Microarray and reverse transcriptase PCR studies revealed an upregulation of the oxidative stress response upon addition of AI-2. This suggests that the response to AI-2 might be related to oxidative stress, rather than quorum sensing. Consistent with this model, addition of hydrogen peroxide, a known stimulus of the oxidative stress response, to *M. avium* cultures resulted in elevated biofilm formation. These results suggest that AI-2 does not act as a quorum-sensing signal in *M. avium*. Instead, biofilm formation is triggered by environmental stresses of biotic and abiotic origin, and AI-2 may exert effects on that level.

Introduction

M. avium is ubiquitous in the environment, occurring in natural and urban water sources as well as in soil, but also can act as an opportunistic pathogen (21, 37). It is related to the intracellular pathogens *M. tuberculosis* and *M. leprae,* the causative agents of tuberculosis and leprosy. The species *M. avium* is comprised of several subspecies, including *M. avium* subspecies *hominissuis,* which causes disease in humans and other mammals; and *M. avium* subspecies *paratuberculosis,* a significant pathogen of livestock with uncertain etiology in human disease (53).

Although *M. avium* mainly infects the immunocompromised, there have been increased reports of infections in people with no obvious predisposing factors (12). For example, hypersensitivity pneumonitis (HP) has been reported in otherwise healthy individuals. These infections occur due to exposure to aerosolized mycobacteria in indoor swimming pools, hot tubs and metalworking fluids in industrial settings (1, 29, 45).

In most environmental settings, bacteria are found in biofilms, complex communities that colonize all kinds of surfaces (6). Non-paratuberculous *M. avium* is among the most commonly isolated species in biofilm samples taken from drinking water distribution systems (11). *M. avium* occupies a broad range of habitats in the environment, and therefore needs to adapt to different environmental conditions. It is very important for the bacterium to sense and process the gathered information. This is accomplished by signal transduction mechanisms, enabling the bacterium to monitor pH, temperature, nutrient availability, and also the presence of other bacteria, which might compete with the slow-growing mycobacteria for food and space. Bacterial adaptation to

environmental changes most commonly follows a general scheme: An environmental cue is sensed by a membrane protein in a two-component system and transferred via phosphorylation to a response regulator or, if the cue is diffusible, it may bind directly to a transcriptional regulator which then alters gene expression (16, 50). Cues can include environmental conditions, such as osmolarity, nutrient availability, temperature or ions. In the case of the oxidative stress response, the reactive oxygen species binds directly to the OxyR transcriptional regulator and activates gene transcription (51).

Social interactions among bacteria are more specific than interactions with the environment. The bacteria sense self-produced signaling compounds at well-defined concentrations, the so-called autoinducers (AIs). This process, termed quorum sensing, enables bacteria to monitor the environment for other bacteria and to react by changing their behavior (54). This is especially important to cell-density dependent cellular functions, such as light production, virulence, sporulation or biofilm formation. Intraspecies signal molecules are often *N*-acylhomoserine lactones (AHLs) or signaling peptides. The only universal signal identified to date is autoinducer-2 (AI-2) (5). AI-2 is a collective term for the molecules derived from the same precursor, 4,5-dihydroxy-2,3-pentanedione (DPD), which is produced by the LuxS synthase. These molecules are in equilibrium and can convert into each other; and each molecule binds to a different receptor in different bacterial species (28). AI-2 is a metabolic by-product in the detoxification of *S*-adenosylhomocysteine (SAH) in the activated methyl cycle of bacterial cells. Therefore, the question of whether AI-2 is a true specific signaling compound has been raised. It is important to distinguish between signaling, which is a social interaction between bacteria, and cues, the interaction of bacteria with the

environment (22). As defined by Diggle et al, signaling occurs when a cell secretes a molecule owing to the effect on the sender. If the receiving cell only benefits from the compound, it is called a cue (9). De Keersmaecker *et al.* suggested that there is not sufficient evidence for the assignment of a signaling role for AI-2 in all bacteria that possess the *luxS* gene (7). Given that interspecies signaling cannot always be explained from an evolutionary standpoint, it is possible that AI-2 might not always act as a signaling compound. Quorum sensing describes the bacterium's ability to cooperate, whereas a cue induces reactive behavior in a cell. Both cooperation and reaction contribute to the bacteria's fitness and ability to adapt to environmental conditions. Therefore, even if a molecule does not elicit a response from a cell that owes to the emitting cell, it still might help the receiving cell to monitor its physical and microbiological environment. In the case of *M. avium*, which is unable to produce AI-2, the molecule might act as a parainducer rather than autoinducer. One example for parainduction was described by Duan *et al.* It shows that AI-2 induces certain virulence genes in *Pseudomonas aeruginosa*, which is also unable to produce AI-2 (10). AI-2 has been shown to be involved in biofilm formation in many bacterial species. The effect of AI-2 can be inhibitory on biofilm formation, as shown for *Bacillus cereus* (2), *Vibrio cholerae* (19) and *Eikenella corrodens* (3), and AI-2 promotes biofilm formation in *Escherichia coli* (17, 20, 38), *Streptococcus mutans* (24, 27, 55, 57) and *Aggregatibacter actinomycetemcomitans* (13, 44). Furthermore, the molecule seems to play an important ecological role in the formation of multispecies biofilms (26, 39, 57).

Because *M. avium* has been isolated from environmental biofilms we sought to investigate the influences that favor biofilm formation, especially in the presence of AI-2.

The present study shows that AI-2 increases biofilm formation in *M. avium* cultures, when present at high concentrations. Microarray and RT-PCR studies show that the oxidative stress response is upregulated in response to the addition of AI-2. Hydrogen peroxide also increased biofilm formation, indicating that oxidative stress stimulates biofilm formation by this pathogen.

Materials and Methods

Bacterial Strains and Culture Conditions

M. avium strain W2001 was isolated from drinking water in the Boston area. The strain was classified as *M. avium* subsp. *hominissuis* with an hsp65 Code 1 sequevar (52, 53), which is the same as the recently sequenced *M. avium* 104.

The organism was grown at 37°C in Middlebrook 7H9 medium supplemented with 10% albumin-dextrose-catalase enrichment and 0.2% glycerol.

Assessment of Biofilm Formation in 96-well Microtiter Plates in the Presence of AI-2 and Hydrogen Peroxide

Bacterial cultures were grown in Middlebrook 7H9 broth to an optical density at 600 nm (OD_{600}) of 0.5 to 1.0, centrifuged, and resuspended in equal amounts of sterile milliQ deionized water. DPD, a generous gift from Prof. Shoolingin-Jordan, was dissolved in water at concentrations indicated below. The microtiter plates were inoculated with 150 µl of bacterial suspension and 50 µl DPD solution, bacterial culture supernatant or H_2O_2, respectively. The negative controls contained 50 µl water or 50 µl LB medium in the supernatant experiment. The final concentrations of DPD per well

were 0.25 μM, 2.5 μM, 25 μM, 250 μM and 2500 μM. The final H_2O_2 concentrations were 0.05 mM, 0.5 mM, 5 mM, and 50 mM. After 10 days of incubation, the biofilm mass was assessed using an adapted version of the crystal violet (CV) staining method (34). In brief, the biofilms were stained with crystal violet and incubated at room temperature for 45 min. Rinsing off the residual dye left only the stained cells attached to the surface of the wells. The CV was solubilized with 80% (v/v) ethanol and OD_{550} readings were taken for quantification. The biofilms grown in the presence of AI-2 were observed using a Leica TCS NT confocal laser scanning microscope.

Microarray Studies

Planktonic *M. avium* cultures were grown to OD_{600} 0.3-0.5 in the absence and presence of 0.5 mM AI-2. The optical density was determined to ensure that AI-2 did not influence the growth of the planktonic culture. Total RNA was extracted using a phenol-chloroform extraction protocol (47). Microarray hybridization and analysis were performed as described previously (30). In brief, the extracted RNA from both AI-2 treated cultures and the negative control was labeled with Cy3 or Cy5 dUTP by reverse-transcriptase (Amersham Biosciences). The three-day induction with AI-2 was performed in triplicate and once with an induction time of 2.5 hours to study the immediate change in gene expression. The labeled cDNA was hybridized to microarrays composed of oligonucleotide probes that were designed based on the annotation of the *M. avium* subsp. *avium* strain 104 sequence (provided by the Institute for Genomic Research [http://www.tigr.org]. Each of the 4,158 probes (MetaBion GmbH, Martinsried, Germany) was printed in duplicate onto microarray slides (SigmascreenTM; Sigma). The

comparison AI-2 versus control was performed for each dye combination (Cy3/Cy5 and Cy5/Cy3) resulting in two hybridizations per experiment and eight hybridizations in total. The hybridized arrays were scanned with Scanarray 5000XL (PerkinElmer, Freemont, CA) and hybridization results were quantified with Scanalyze software (http://rana.lbl.gov/EisenSoftware.htm/). Array analysis was performed as previously described (4, 31). *z*-scores were determined for each data point to calculate how many standard deviations a data point lies from the population mean. *z*-scores for each gene were averaged and genes with *z*-scores of 2 or greater were considered up-or downregulated.

Real-time Reverse Transcription (RT)-PCR

For real-time RT-PCR, cultures were grown in the presence and absence of 0.5mM AI-2 for 3h. Total RNA was isolated and residual DNA was removed using the Ambion DNAse-I kit according to the manufacturer's instructions.

The real-time RT-PCR was carried out using the Corbett Rotor-Gene 3000 real-time DNA detection system and the QuantiTect™ SYBR® Green RT-PCR kit (QIAGEN) in 25µl (total volume) reactions. These mixtures contained 12.5µl SYBR® Green mix, 0.25µl QuantiTect™ RT mix, 2µM primer and 10ng RNA. Primers were designed using the Frodo software (40) (Table 3.1). The reverse transcriptase reaction was carried out at 50°C for 30 min, followed by denaturation at 95°C for 15min. This was followed by 50 PCR cycles of 15s at 94°C, 30s at 59°C and 30s at 72°C.

Table 3.1. Primers used for RT-PCR

Primers	Oligonucleotide Sequence (5'-3')
AhpC forward	AGCACGAGGACCTCAAGAAC
AhpC reverse	GTGACCGAGACGAACTGGAT
AhpD forward	GTACGCCAAGGATCTCAAGC
AhpD reverse	GTACTTGCCGTCCAAGAAGC
TreS forward	TACGACACCACCGACCACTA
TreS reverse	CGTGATCGTCAGAGTCGATG
MAV_4967 forward	GGATGGCAGTGGGTGACTAC
MAV_4967 reverse	CCGTAGGTGTTGAGGGACAG
MAV_2838 forward	GGATGGCACTGGGTGACTAC
MAV_2838 reverse	CCGTAGGTGTTGAGGGACAG
SigA forward	CCTCAAGCAGATCGGTAAGG
SigA reverse	AGATTCGCTTCCAGCAGATG
16S forward	GCGATATCGGGCAGACTAGAG
16S reverse	AAGGAAGGAAACCCACACCT

For analysis, the C_t values obtained for each gene were converted to linear numbers by calculating $L_t=2^{-Ct}$ and normalized with the SigA and 16S genes. Fold changes were obtained by dividing the normalized AI-2 treated values by the normalized negative control values for each gene.

Results

AI-2 Influences *M. avium* Biofilm Formation

M. avium biofilms grown in microtiter plates responded to the addition of AI-2 in a concentration-dependent manner. The CV stain showed that biofilm formation increased in the presence of 0.25 μM, 2.5 μM and 25 μM AI-2 with 1.2, 1.6, and 1.7-fold increase in biofilm biomass compared to the negative control (Figure 3.1).

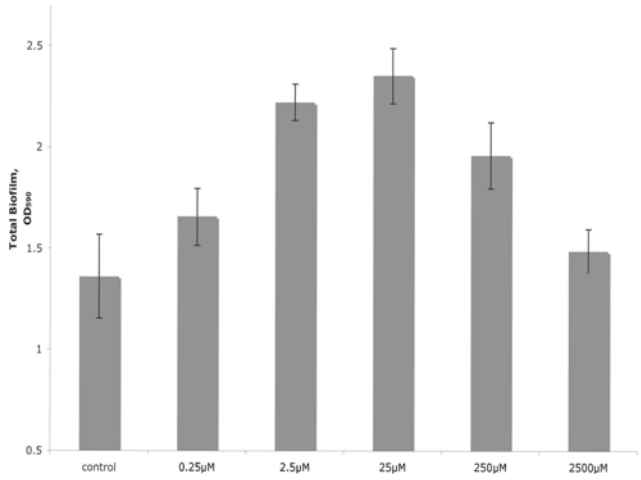

Figure 3.1. Crystal violet stain of *M. avium* biofilms in 96-well microtiter plates. The addition of AI-2 increased biofilm formation in *M.avium* cultures. The optimum concentration for this induction was 25 μM, higher concentration resulted in less or no increase in biofilm biomass compared to the negative control.

This effect was attenuated with even greater concentrations of AI-2. In the presence of 250 μM and 2500 μM AI-2, induction decreased to 1.4 and 1.1 fold, respectively. The fact that biofilm formation occurs at an optimum concentration of AI-2

and declines at higher concentrations indicates that other environmental factors, such as starvation, are not major factors promoting biofilm formation in this study.

Confocal laser scanning microscopy confirmed these findings, and showed that increased AI-2 concentration lead to an increase in biofilm biomass and complexity of the three-dimensional structures of the biofilm (Figure 3.2).

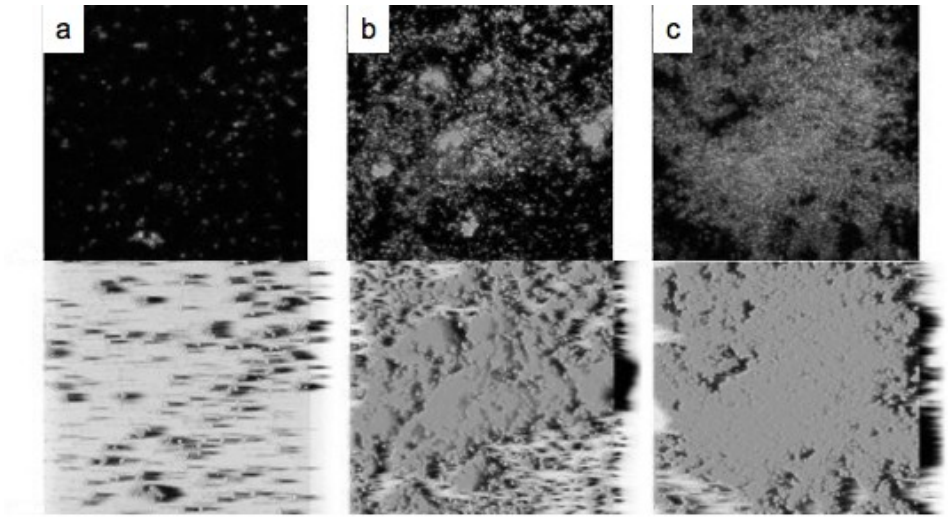

Figure 3.2. Biofilms grown in the presence of AI-2. The negative control (a) shows the smallest amount of AI-2, whereas increasing concentrations to 2.5 μM (b) and 25 μM (c) resulted in increased biofilm formation.

Without AI-2, attachment is sparse and covers only a small portion of the surface. (Figure 3.2a). In the presence of 2.5 μM AI-2, attachment is increased and very dense cell clusters become visible (Figure 3.2b). At 25 μM AI-2, the biofilm height is increased and the surface is almost fully covered. The biofilm surface appears more homogeneous (Figure 3.2c).

AI-2 Induces an Oxidative Stress Response in *M. avium*

The finding that AI-2 influences the biofilm phenotype led to the question of how the gene expression is affected by the molecule. Therefore, differential gene expression was studied using microarray analysis. For this, planktonic *M. avium* cultures were grown in parallel in the presence and absence of AI-2 at 37°C.

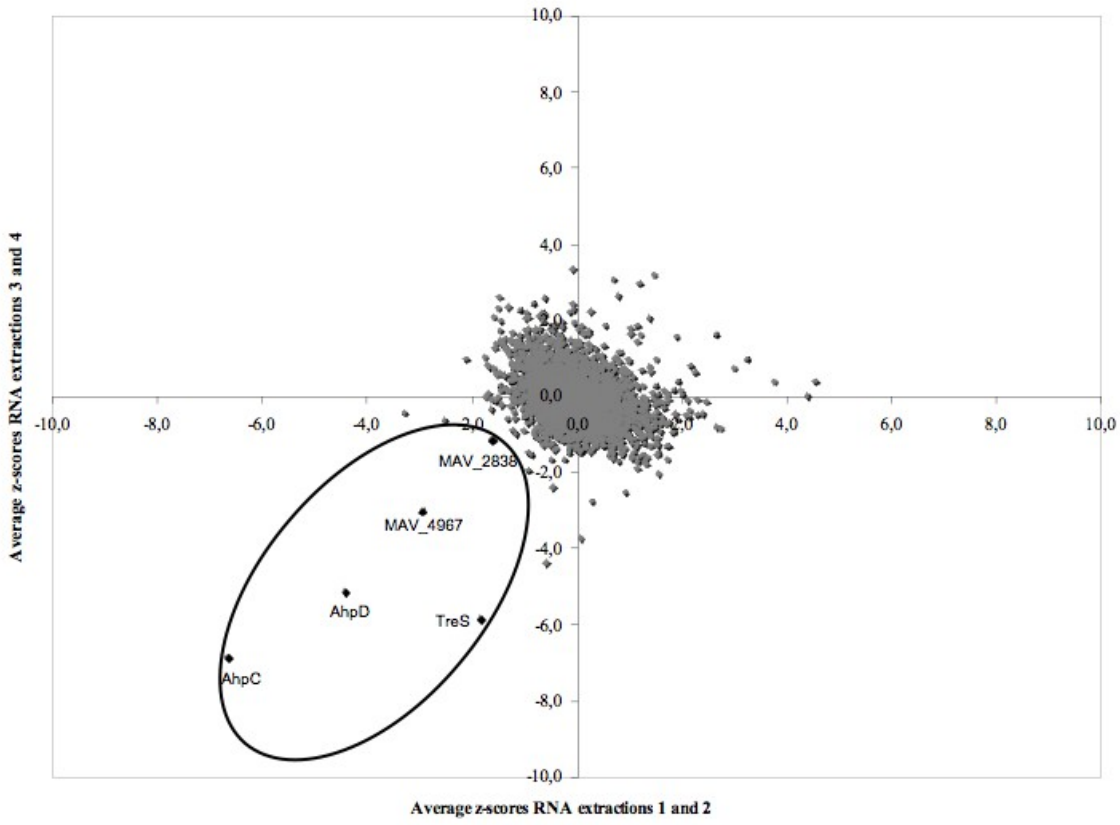

Figure 3.3. Transcriptional profile of AI-2-treated cells versus negative control determined by microarray analysis. Each *z-score* represents the hybridization result for one gene in one experiment with and without AI-2. Normalization using *z-scores* allows comparison of independent experiments. Genes with negative *z*-scores are upregulated in the AI-2 treated samples.

Optical density readings after incubation were taken to demonstrate that AI-2 did not have a growth effect on the planktonic cultures compared to the negative control (data not shown). The RNA of both cultures was extracted and microarray analysis was performed. The experiment was performed in triplicate for an incubation time of three days. Furthermore, an incubation time of 2.5 hours was tested to assess the immediate response of *M. avium* to AI-2.

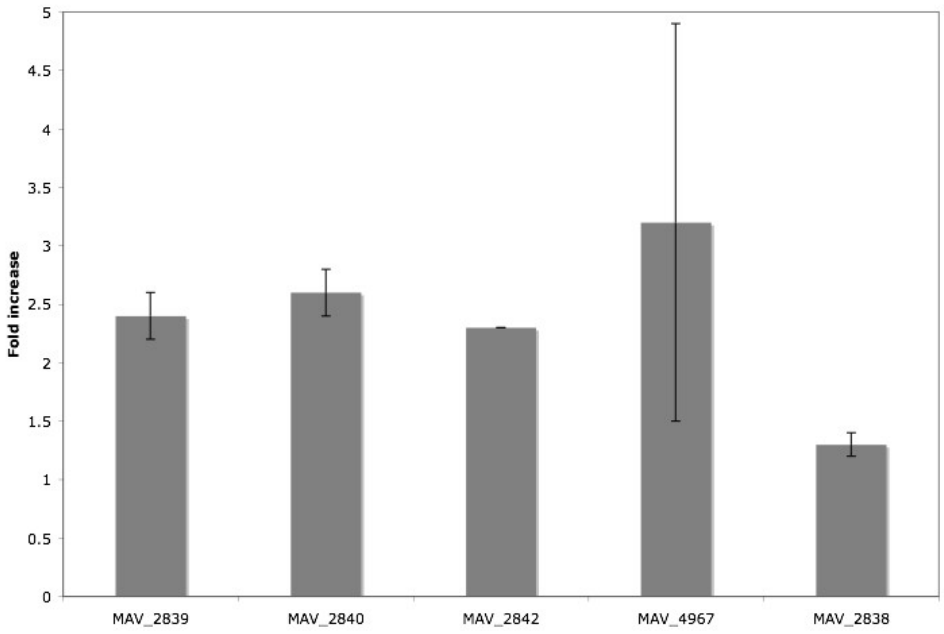

Figure 3.4. Fold inductions determined by qRT-PCR.

Out of the >4000 genes in the *M. avium* genome and independently of the incubation time, five genes were reproducibly upregulated in response to addition of 0.5 mM AI-2 (Figure 3.3).

The upregulation was confirmed by RT-PCR (Figure 3.4). The fold inductions are compared and summarized in Table 3.2. The alkyl hydroperoxidases AhpC and AhpD as well as the trehalose synthase TreS are involved in the bacterial oxidative stress response. AhpC and AhpD provide antioxidant protection by removing peroxides from the environment and, upon macrophage entry, detoxify the reactive oxygen species produced by the host immune system (25, 48). Trehalose is an important cell wall component in mycobacteria since it increases the impermeability and therefore prevents toxic compounds from entering the cell (15, 36).

Table 3.2. Genes upregulated in the presence of AI-2

Gene name	Description	Fold-induction	
		Microarray	RT-PCR
MAV_2839 (ahpC)	Alkyl hydroperoxide reductase C	8.1±2.4	2.4±0.2
MAV_2840 (ahpD)	Alkyl hydroperoxide reductase D	5.8±2.3	2.6±0.2
MAV_2842 (treS)	Trehalose synthase	4.0±1.6	2.3±0.0
MAV_4967	Conserved hypothetical protein	2.5±0.8	3.2±1.7
MAV_2838	LysR transcription regulator	1.8±0.9	1.3±0.1

The three genes are in direct proximity and divergently described from the transcriptional regulator MAV_2838. MAV_2838 is annotated as a homologue to the OxyR transcriptional regulator, based on 38% identity and 53% similarity at the peptide level by BLASTP analysis. MAV_2838 exhibited a modest degree of upregulation, as

seen with many transcriptional regulators. In *Mycobacterium* spp., OxyR is both a sensor of oxidative stress and a transcriptional activator by induction of the *ahpC* and *ahpD genes* (51). OxyR has been shown to be inactive in *M. tuberculosis* due to numerous deletions and frame shift mutations but is functional in *M. avium* (46). The fifth gene, MAV_4967, is a conserved hypothetical protein of unknown function.

Hydrogen Peroxide Induces Biofilm Formation in *M. avium*

The microarray study suggested that AI-2 triggers an oxidative stress response in *M.avium*. Therefore, hydrogen peroxide, known to induce the same genes (35, 46), was used to test the influence of oxidative stress on *M. avium* biofilm formation.

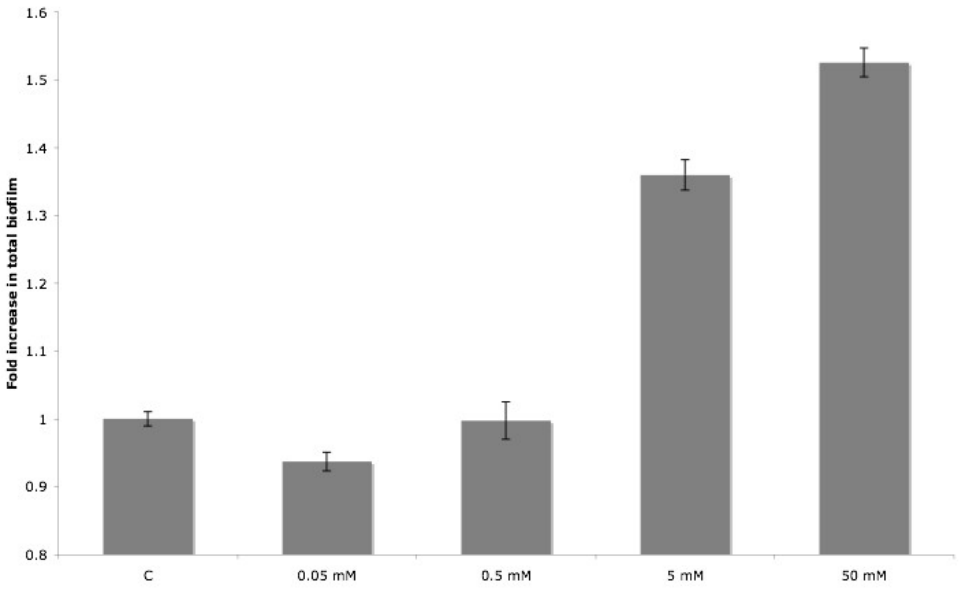

Figure 3.5. Influence of H_2O_2 on *M. avium* biofilm formation.

The CV stain of biofilms grown in the presence of 5 mM and 50 mM hydrogen peroxide showed increased biofilm biomass, while hydrogen peroxide at 0.5 mM and 0.05 mM did not influence this parameter (Figure 3.5).

Discussion

The environmental bacterium *M. avium* is able to adapt to a wide variety of external conditions and can be found in water, soil and animal sources. The bacterium can be found aerosolized, in aqueous suspension or attached to surfaces as biofilms. Due to its enhanced natural resistance to chlorination and heat (33), the bacterium has been isolated from drinking water, hot tubs, sauna walls, shower heads and swimming pools where it represents a health risk to the immunocompromised and others.

Microorganisms in the environment live predominantly in biofilms, which enables them to withstand harsh conditions and increases their resistance to antimicrobial agents. It has been shown that environmental signals and bacterial interactions are very important for biofilm formation (49). Therefore, we studied the influence of the universal quorum-sensing signal AI-2 on *M. avium* biofilm formation. The addition of AI-2 to *M. avium* biofilms led to increased biofilm formation indicating that *M. avium* is able to sense the molecule. The concentration of the added AI-2 was important to this effect. The optimum concentration was between 2.5 μM and 25 μM, whereas addition of 2500 μM did not induce biofilm formation. The concentration-dependent effect of AI-2 on biofilm formation has been shown for the oral bacteria *Actinomyces naeslundii* and *Streptococcus oralis*, where the optimal concentration lies between 0.08 and 0.8 nM (39). One possible explanation for this enormous difference in concentration optima is that AI-2 does not act

as an autoinducer in *M. avium,* which does not have the LuxS synthase. Instead, AI-2 produced by a different bacterial species might act as cross-species signal or parainducer.

Microarray analysis revealed a set of five genes that are consistently upregulated upon the addition of AI-2. *ahpC*, *ahpD* and *treS,* are situated immediately adjacent to each other in the genome. The transcriptional regulator MAV_2838 is situated in close proximity to these three genes and may regulate their transcription. The conserved hypothetical protein MAV_4967 does not cluster with these genes. *ahpC* and *ahpD* are alkyl hydroperoxidases and contribute to the mycobacterial oxidative stress response by detoxifying reactive oxygen species (ROS), especially organic peroxides and hydrogen peroxide. This defense mechanism is crucial for survival and persistence inside the macrophages. The upregulation of *ahpC* and *ahpD* upon addition of AI-2 suggests that these proteins may also have important roles outside the mammalian host.

The trehalose synthase TreS converts maltose to trehalose and is capable of catalyzing the reaction in both directions (8). Trehalose is a non-reducing disaccharide and has a protective effect on proteins and biological membranes exposed to environmental stresses by increasing cell wall impermeability. Trehalose is a major constituent of many glycolipids in the mycobacterial cell wall, such as trehalose 6,6'-dimycolate (cord factor) (15). Furthermore, the sugar plays a role in mycolic acid transport during cell wall biogenesis.

The transcriptional regulator MAV_2838 is a homologue to the *oxyR* gene, the mycobacterial equivalent of the central regulator of oxidative stress response in gram-negative bacteria. OxyR is a peroxide-sensing positive regulator and is tightly linked to and divergently described from ahpC (18). These five genes are not biofilm specific since

the array studies were performed with planktonic *M. avium* cultures. However, the results show that AI-2 triggers an oxidative stress response in *M. avium* suggesting that AI-2 does not act as a quorum-sensing signal per se but as an environmental cue. This model is supported by the fact that addition of hydrogen peroxide also resulted in increased biofilm formation. Kovacic (23) suggested that AI-2 could act as a ROS. Analysis of the chemical structure of DPD, the AI-2 precursor, suggests a possible role in electron transfer (ET), a process generating ROS. Therefore, it is possible that the OxyR regulator senses AI-2 and triggers the oxidative stress response. Biofilm formation has been shown to be a response to oxidative stress in the mammalian host as well as in the environment. The oxidative stress response is crucial for the survival of the pathogen within the host. A recent study correlated the ability to form biofilms with the invasiveness of *M. avium* cells (56). This may be associated with the morphotypic expression of cell wall structures (14), however the oxidative stress response may also play an important role.

A study by Seib et al. (43) shows that OxyR is necessary for biofilm formation in *Neissaria gonorrhoeae*. The link between biofilm formation and oxidative stress has been shown in a number of bacteria including *E. coli* (42) *Helicobacter influenzae* (32), *Campylobacter jejuni* (41) and *Streptococcus mutans* (55). These examples show that biofilm formation can be linked to oxidative stress as a reaction to changing environmental conditions.

References

1. Angenent, L. T., S. T. Kelley, A. St Amand, N. R. Pace, and M. T. Hernandez. 2005. Molecular identification of potential pathogens in water and air of a hospital therapy pool. Proc Natl Acad Sci U S A 102:4860-5.

2. Auger, S., E. Krin, S. Aymerich, and M. Gohar. 2006. Autoinducer 2 affects biofilm formation by Bacillus cereus. Appl Environ Microbiol 72:937-41.

3. Azakami, H., I. Teramura, T. Matsunaga, H. Akimichi, Y. Noiri, S. Ebisu, and A. Kato. 2006. Characterization of autoinducer 2 signal in Eikenella corrodens and its role in biofilm formation. J Biosci Bioeng 102:110-7.

4. Charlet, D., S. Mostowy, D. Alexander, L. Sit, H. G. Wiker, and M. A. Behr. 2005. Reduced expression of antigenic proteins MPB70 and MPB83 in Mycobacterium bovis BCG strains due to a start codon mutation in sigK. Mol Microbiol 56:1302-13.

5. Chen, X., S. Schauder, N. Potier, A. Van Dorsselaer, I. Pelczer, B. L. Bassler, and F. M. Hughson. 2002. Structural identification of a bacterial quorum-sensing signal containing boron. Nature 415:545-9.

6. Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott. 1995. Microbial biofilms. Annu Rev Microbiol 49:711-45.

7. De Keersmaecker, S. C., K. Sonck, and J. Vanderleyden. 2006. Let LuxS speak up in AI-2 signaling. Trends Microbiol 14:114-9.

8. De Smet, K. A., A. Weston, I. N. Brown, D. B. Young, and B. D. Robertson. 2000. Three pathways for trehalose biosynthesis in mycobacteria. Microbiology 146 (Pt 1):199-208.

9. Diggle, S. P., A. Gardner, S. A. West, and A. S. Griffin. 2007. Evolutionary theory of bacterial quorum sensing: when is a signal not a signal? Philos Trans R Soc Lond B Biol Sci 362:1241-9.

10. Duan, K., C. Dammel, J. Stein, H. Rabin, and M. G. Surette. 2003. Modulation of Pseudomonas aeruginosa gene expression by host microflora through interspecies communication. Mol Microbiol 50:1477-91.

11. Falkinham, J. O., 3rd, C. D. Norton, and M. W. LeChevallier. 2001. Factors influencing numbers of Mycobacterium avium, Mycobacterium intracellulare, and other Mycobacteria in drinking water distribution systems. Appl Environ Microbiol 67:1225-31.

12. Falkinham, J. O., 3rd. 2003. Mycobacterial aerosols and respiratory disease. Emerg Infect Dis 9:763-7.

13. Fong, K. P., W. O. Chung, R. J. Lamont, and D. R. Demuth. 2001. Intra- and interspecies regulation of gene expression by Actinobacillus actinomycetemcomitans LuxS. Infect Immun 69:7625-34.

14. Freeman, R., H. Geier, K. M. Weigel, J. Do, T. E. Ford, and G. A. Cangelosi. 2006. Roles for cell wall glycopeptidolipid in surface adherence and planktonic dispersal of Mycobacterium avium. Appl Environ Microbiol 72:7554-8.

15. Fujita, Y., T. Naka, M. R. McNeil, and I. Yano. 2005. Intact molecular characterization of cord factor (trehalose 6,6'-dimycolate) from nine species of mycobacteria by MALDI-TOF mass spectrometry. Microbiology 151:3403-16.

16. Fuqua, C., M. R. Parsek, and E. P. Greenberg. 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. Annu Rev Genet 35:439-68.

17. Gonzalez Barrios, A. F., R. Zuo, Y. Hashimoto, L. Yang, W. E. Bentley, and T. K. Wood. 2006. Autoinducer 2 controls biofilm formation in Escherichia coli through a novel motility quorum-sensing regulator (MqsR, B3022). J Bacteriol 188:305-16.

18. Hahn, J. S., S. Y. Oh, and J. H. Roe. 2002. Role of OxyR as a peroxide-sensing positive regulator in Streptomyces coelicolor A3(2). J Bacteriol 184:5214-22.

19. Hammer, B. K., and B. L. Bassler. 2003. Quorum sensing controls biofilm formation in Vibrio cholerae. Mol Microbiol 50:101-4.

20. Herzberg, M., I. K. Kaye, W. Peti, and T. K. Wood. 2006. YdgG (TqsA) controls biofilm formation in Escherichia coli K-12 through autoinducer 2 transport. J Bacteriol 188:587-98.

21. Inderlied, C. B., C. A. Kemper, and L. E. Bermudez. 1993. The Mycobacterium avium complex. Clin Microbiol Rev 6:266-310.

22. Keller, L., and M. G. Surette. 2006. Communication in bacteria: an ecological and evolutionary perspective. Nat Rev Microbiol 4:249-58.

23. Kovacic, P. 2007. Unifying mechanism for bacterial cell signalers (4,5-dihydroxy-2,3-pentanedione, lactones and oligopeptides): Electron transfer and reactive oxygen species. Practical medical features. Med Hypotheses.

24. Lemos, J. A., T. A. Brown, Jr., and R. A. Burne. 2004. Effects of RelA on key virulence properties of planktonic and biofilm populations of Streptococcus mutans. Infect Immun 72:1431-40.

25. Master, S. S., B. Springer, P. Sander, E. C. Boettger, V. Deretic, and G. S. Timmins. 2002. Oxidative stress response genes in Mycobacterium tuberculosis: role of ahpC in resistance to peroxynitrite and stage-specific survival in macrophages. Microbiology 148:3139-44.

26. McNab, R., and R. J. Lamont. 2003. Microbial dinner-party conversations: the role of LuxS in interspecies communication. J Med Microbiol 52:541-5.

27. Merritt, J., F. Qi, S. D. Goodman, M. H. Anderson, and W. Shi. 2003. Mutation of luxS affects biofilm formation in Streptococcus mutans. Infect Immun 71:1972-9.

28. Miller, S. T., K. B. Xavier, S. R. Campagna, M. E. Taga, M. F. Semmelhack, B. L. Bassler, and F. M. Hughson. 2004. Salmonella typhimurium recognizes a chemically distinct form of the bacterial quorum-sensing signal AI-2. Mol Cell 15:677-87.

29. Moore, J. S., M. Christensen, R. W. Wilson, R. J. Wallace, Jr., Y. Zhang, D. R. Nash, and B. Shelton. 2000. Mycobacterial contamination of metalworking fluids: involvement of a possible new taxon of rapidly growing mycobacteria. Aihaj 61:205-13.

30. Mostowy, S., C. Cleto, D. R. Sherman, and M. A. Behr. 2004. The Mycobacterium tuberculosis complex transcriptome of attenuation. Tuberculosis (Edinb) 84:197-204.

31. Mostowy, S., A. Onipede, S. Gagneux, S. Niemann, K. Kremer, E. P. Desmond, M. Kato-Maeda, and M. Behr. 2004. Genomic analysis distinguishes Mycobacterium africanum. J Clin Microbiol 42:3594-9.

32. Murphy, T. F., C. Kirkham, S. Sethi, and A. J. Lesse. 2005. Expression of a peroxiredoxin-glutaredoxin by Haemophilus influenzae in biofilms and during human respiratory tract infection. FEMS Immunol Med Microbiol 44:81-9.

33. Norton, C. D., M. W. LeChevallier, and J. O. Falkinham, 3rd. 2004. Survival of Mycobacterium avium in a model distribution system. Water Res 38:1457-66.

34. O'Toole, G. A., L. A. Pratt, P. I. Watnick, D. K. Newman, V. B. Weaver, and R. Kolter. 1999. Genetic approaches to study of biofilms. Methods Enzymol 310:91-109.

35. Pagan-Ramos, E., J. Song, M. McFalone, M. H. Mudd, and V. Deretic. 1998. Oxidative stress response and characterization of the oxyR-ahpC and furA-katG loci in Mycobacterium marinum. J Bacteriol 180:4856-64.

36. Pan, Y. T., V. Koroth Edavana, W. J. Jourdian, R. Edmondson, J. D. Carroll, I. Pastuszak, and A. D. Elbein. 2004. Trehalose synthase of Mycobacterium smegmatis: purification, cloning, expression, and properties of the enzyme. Eur J Biochem 271:4259-69.

37. Primm, T. P., C. A. Lucero, and J. O. Falkinham, 3rd. 2004. Health impacts of environmental mycobacteria. Clin Microbiol Rev 17:98-106.

38. Reisner, A., J. A. Haagensen, M. A. Schembri, E. L. Zechner, and S. Molin. 2003. Development and maturation of Escherichia coli K-12 biofilms. Mol Microbiol 48:933-46.

39. Rickard, A. H., R. J. Palmer, Jr., D. S. Blehert, S. R. Campagna, M. F. Semmelhack, P. G. Egland, B. L. Bassler, and P. E. Kolenbrander. 2006. Autoinducer 2: a concentration-dependent signal for mutualistic bacterial biofilm growth. Mol Microbiol 60:1446-56.

40. Rozen, S., and H. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132:365-86.

41. Sampathkumar, B., S. Napper, C. D. Carrillo, P. Willson, E. Taboada, J. H. Nash, A. A. Potter, L. A. Babiuk, and B. J. Allan. 2006. Transcriptional and translational expression patterns associated with immobilized growth of Campylobacter jejuni. Microbiology 152:567-77.

42. Schembri, M. A., L. Hjerrild, M. Gjermansen, and P. Klemm. 2003. Differential expression of the Escherichia coli autoaggregation factor antigen 43. J Bacteriol 185:2236-42.

43. Seib, K. L., H. J. Wu, Y. N. Srikhanta, J. L. Edwards, M. L. Falsetta, A. J. Hamilton, T. L. Maguire, S. M. Grimmond, M. A. Apicella, A. G. McEwan, and M. P. Jennings. 2007. Characterization of the OxyR regulon of Neisseria gonorrhoeae. Mol Microbiol 63:54-68.

44. Shao, H., R. J. Lamont, and D. R. Demuth. 2007. Autoinducer 2 Is Required for Biofilm Growth of Aggregatibacter (Actinobacillus) actinomycetemcomitans. Infect Immun 75:4211-8.

45. Shelton, B. G., W. D. Flanders, and G. K. Morris. 1999. Mycobacterium sp. as a possible cause of hypersensitivity pneumonitis in machine workers. Emerg Infect Dis 5:270-3.

46. Sherman, D. R., P. J. Sabo, M. J. Hickey, T. M. Arain, G. G. Mahairas, Y. Yuan, C. E. Barry, 3rd, and C. K. Stover. 1995. Disparate responses to oxidative stress in saprophytic and pathogenic mycobacteria. Proc Natl Acad Sci U S A 92:6625-9.

47. Sherman, D. R., M. Voskuil, D. Schnappinger, R. Liao, M. I. Harrell, and G. K. Schoolnik. 2001. Regulation of the Mycobacterium tuberculosis hypoxic response gene encoding alpha -crystallin. Proc Natl Acad Sci U S A 98:7534-9.

48. Springer, B., S. Master, P. Sander, T. Zahrt, M. McFalone, J. Song, K. G. Papavinasasundaram, M. J. Colston, E. Boettger, and V. Deretic. 2001. Silencing of oxidative stress response in Mycobacterium tuberculosis: expression patterns of ahpC in virulent and avirulent strains and effect of ahpC inactivation. Infect Immun 69:5967-73.

49. Stanley, N. R., and B. A. Lazazzera. 2004. Environmental signals and regulatory pathways that influence biofilm formation. Mol Microbiol 52:917-24.

50. Stock, A. M., V. L. Robinson, and P. N. Goudreau. 2000. Two-component signal transduction. Annu Rev Biochem 69:183-215.

51. Storz, G., and J. A. Imlay. 1999. Oxidative stress. Curr Opin Microbiol 2:188-94.

52. Turenne, C. Y., M. Semret, D. V. Cousins, D. M. Collins, and M. A. Behr. 2006. Sequencing of hsp65 distinguishes among subsets of the Mycobacterium avium complex. J Clin Microbiol 44:433-40.

53. Turenne, C. Y., R. Wallace, Jr., and M. A. Behr. 2007. Mycobacterium avium in the postgenomic era. Clin Microbiol Rev 20:205-29.

54. Waters, C. M., and B. L. Bassler. 2005. Quorum sensing: cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol 21:319-46.

55. Wen, Z. T., P. Suntharaligham, D. G. Cvitkovitch, and R. A. Burne. 2005. Trigger factor in Streptococcus mutans is involved in stress tolerance, competence development, and biofilm formation. Infect Immun 73:219-25.

56. Yamazaki, Y., L. Danelishvili, M. Wu, E. Hidaka, T. Katsuyama, B. Stang, M. Petrofsky, R. Bildfell, and L. E. Bermudez. 2006. The ability to form biofilm influences Mycobacterium avium invasion and translocation of bronchial epithelial cells. Cell Microbiol 8:806-14.

57. Yoshida, A., T. Ansai, T. Takehara, and H. K. Kuramitsu. 2005. LuxS-based signaling affects Streptococcus mutans biofilm formation. Appl Environ Microbiol 71:2372-80.

CHAPTER 4

THE MYCOBACTERIAL CELL WALL IS A CRUCIAL FACTOR FOR BIOFILM FORMATION

Abstract

The opportunistic pathogen *Mycobacterium avium* is a significant inhabitant of biofilms in drinking water distribution systems. *M.avium* expresses on its cell surface serovar-specific glycopeptidolipids (ssGPLs). Studies have implicated the core GPL in biofilm formation by *M. avium* and by other *Mycobacterium* species. In order to test this hypothesis in a directed fashion, three model systems were used to examine biofilm formation by mutants of *M. avium* with transposon insertions into *pstAB* (also known as *nrp* and *mps*). *pstAB* encodes the nonribosomal peptide synthetase that catalyzes the synthesis of the core GPL. The mutants did not adhere to polyvinyl chloride plates; however, they adhered well to plastic and glass chamber slide surfaces, albeit with different morphologies from the parent strain. In a model that quantified surface adherence in recirculating water, wild-type and *pstAB* mutant cells accumulated on stainless steel surfaces in equal numbers. Unexpectedly, *pstAB* mutant cells were >10-fold less abundant in the recirculating-water phase than parent strain cells. These observations show that GPLs are directly or indirectly required for colonization of some, but by no means all, surfaces. Under some conditions, GPLs may play an entirely different role by facilitating the survival of dispersed nonadherent *M. avium* cells in circulating water. Such a function could contribute to waterborne *M.avium* infection.

Introduction

Mycobacterium avium is an opportunistic pathogen that causes primarily pulmonary, but also cutaneous diseases. It mainly infects the immuno-compromised, such as AIDS patients with a CD-4 cell count below 50 (3). Cases of hypersensitivity pneumonitis (HP) were reported in otherwise healthy individuals due to exposure to aerosolized mycobacteria from indoor swimming pools, hot tubs and metalworking fluids in industrial settings (1, 18, 24). Person-to-person transmission is thought to be minimal, with the main source of infection from the environment (26). Mycobacteria are significant inhabitants of biofilms in a number of environmental settings. The bacterium is found in soils, water, the air, and in food. Due to its resistance to disinfection, it can also be found in drinking water (11, 25). It has been shown that *M. avium* strains recovered from municipal water systems were identical to those recovered from AIDS patients exposed to such sources – implicating potable water as a possible source for *M. avium* infection (26).

Mycobacteria show very high intrinsic resistance to environmental insults such as high temperatures, antibiotics and disinfectants (16, 25). This is true mainly due to the unique morphology of the mycobacterial cell wall, which is very complex, lipid-rich and hydrophobic. The cell wall characteristics contribute to its virulence, persistence within macrophages and to its ability to form biofilms (22).

The mycobacterial cell wall consists of an arabinogalactan-petidoglycan-mycolic acid core and is surrounded by a second electron-dense layer, which is made up, in part of serovar-specific glycopeptidolipids (ssGPL).

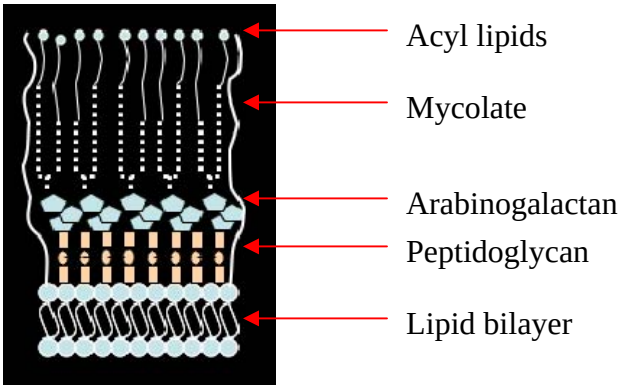

Figure 4.1. Morphology of the mycobacterial cell wall.

GPLs are surface antigens whose lipotetrapeptide core consists of fatty acyl-*NH*-D-phenylalanine-D-allothreonine-D-alanine-L-alaninol (2, 7). The alaninyl C terminus is rhamnosylated by the rhamnosyltransferase RtfA (9), and the allothreonine residue is linked to a 6-deoxy-L-talose. Modification of the latter with variant oligosaccharide structures accounts for the distinct serovars of the MAC. Loss of the ssGPL gene cluster, named *ser2*, leads to the rough colony morphotype (3). GPLs seem to be involved in mycobacterial virulence and invasiveness, although the mechanisms remain unclear.

Contradictory studies have been published regarding the connection between biofilm formation and virulence. Howard et al. reported that in *M. abscessus* the loss of GPLs results in an increase in virulence whereas the ability to form biofilms decreases (13).

On the other hand, Yamazaki et al. reported that biofilm-deficient *M. avium* strains showed impaired virulence in *M. avium* (27).

However, the importance of GPLs for mycobacterial biofilm formation has been shown in a number of examples. The loss of GPLs in *M. smegmatis* resulted in both loss of motility and biofilm formation (23). It was also shown that GPL biosynthesis is a crucial pathway involved in the formation of a biofilm (28).

The genetic analysis of mycobacteria is a challenging task because of the bacterium's slow growth, its genetic instability and the intrinsic multidrug resistance (15). In order to study the mycobacterial cell wall as a key player of colony morphology, virulence, drug resistance and biofilm formation, a random transposon mutagenesis approach was used. The transposome complex carries a Kan^r gene and a transposase that recognizes its terminal inverted repeats (12, 15) and randomly integrates in the bacterial genome.

Using this technique, a white opaque (WO) *M. avium* strain was mutagenized and mutants with altered colony morphology were isolated and characterized. The peptide synthetases pstA and pstB were identified to be involved in morphotypic switching. The genes are situated directly next to the *ser2* gene cluster, which is responsible for GPL synthesis in mycobacteria.

This study was performed in collaboration with Gerard Cangelosi's group in order to examine the ability of GPL mutants to form biofilms on diverse surfaces in three different model systems. These mutations were located in the nonribosomal peptide synthetase gene *pstAB* and in the hypothetical protein Maa0846. Previous studies showed that mutations in *pstAB* were impaired in mycobacterial biofilm formation (6, 13, 23, 28).

The part of the project conducted at Montana State University, and a component of this thesis work, describes the ability of several, independently isolated *M. avium pstAB* mutants as well as Maa0846 mutants to adhere to stainless steel coupons compared to the parent strain in a recirculating water system.

Methods and Materials

Bacterial Strains and Culture Conditions

M. avium subspecies *hominissuis* strain HMC02 is a clinical isolate from an anonymous patient in Seattle, Washington. Mutants were isolated from an EZ-TN (KAN-2) transposon insertion library as described previously (4, 21, 10). *M. avium* HMC02 shows the white opaque (WO) morphotype, which is associated with increased virulence and multidrug resistance relative to the red opaque (RO) morphotype. Mutants that showed red, rough colonies on Congo red agar were isolated and shown to have insertions into the peptide synthetases *pstA* and *pstB.*

Accumulation of Viable *M. avium* Subspecies *hominissuis* on Stainless Steel Surfaces and in Recirculating Water

The recirculating system is shown in Figure 4.2. Water was circulated through the system using a Masterflex pump head (Cole-Parmer model 7518-00). The tubing used was Masterflex Norprene (06402-25), with an inner diameter of 0.5 mm. The reservoir vessel was filled with 500 ml of sterile Milli-Q water and connected to both sides of a 23-in. glass tube with an inner diameter of 1.5 cm. All connections were made with silicone stoppers. Contained within each tube were four stainless steel coupons with dimensions

of 1.3 by 8.0 cm, held together by stainless steel wire. After assembly, the entire system was autoclaved.

Cells taken from a Middlebrook 7H10 agar plate were suspended in 1 ml sterile Milli-Q purified water. These suspensions were added to 2-ml screw-cap tubes containing 600 to 800 μl glass beads (2-mm diameter). To ensure uniform dispersal of variably flocculent *M. avium* subspecies *hominissuis* cell suspensions, the cells were mechanically dispersed by agitation with a Biospec Products Mini-Beadbeater. Bead beating was performed at the lowest power in two 15-s bursts. The density of each culture was read by using a Klett Photoelectric Colorimeter Model 800-3 and normalized to 75 Klett units (equivalent to an OD_{660} of approximately 0.15) prior to inoculation. Eight milliliters of each adjusted cell suspension was then injected into a recirculation system. The pump was adjusted to a flow rate of 38 ml/min, and the system was allowed to run at ambient temperature for 2 weeks.

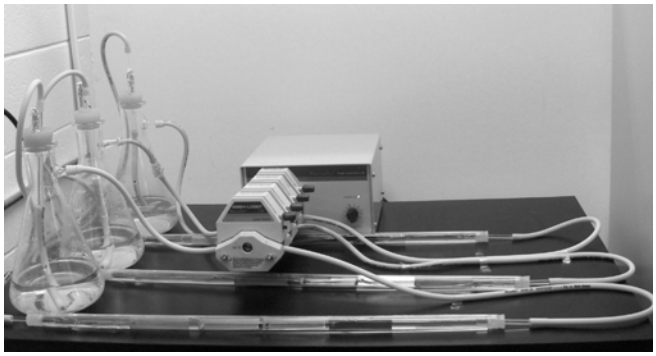

Figure 4.2. Recirculating model system. A bank of three replicate systems is shown, each with four coupons in a glass tube.

At the end of the incubation period, four coupons and two water samples were taken for analysis as follows. Glass tubes were aseptically removed from the system and

transferred to a hood. The coupons were pulled out of the cartridge and extensively rinsed to remove nonadherent cells. Adherent cells were scraped off of the coupons with a rubber policeman and suspended in 10 ml of sterile water. After bead beating to disperse clumps as described above, replicate samples were serially diluted onto 7H10 agar plates. Ten replicates were plated per dilution. Bacteria in the water (planktonic) phase of the samples in glass tubes were also analyzed. Two 1-ml samples per tube were serially diluted onto 7H10 agar in replicates of 10, as described above.

Results

Biofilm Formation on a Stainless Steel Surface In a Recirculating-Water Distribution Model

In order to model flow conditions of a drinking water distribution system, a laboratory-based recirculating water system was used to quantify biofilm adherence to stainless steel surfaces (Figure 4.2). Replicate systems were inoculated with the parent HMC02-WO clone and a suite of the following mutants: three *pstA* mutants, 13.1641, 15.1899, and 20.2439 and two *pstB* mutants, 6.418 and 24.3411. Two mutants in the hypothetical protein, Maa0846, were also studied. At the conclusion of the 14-day incubation period, recirculating, as well as adherent, bacteria were quantified by plating and colony counting.

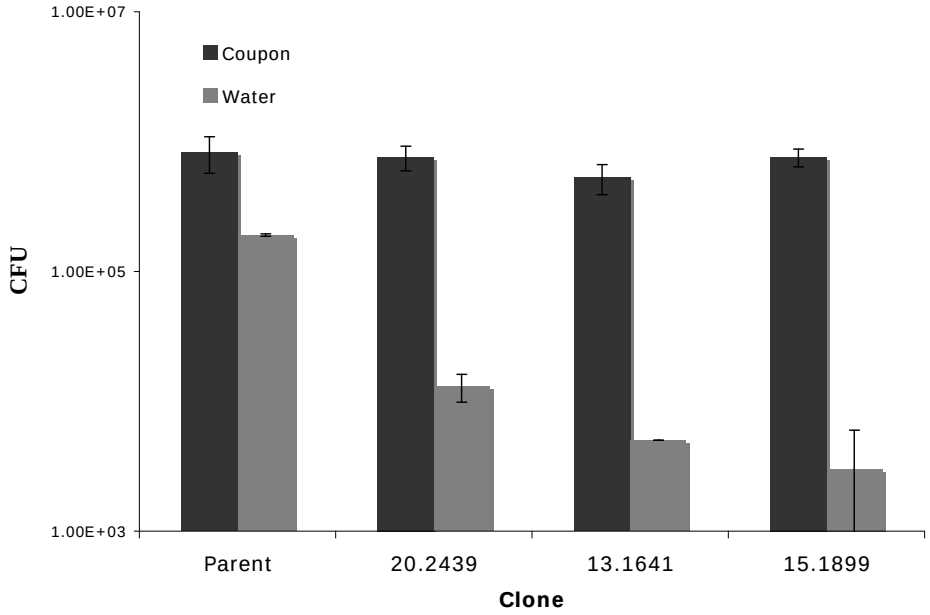

Figure 4.3. Recirculation system cell counts for PstA mutants. Viable bacteria were quantified as cells/coupon or cells/mL in the water.

The parent and mutant strains did not differ quantitatively in their adherence to stainless steel coupons under these conditions. However, a consistent quantitative difference was seen in the recirculating phase, where the parent strain was found in ≥10-fold greater numbers than the mutants after 14 days (Figure 4.3).

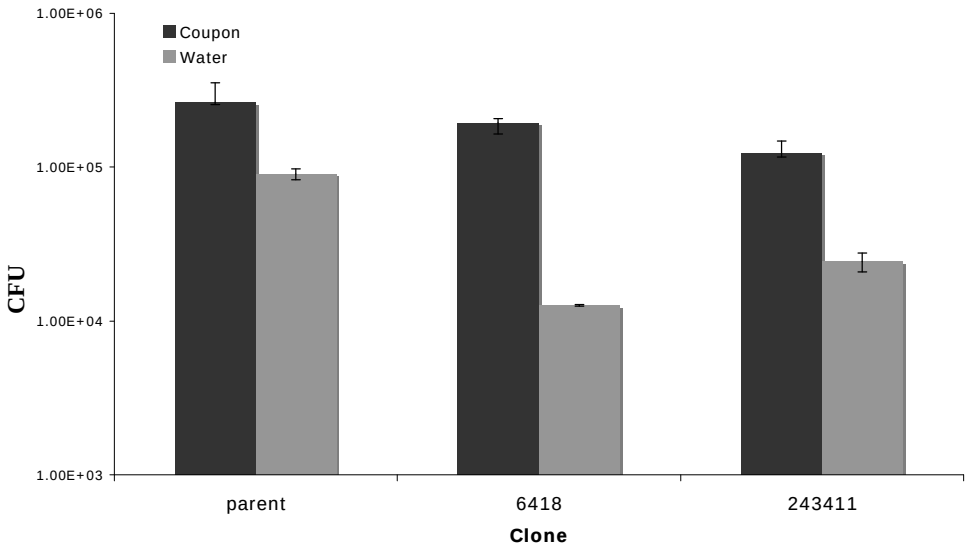

Figure 4.4. Recirculation system cell counts for PstB mutants. Viable bacteria were quantified as cells/coupon or cells/mL in the water.

Similar results were obtained in an experiment conducted on the parent strain, HMC02-WO, and the two *pstB* mutants (Figure 4.4). In both cases, mutant and wild type adherences to coupons were indistinguishable (within two standard deviations), while wild-type colony counts in the recirculationg-water phase were ≥10-fold greater than those of the mutants.

A third experiment conducted with two mutants of the hypothetical protein Maa0846, showed slightly less attachment to the coupons than the parent strain (Figure 4.5). However, the number of parent cells in the recirculating phase was much lower than in previous experiments.

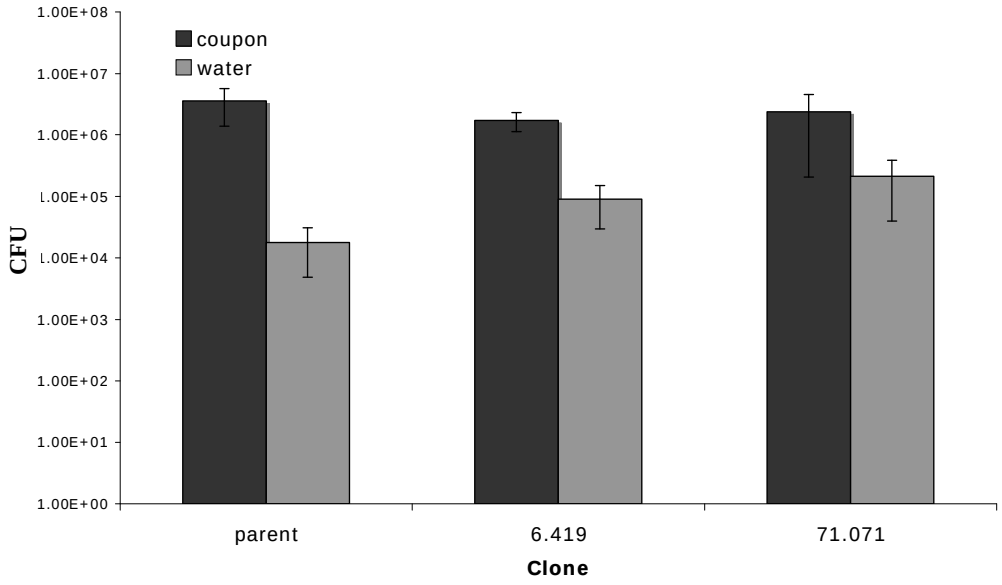

Figure 4.5. Recirculation system cell counts for conserved hypotheticals.

The number of mutant cells in the recirculating phase was much higher than in the *pstAB* experiments. This can be due to different strategies during inoculation, as the inoculum contained about twice as many cells as in previous experiments. Although there were more mutant cells accumulated in the recirculating phase, the cells adhered to a slightly lesser extent than the parent strain to the stainless steel coupons.

Table 4.1. Summary of all recirculation system runs conducted

	PstA		**PstB**		**Maa0846**	
Phase	Coupon (cells)	Water (cells/ml)	Coupon (cells)	Water (cells/ml)	Coupon (cells)	Water (cells/ml)
Parent	$8.3x10^5$	$1.9x10^5$	$2.6x10^5$	$9.0x10^4$	$3.5x10^6$	$1.8x10^4$
Mutants[1]	$6.8x10^5$	$7.0x10^3$	$1.6x10^5$	$1.9x10^4$	$2.0x10^6$	$1.5x10^5$

[1]The mutants within the same proteins were averaged.

The summary presented in Table 4.1 shows that the mutants in both the *pstA* and *pstB* genes show similar behavior in the recirculation system. The Maa0846 mutant however gave different results; the mutant cells accumulated in higher numbers in the recirculating phase than the parent.

Influence of the Water Type on the Recirculation System

Most of the experiments described above were conducted with sterile milliQ water. However, for some experiments, autoclaved tap water was used. Therefore, two recirculation runs were carried out to compare the parent strain in both milliQ and tap water. The results are shown in Figure 4.6.

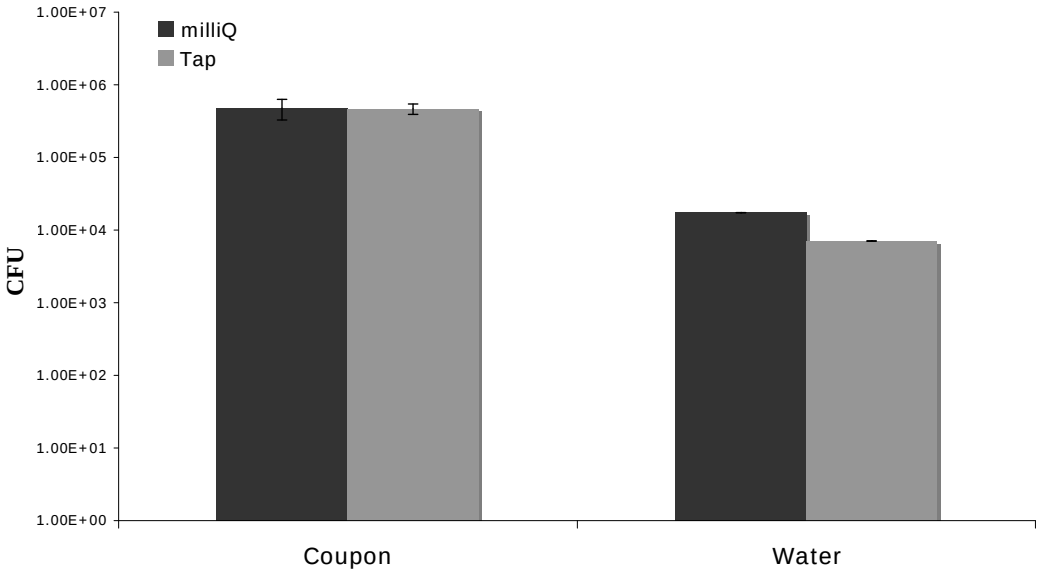

Figure 4.6. Recirculation system cell counts for the parent strain in milliQ and tap water.

Both adherence to the coupon as well as accumulation of the cells in the water phase are very similar indicating that the cells' behavior is comparable in milliQ and tap water.

Discussion

M. avium represents a significant health risk and has been isolated from biofilms in a number of different environments. The bacterium features an exceptionally thick and hydrophobic cell wall, which is one of the key elements in bacterial attachment. The experiments described above were part of a project aimed at assessing the bacterium's attachment to different surfaces and the influence of the cell wall properties in biofilm formation. Previous studies have shown that GPLs are involved in sliding motility and biofilm formation in *M. smegmatis* (23). Other studies have been performed to confirm these findings in *M. avium* (6, 28). However, these studies only used single mutations and under one experimental set-up. This study used multiple, independently isolated mutants of *M. avium* subspecies *hominissuis* in three different model systems. Two of these model systems were tested in the Cangelosi lab: the first system used PVC microtiter plates, a static system that enables screening for biofilm formation by crystal violet staining on a large scale. The requirement for *pstAB* in binding of *M. avium* subspecies *hominissuis* to these PVC plates was confirmed. The results also indicated that the colony type has an impact on biofilm formation, with white and transparent variants adhering to PVC wells in greater amounts than their red and opaque counterparts (10). Interestingly, white and transparent variants are isolated more frequently from patients, and are more virulent in animal models, than red and opaque variants (5, 19), hinting a connection between virulence and biofilm formation. The present study could not confirm the connection between sliding motility and biofilm formation as has been shown in studies on *M.*

smegmatis (14, 17, 23) and *M. abscessus* (13). The RO variant, which naturally shows greater sliding motility than other morphotypes, adhered very poorly to PVC plates.

The second model system tested by Jerry Cangelosi and co-workers consisted of chamber slides, made from silanized glass and plastic (Permanox), respectively. The chamber slides provide a larger surface for bacterial attachment and therefore enabled microscopic observation of the biofilms. The microscopy revealed that the *pstAB* mutants adhered at least equally well to the surface as the parent strain (10). However, the colony morphology was altered. The *pstAB* mutants adhered in large clumps rather than isolated cells in a monolayer, suggesting that the cell-cell interactions become stronger with the GPL mutation. These cell clumps attached very strongly to the surface and withstood even vigorous washing.

The third model system, the recirculation system, is part of this thesis work. It was designed to model drinking water distribution conditions. The model had limitations, including the use of a single-species inoculum and purified water (in order to maximize reproducibility; although attachment was very similar in comparison to autoclaved tap water). Stainless steel surfaces are found in point-of-use fixtures but are not the major habitat in water distribution pipes within the home, which typically have PVC or copper surfaces overlaid with multispecies bacterial biofilms. Nonetheless, the recirculating model is in many ways more authentic than static models used in previous analyses, and it yielded unexpected results. Mutant and wild-type cells bound equally well to the stainless steel coupons, indicating that the core GPL is dispensable in the primary stages of biofilm formation under these conditions. However, the mutants were present in relatively small numbers in the recirculating-water (planktonic) phase. The basis for this

observation is not known. Explanations could include (i) inefficient detachment of mutant cells from biofilms due to enhanced cell-to-cell interactions; (ii) poor survival in purified water for nutritional, structural, or other reasons; and (iii) inefficient suspension in water due to hydrophobic surface properties.

These experiments showed that the cell wall properties are very important for *M. avium* attachment to surfaces. Also, the characteristics of the different surfaces play an essential role. The physico-chemical properties of different surfaces can be very different and therefore, it is hard to generalize about the bacterial behavior. Many factors play important roles in bacterial attachment, such as surface conditioning, mass transport, surface charge, hydrophobicity, surface roughness and more (20). It has been speculated that hydrophobicity results from protein and lipids on the cell surface (8). Therefore, knocking out the GPLs on the cell surface could alter the cells' hydrophobicity, which can change the electrostatic forces leading to attachment.

References

1. Angenent, L. T., S. T. Kelley, A. St Amand, N. R. Pace, and M. T. Hernandez. 2005. Molecular identification of potential pathogens in water and air of a hospital therapy pool. Proc Natl Acad Sci U S A 102:4860-5.

2. Belisle, J. T., M. R. McNeil, D. Chatterjee, J. M. Inamine, and P. J. Brennan. 1993. Expression of the core lipopeptide of the glycopeptidolipid surface antigens in rough mutants of Mycobacterium avium. J Biol Chem 268:10510-6.

3. Belisle, J. T., L. Pascopella, J. M. Inamine, P. J. Brennan, and W. R. Jacobs, Jr. 1991. Isolation and expression of a gene cluster responsible for biosynthesis of the glycopeptidolipid antigens of Mycobacterium avium. J Bacteriol 173:6991-7.

4. Cangelosi, G. A., J. S. Do, R. Freeman, J. G. Bennett, M. Semret, and M. A. Behr. 2006. The two-component regulatory system mtrAB is required for morphotypic multidrug resistance in Mycobacterium avium. Antimicrob Agents Chemother 50:461-8.

5. Cangelosi, G. A., C. O. Palermo, and L. E. Bermudez. 2001. Phenotypic consequences of red-white colony type variation in Mycobacterium avium. Microbiology 147:527-33.

6. Carter, G., M. Wu, D. C. Drummond, and L. E. Bermudez. 2003. Characterization of biofilm formation by clinical isolates of Mycobacterium avium. J Med Microbiol 52:747-52.

7. Chatterjee, D., and K. H. Khoo. 2001. The surface glycopeptidolipids of mycobacteria: structures and biological properties. Cell Mol Life Sci 58:2018-42.

8. Davies, D.G., 2000. Physiological events in biofilm formation. In: Allison, D., P. Gilbert, H. Lappin-Scott, and M. Wilson (eds): Community structure and co-operation in biofilms:37-51.

9. Eckstein, T. M., F. S. Silbaq, D. Chatterjee, N. J. Kelly, P. J. Brennan, and J. T. Belisle. 1998. Identification and recombinant expression of a Mycobacterium avium rhamnosyltransferase gene (rtfA) involved in glycopeptidolipid biosynthesis. J Bacteriol 180:5567-73.

10. Freeman, R., H. Geier, K. M. Weigel, J. Do, T. E. Ford, and G. A. Cangelosi. 2006. Roles for cell wall glycopeptidolipid in surface adherence and planktonic dispersal of Mycobacterium avium. Appl Environ Microbiol **72:**7554-8.

11. Falkinham, J. O., 3rd, C. D. Norton, and M. W. LeChevallier. 2001. Factors influencing numbers of Mycobacterium avium, Mycobacterium intracellulare, and other Mycobacteria in drinking water distribution systems. Appl Environ Microbiol 67:1225-31.

12. Goryshin, I. Y., J. Jendrisak, L. M. Hoffman, R. Meis, and W. S. Reznikoff. 2000. Insertional transposon mutagenesis by electroporation of released Tn5 transposition complexes. Nat Biotechnol 18:97-100.

13. Howard, S. T., E. Rhoades, J. Recht, X. Pang, A. Alsup, R. Kolter, C. R. Lyons, and T. F. Byrd. 2006. Spontaneous reversion of Mycobacterium abscessus from a smooth to a rough morphotype is associated with reduced expression of glycopeptidolipid and reacquisition of an invasive phenotype. Microbiology 152:1581-90.

14. Kolter, R. 1999. Growth in studying the cessation of growth. J Bacteriol 181:697-9.

15. Laurent, J. P., K. Hauge, K. Burnside, and G. Cangelosi. 2003. Mutational analysis of cell wall biosynthesis in Mycobacterium avium. J Bacteriol 185:5003-6.

16. Le Dantec, C., J. P. Duguet, A. Montiel, N. Dumoutier, S. Dubrou, and V. Vincent. 2002. Occurrence of mycobacteria in water treatment lines and in water distribution systems. Appl Environ Microbiol 68:5318-25.

17. Mathew, R., R. Mukherjee, R. Balachandar, and D. Chatterji. 2006. Deletion of the rpoZ gene, encoding the omega subunit of RNA polymerase, results in pleiotropic surface-related phenotypes in Mycobacterium smegmatis. Microbiology 152:1741-50.

18. Moore, J. S., M. Christensen, R. W. Wilson, R. J. Wallace, Jr., Y. Zhang, D. R. Nash, and B. Shelton. 2000. Mycobacterial contamination of metalworking fluids: involvement of a possible new taxon of rapidly growing mycobacteria. Aihaj 61:205-13.

19. Mukherjee, S., M. Petrofsky, K. Yaraei, L. E. Bermudez, and G. A. Cangelosi. 2001. The white morphotype of Mycobacterium avium-intracellulare is common in infected humans and virulent in infection models. J Infect Dis 184:1480-4.

20. Palmer, J., S. Flint, and J. Brooks. 2007. Bacterial cell attachment, the beginning of a biofilm. J Ind Microbiol Biotechnol 34:577-88.

21. Philalay, J. S., C. O. Palermo, K. A. Hauge, T. R. Rustad, and G. A. Cangelosi. 2004. Genes required for intrinsic multidrug resistance in Mycobacterium avium. Antimicrob Agents Chemother 48:3412-8.

22. Primm, T. P., C. A. Lucero, and J. O. Falkinham, 3rd. 2004. Health impacts of environmental mycobacteria. Clin Microbiol Rev 17:98-106.

23. Recht, J., and R. Kolter. 2001. Glycopeptidolipid acetylation affects sliding motility and biofilm formation in Mycobacterium smegmatis. J Bacteriol 183:5718-24.

24. Shelton, B. G., W. D. Flanders, and G. K. Morris. 1999. Mycobacterium sp. as a possible cause of hypersensitivity pneumonitis in machine workers. Emerg Infect Dis 5:270-3.

25. Taylor, R. H., J. O. Falkinham, 3rd, C. D. Norton, and M. W. LeChevallier. 2000. Chlorine, chloramine, chlorine dioxide, and ozone susceptibility of Mycobacterium avium. Appl Environ Microbiol 66:1702-5.

26. von Reyn, C. F., J. N. Maslow, T. W. Barber, J. O. Falkinham, 3rd, and R. D. Arbeit. 1994. Persistent colonisation of potable water as a source of Mycobacterium avium infection in AIDS. Lancet 343:1137-41.

27. Yamazaki, Y., L. Danelishvili, M. Wu, E. Hidaka, T. Katsuyama, B. Stang, M. Petrofsky, R. Bildfell, and L. E. Bermudez. 2006. The ability to form biofilm influences Mycobacterium avium invasion and translocation of bronchial epithelial cells. Cell Microbiol 8:806-14.

28. Yamazaki, Y., L. Danelishvili, M. Wu, M. Macnab, and L. E. Bermudez. 2006. Mycobacterium avium genes associated with the ability to form a biofilm. Appl Environ Microbiol 72:819-25.

CHAPTER 5

THE PRESENCE OF SOME BACTERIAL SPECIES AND THEIR METABOLIC BY-PRODUCTS INFLUENCE *MYCOBACTERIUM AVIUM* BIOFILM FORMATION

Introduction

Mycobacterium avium is an environmental opportunistic pathogen, which is able to thrive in a wide variety of environments. It can be found in soils, in natural fresh water, brackish water and also in animals such as birds or swine. It causes predominantly respiratory diseases, mainly in immunocompromised persons, but otherwise healthy people have been reported with *M. avium* infections (2, 11, 24, 36). Routes of exposure include ingestion and inhalation of aerosolized bacteria. Most of the *M. avium* infections are acquired from the environment; person-to-person transmission is unlikely (21, 28, 44).

Due to its many different reservoirs, *M. avium* has developed excellent strategies to adapt to adverse environmental conditions. It is highly resistant to antibiotics and disinfectants and is able to withstand the immune response of a mammalian host (19, 29, 40). This high degree of resistance is mostly due to the bacterium's slow growth rate and the thick and waxy mycobacterial cell wall. It consists of an arabinogalactan-peptidoglycan-mycolic acid core, which renders the cells impermeable and hydrophobic (5, 6). The elaborate cell wall architecture also contributes to the slow growth of *M. avium.*

M. avium thrives in environments where a myriad of different bacterial species are encountered in competition for nutrients and ecological niches. Therefore, it is very important for the bacterium to monitor its surroundings. The presence of other bacteria can be both beneficial and detrimental. The co-habitation of different bacterial species can lead to the exchange of metabolic by-products but it can also mean competition for food and space. Bacteria produce a multitude of signaling molecules and metabolites that can serve as signals and cues for other bacteria. Signals are small, diffusible molecules produced by the cells to communicate with one another. In a process called quorum sensing (QS), bacteria produce autoinducer (AI) molecules in order to co-ordinate their behavior within a population (14). Once a certain threshold concentration is reached, the AI binds to a histidine sensor kinase of a two-component system or directly to a response regulator, which then activates gene transcription of a target gene. QS in gram-negative bacteria is accomplished via acylated homoserine lactones (AHLs). The AHLs consist of a homoserine lactone core and acyl side chain, the length of which determines the species-specificity (45). In gram-positive bacteria, QS is accomplished via small peptides that are also species-specific (18). One class of AI, called AI-2, is recognized by both gram-positive and gram-negative bacteria (33, 39). It is therefore considered a universal signal. AI-2 is derived from 4,5-dihydroxy-2,3-pentonedione (DPD), the precursor for a family of spontaneously interconverting molecules (33). DPD is produced by the LuxS enzyme, which is a part of the *S*-adenosyl-L-methionine (SAM) pathway. The toxic byproduct *S*-adenosylhomocysteine (SAH) is converted to adenine, homocysteine and the AI-2 precursor 4,5-dihydroxy-2,3-pentonedione (DPD), in a concerted action of the Pfs and LuxS enzymes (33). Two of the structures derived from DPD have been identified as

active molecules bound to their cognate receptors, (2*S*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate (*S*-THMF borate) in *V. harveyi* (8) and (2*R*,4*S*)-2-methyl-2,3,3,4-tdtrahydroxytetrahydrofuran (*R*-THMF) in *Salmonella typhimurium* (23). Since LuxS plays an important role in the activated methyl cycle and detoxification, it is not clear whether AI-2 evolved as a true signal. A signal is, according to the definition, a compound that was produced in order to trigger a certain effect in both the signal producing and receiving cell. If only the receiving cell profits from the compound, it is not called a signal but a cue (10).

However, not only a signal produced for this purpose, but also the waste product of a species could lead to the advantage of a second species and therefore the second species could be selected to cooperatively help the first species in order to increase the by-product benefits (46).

For some processes, it is difficult to define whether true signaling is involved. For example, *E. coli* is in possession of the *luxS* gene and produces AI-2. The bacterium is able to induce bioluminescence in *V. harveyi*, albeit *E. coli* has a different receptor (48). Since the members of the AI-2 family are interconvertible, it can induce bioluminescence in the marine organism and therefore every bacterium in possession of the *luxS* gene is able to induce bioluminescence in *V. harveyi*.

Other examples for the interspecies interaction via AI-2 have been described in the literature. McNab and co-workers showed that AI-2 is necessary for mixed species biofilm formation between the oral bacteria *Porphorymonas gingivalis* and *Streptococcus gordonii*. In *luxS* null mutants, these two strains did not form biofilms (20). Rickard et al. demonstrated that the oral commensal bacteria *Actinomyces naeslundii* and *Streptococcus*

oralis form biofilms dependent on AI-2 at a certain optimal concentration, below and above which biofilms do not form (31).

AI-2 has been shown to be involved in biofilm formation, virulence, iron uptake and other functions; however true signaling could not always be proven (7, 37). Transcriptome studies have shown that about 10% of all genes are disregulated when LuxS is knocked out in *E.coli* (37) and about 5% are upregulated when AI-2 is added (9). These findings lead to the conclusion that AI-2 is relevant in bacterial gene regulation beyond the activated methyl cycle. However, AI-2 is not a signal in the classical notion because (i) it is not strictly cell-density dependent, (ii) *luxS* is not among its target genes and therefore the positive feedback loop (=autoinduction loop) is absent and (iii) the large background noise in environments with high cell densities of AI-2 producing cells, such as in the human intestinal tract leads to interference with other signals, as has been shown for *Vibrio cholerae* (16).

Another factor that is related to the cells' metabolism and has been linked to biofilm formation is iron. Iron is an essential factor for microbial growth. It is abundant in the environment, in soils, and source waters, but also in animals and plants. However, iron in its ferrous state is soluble and toxic. In order to prevent iron toxicity, the cells produce siderophores, which bind iron and neutralize it. The siderophores' affinity for iron is very high, therefore enabling the cells access even in low-iron environments. However, in situations where iron is found in excess, it can generate reactive oxygen species (ROS), causing oxidative stress to the cells. Two important iron-dependent regulator proteins are Fur and IdeR. Fur is active as a dimer, with one Fe^{2+} per monomer as a cofactor (41). It is thought to regulate iron homeostasis in a wide variety of bacteria.

It is also involved in protection against oxidative stress. IdeR is involved in the regulation of the *mtb* genes in mycobacteria, which code for mycobactin synthesis.

This study investigated the effect of a number of bacterial strains on *M. avium* biofilm formation and focused on the role of two factors in this process.

AI-2 is a suitable candidate to be involved in the formation of environmental biofilms since the molecule is not species-specific and is a metabolic by-product. It can act either as a signal or a cue in order to regulate the co-habitation of many species.

AI-2 triggers the oxidative stress response in *M. avium*, which ultimately leads to biofilm formation. Iron is an important cofactor that also triggers oxidative stress and leads to biofilm formation. The present study was conducted using cell-free supernatants in order to investigate the role of other bacteria and their metabolism in this process. Time-course experiments revealed that *M. avium* responded differently to culture supernatants produced at different growth stages, hinting that metabolic by-products from other bacteria as well as scavenging of components from the growth medium, i.e. iron, play a role in biofilm formation by *M. avium*.

This study also demonstrates another aspect of ecological relevance: the presence of many factors involved in biofilm formation acting either in a synergistic or inhibitory role.

Methods and Materials

Bacterial Strains and Culture Conditions

M. avium strain W2001 was isolated from drinking water in the Boston area. The strain was classified as *M. avium* subsp. *hominissuis* with an hsp65 Code 1 sequevar (42, 43), which is the same as the recently sequenced *M. avium* 104. The organism was grown at 37°C in Middlebrook 7H9 medium supplemented with 10% ADC enrichment and 0.2% glycerol. *Pseudomonas aeruginosa* PAO1, *Escherichia coli* O157:H7, *Bacillus cereus, Salmonella enterica* serovar *typhimurium, Vibrio harveyi* BB170 and *V. harveyi* MM32 were grown in Luria-Bertani (LB) medium at 37°C in a shaker at 150 rpm.

These cultures were chosen because of their different abilities to produce AI-2. *P. aeruginosa* PAO1 does not possess the *luxS* gene (38), *E. coli* O157:H7 and *S. typhimurium* respond the *R*-THMF form of AI-2 (23). *B. cereus* is a gram-positive bacterium that produces AI-2 (which form is unknown) (1). *V. harveyi* BB170 responds to the *S*-THMF-borate form of AI-2 and *V. harveyi* MM32 is a *luxS* knockout (3).

Assessment of *M. avium* Biofilm Formation in 96-well Microtiter Plates in the Presence of Culture Supernatants

Bacterial cultures of *M. avium* were grown in Middlebrook 7H9 broth to an optical density at 600 nm (OD_{600}) of 0.5 to 1.0, centrifuged for 15 minutes at 3000 rpm, and resuspended in equal amounts of sterile milliQ deionized water. *P. aeruginosa* PAO1, *E. coli* O157:H7, *B. cereus, S. typhimurium, V. harveyi* BB170 and *V. harveyi* MM32 were grown in identical medium over night at 37°C and cell-free supernatants were prepared. For time-course experiments, *E. coli* O157:H7 and *P. aeruginosa* were

grown in LB broth and samples were taken hourly between 1-7h and at 24h of growth. The samples were centrifuged for 15 min at 3000 rpm and the supernatants were filtered through Millex® GP filter with a pore size of 0.22 μm. The supernatants were tested for their iron (Fe^{2+}) content using the Hach iron test kit Model IR-18 and frozen at -20°C. The iron test kit measures from 0-5 mg/L iron, and is a colorimetric test, which is not designed to determine the exact iron content of the sample. However, it does allow reasonably accurate comparisons between experiments.

Microtiter plates were inoculated with 150 μl of bacterial suspension and 50 μl bacterial culture supernatant or $FeSO_4$ solution, respectively. The negative controls contained 50 μl water or 50 μl LB medium in supernatant experiments. The final concentration of $FeSO_4$ per well was 2 μM. After 10 days of incubation, biofilm mass was measured using an adaptation of the crystal violet (CV) staining method (27). In brief, the biofilms were stained with crystal violet and incubated at room temperature for 45 min. Residual dye was rinsed off the plates, leaving only the stained cells attached to the surface of the wells. The CV was solubilized with 80% (v/v) ethanol and OD_{550} readings were taken for quantification.

Real-time Reverse Transcription (RT)-PCR

For real-time RT-PCR, planktonic cultures were grown in the presence and absence of 2 μM iron for three days. Biofilms were grown on Permanox chamber slides for 10 days in the presence and absence of 2 μM iron. For RNA isolation, the biofilms were removed from the slides by sonication and RNA extraction was performed.

Residual DNA was removed using the Ambion DNAse-I kit according to the manufacturer's instructions.

Table 5.1. Primers used for RT-PCR

Primers	Oligonucleotide Sequence (5'-3')
AhpC forward	AGCACGAGGACCTCAAGAAC
AhpC reverse	GTGACCGAGACGAACTGGAT
AhpD forward	GTACGCCAAGGATCTCAAGC
AhpD reverse	GTACTTGCCGTCCAAGAAGC
TreS forward	TACGACACCACCGACCACTA
TreS reverse	CGTGATCGTCAGAGTCGATG
IdeR forward	CTGGTCAAGGTGCTGAACAA
IdeR reverse	CGAGATCAGGTCGATGTCAC
MAV_2838 forward	GGATGGCACTGGGTGACTAC
MAV_2838 reverse	CCGTAGGTGTTGAGGGACAG
KatG forward	ACTACGGTGGCCTGTTCATC
KatG reverse	TCTTGAATCCCATCGACTCC
FurA forward	CGACAATCACCACCACGTC
FurA reverse	CACAGGCCCCAGTAGATGAC
16S forward	GCGATATCGGGCAGACTAGAG
16S reverse	AAGGAAGGAAACCCACACCT

The real-time RT-PCR was carried out using the Corbett Rotor-Gene 3000 real-time DNA detection system and the QuantiTect™ SYBR® Green RT-PCR kit (QIAGEN) in 25µl (total volume) reactions. These mixtures contained 12.5µl SYBR® Green mix, 0.25µl QuantiTect™ RT mix, 2µM primer and 10ng RNA. Primers were designed using the Frodo software (32) (Table 5.1). The reverse transcriptase reaction was carried out at 50°C for 30 min, followed by denaturation at 95°C for 15min. This was followed by 50 PCR cycles of 15s at 94°C, 30s at 59°C and 30s at 72°C.

For analysis, the C_t values obtained for each gene were converted to linear numbers by calculating $L_t=2^{-Ct}$ and normalized with the 16S gene. Fold changes were obtained by dividing the normalized iron-treated values by the normalized negative-control values for each gene.

Results

Influence of Bacterial Culture Supernatants on *M. avium* Biofilm Formation

In order to assess whether the presence of other bacteria and their products alters *M. avium* biofilm formation, six bacterial strains were chosen and cell-free supernatants were prepared. Initial experiments were very inconsistent in their outcome (data not shown). These experiments were carried out with all bacterial cultures grown overnight and biofilm formation by *M. avium* seemed to be randomly inhibited or enhanced. Closer observation of these initial experiments led to the conclusion that *M. avium* biofilm formation seems to be dependent on the growth stage of the bacterial cultures. It has also been reported that 2 μM iron is necessary for biofilm growth, but not for planktonic growth in *M. smegmatis* (26). Therefore, iron was considered as a potential factor in the influence of cell-free supernatants on *M. avium* biofilm formation. It was hypothesized that the growing cells depleted the medium of important growth factors, including iron. To test this hypothesis, the six bacterial strains were grown for 5 hours and the iron content of the cell-free culture supernatant was measured. The results are shown in Figure 5.1. The experiment showed that with the exception of *E. coli*, there seemed to be a

correlation between the iron content of the medium and the induction of biofilm formation in *M. avium.*

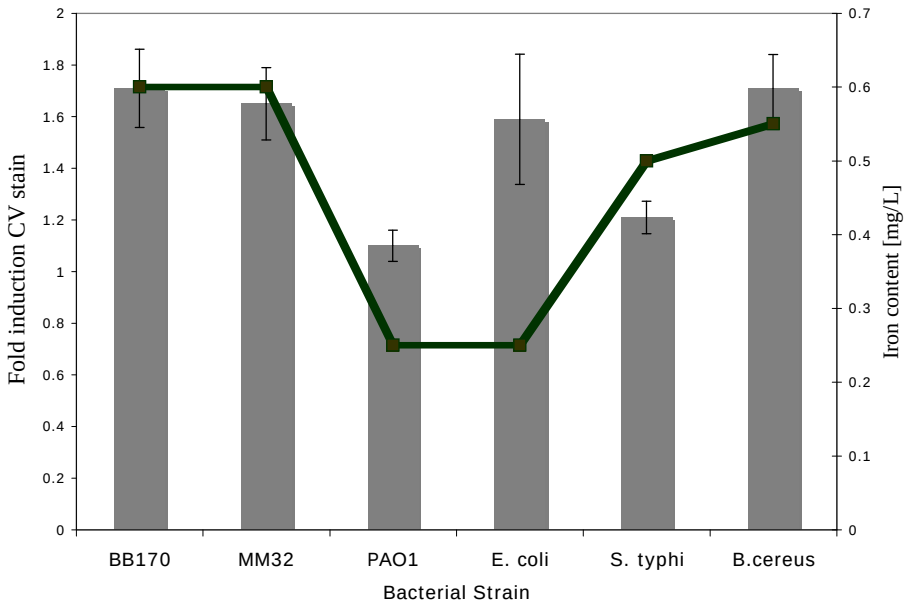

Figure 5.1. The influence of cell-free supernatants and their iron content on M. avium biofilm formation. The grey bars indicate biofilm formation as determined by crystal violet staining and the line indicates iron content in the medium. BB170: *V. harveyi* BB170, MM32: *V. harveyi* MM32, PAO1: *P. aeruginosa* PAO1, S.typhi: *S. typhimurium.*

Time-course Supernatant Experiments

In order to further study the influence of growth stage, two bacterial strains, *E. coli* and *P. aeruginosa*, were selected and cell-free supernatants were prepared every hour for 7 hours and once after 24 hours of growth. Three independent experiments were averaged, and the results are shown in Figures 5.2 and 5.3.

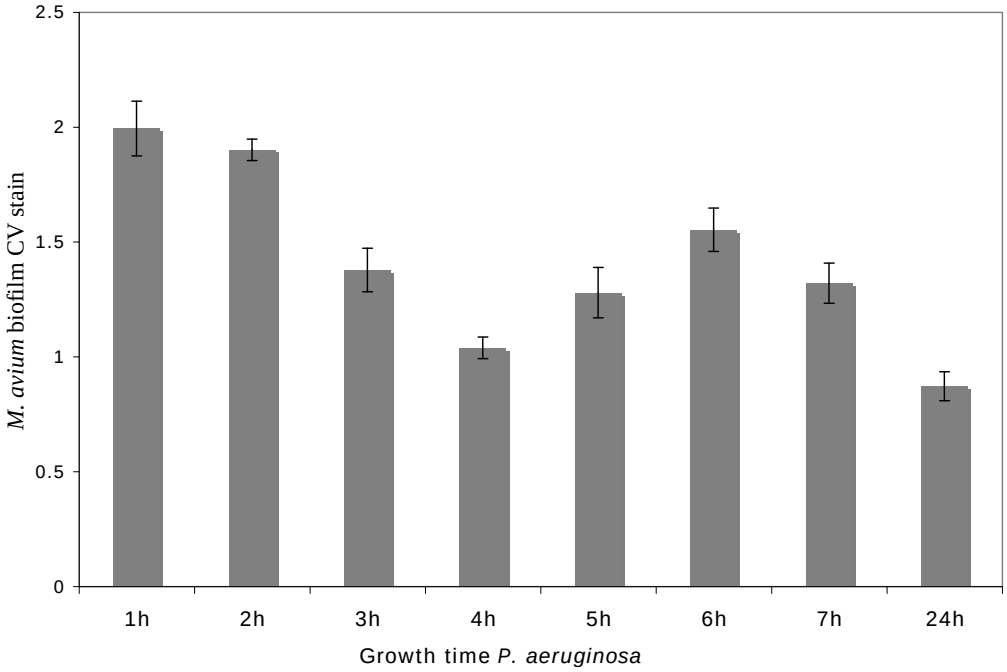

Figure 5.2. *P. aeruginosa* cultures influence *M. avium* biofilm formation in a growth-stage dependent manner.

In the *P. aeruginosa* supernatants, the influence of the growth stage on *M. avium* biofilm formation could clearly be shown. For the first four hours, the biofilm induction decreased with increasing time, indicating that the medium was depleted of one or more factors necessary for *M. avium* biofilm formation. However, at 5 hours and 6 hours of growth, the biofilm induction increased, maybe due to metabolic byproducts secreted by the *P. aeruginosa* cells (Figure 5.2). The 24-hour supernatant showed very low induction of biofilm formation, potentially due to low metabolic activity in late stationary phase of the culture (metabolic activity was not measured).

The *E. coli* culture supernatants had a very similar effect on *M. avium* biofilm formation (Figure 5.3). For the first four hours, the culture supernatants produced a decrease in biofilm formation, whereas increased *M. avium* biofilm was seen with

supernatants from the late exponential phase of the culture. Although cycling effects on biofilm formation were not measured between 7 and 24 hours, it seems reasonable to speculate that the effect levels off when the culture reaches stationary phase.

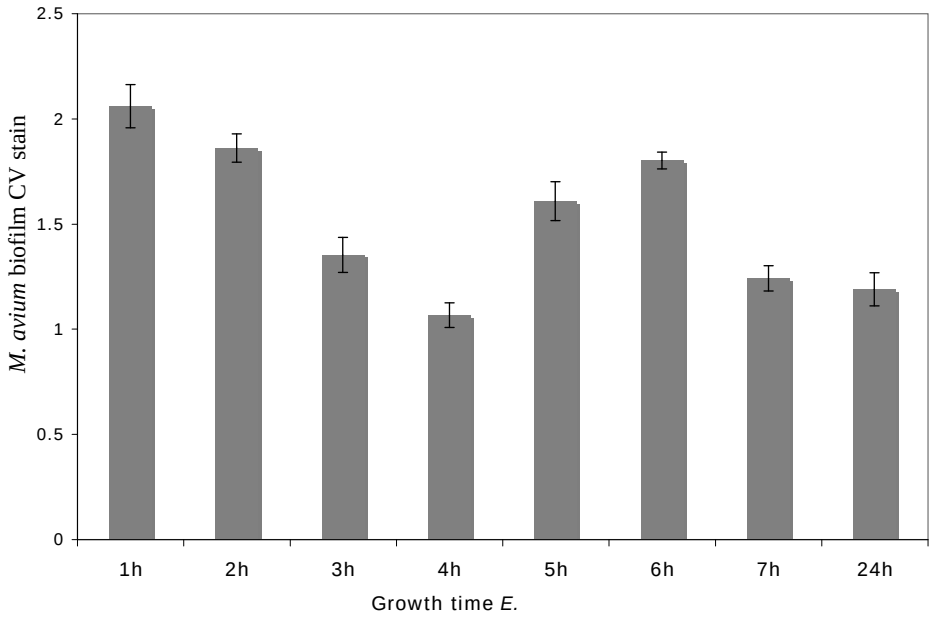

Figure 5.3. *E. coli* cultures influence *M. avium* biofilm formation in a growth-stage dependent manner, suggesting that a growth factor in the medium and metabolic byproducts from late exponential phase are involved.

In order to study the effect of iron on *M. avium* biofilm formation, a $FeSO_4$ solution was added to the biofilms at a final concentration of 2 μM; the concentration that has been shown to be necessary for biofilm formation in *M. smegmatis* (26). Furthermore, the effect of LB medium, the medium used for the culture supernatants, was studied. The iron solution lead to more than two-fold increase in biofilm formation, and LB medium led to a 1.8 fold increase (Figure 5.4).

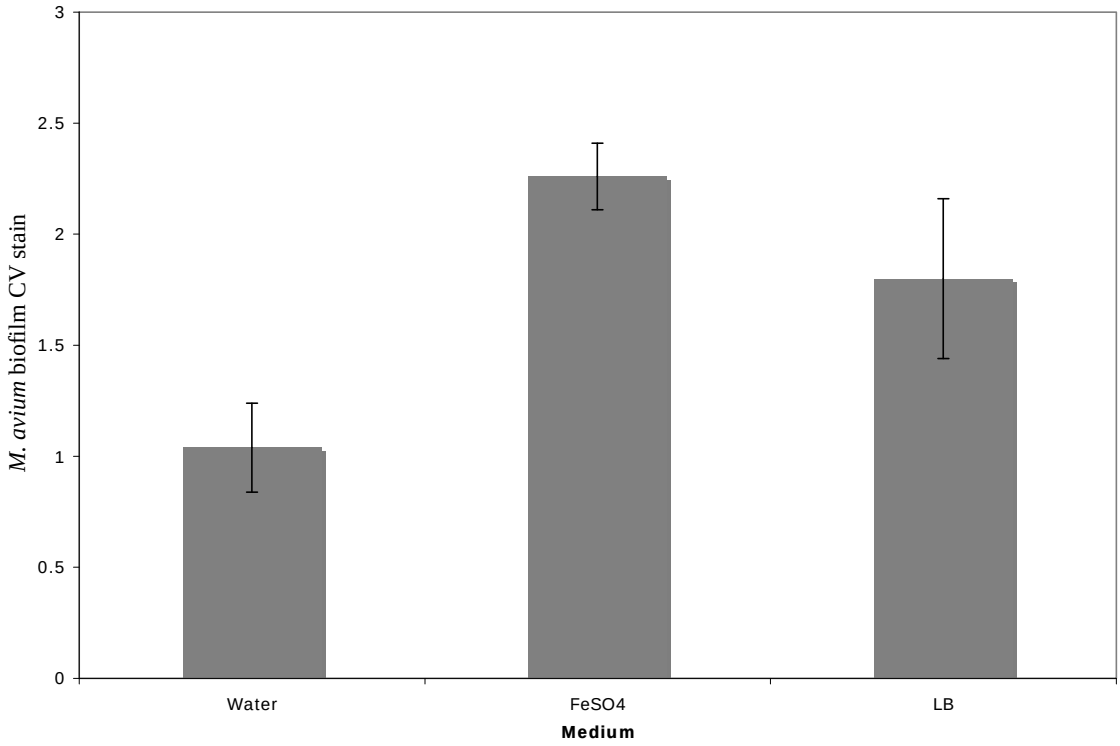

Figure 5.4. Iron solution and LB medium increase *M. avium* biofilm formation.

The observation that iron increases biofilm formation led to the question of whether this phenomenon was simply the result of an increase in oxidative stress, which leads to biofilm formation in *M. avium* (15), or whether the iron itself is the cue for biofilm formation. Therefore, quantitative RT-PCR was performed with RNA isolated from planktonic cultures and biofilms grown in the absence and presence of 2 μM added iron. The genes chosen for this study were the oxidative stress response genes *ahpC, ahpD, treS* and their transcriptional regulator *oxyR* as well as the important regulators of the iron response *ideR* and *furA; katG* which is under FurA control and finally the heat shock protein *groEL1*.

The qRT-PCR revealed that gene transcription during the biofilm mode of growth differs from that during planktonic growth. All of the tested genes were upregulated in the presence of iron in planktonic cultures. However, no difference in transcription was detected between biofilm grown in the presence and absence of added iron (table 5.2).

Table 5.2. Gene regulation in the presence of iron

Gene name	Description	Fold-induction	
		Biofilm + iron vs. biofilm	Plankt. + iron vs. plankt.
ahpC	Alkyl hydroperoxide reductase C	1.0±0.02	2.6±0.8
ahpD	Alkyl hydroperoxide reductase D	1.0±0.05	1.4±0.3
treS	Trehalose synthase	1.0±0.15	2.0±0.1
oxyR	Transcriptional regulator	0.7±0.14	2.0±0.4
katG	Catalase-peroxidase	0.9±0.06	2.0±0.4
ideR	Transcriptional regulator	0.9±0.1	2.4±0.2
furA	Transcriptional regulator	1.0±0.1	2.2±0.2
groEL1	Heat shock protein	1.0±0.2	1.8±0.1

Discussion

M. avium is a very versatile pathogen that can survive in many environments such as soils, water and also inside the mammalian host. The bacterium forms biofilms in drinking water systems (12), showerheads and also within the human body (49). The bacterium is able to adapt to these very diverse conditions, but little is known about the adaptive mechanisms. In the environment, *M. avium* encounters many different bacterial species, which can interact with *M. avium* by means of signaling or metabolic byproducts. These interactions can be advantageous if an exchange of metabolites such as siderophores or antibiotics is involved. They can also be deleterious if the bacteria compete for resources such as food and space. This study examined the influence of cell-free spent supernatants from six different bacterial strains on *M. avium* biofilm formation. There seemed to be a correlation between the iron content in the medium and its influence on biofilm formation. Slight differences may be due to slow growth, different growth rates and different iron uptake rates of the cells.

In order to investigate this effect in more detail, supernatants from *E. coli* and *P. aeruginosa* were prepared from different growth stages.

Sterile LB medium added to *M. avium* cultures increased biofilm formation almost two-fold. However, although *E. coli* and *P. aeruginosa* cultures grown in LB medium increased biofilm formation, this effect decreased with time as the medium became depleted of its nutrients. Iron content decreased, for example, hourly by approximately 0.1 mg/L per hour. After approximately 4 hours of growth, the cultures started to induce biofilm formation again, with an hourly increase from hour 4-6, followed by a second

decrease in induction. The increase may be due to production of metabolic byproducts followed by a decrease as the culture population enters stationary phase. If depletion of a nutrient due to uptake by the cells decreases linearly, and production of metabolic byproducts, sets in later and then increases, a cycling effect like the one observed could take place as theoretically shown in Figure 5.5.

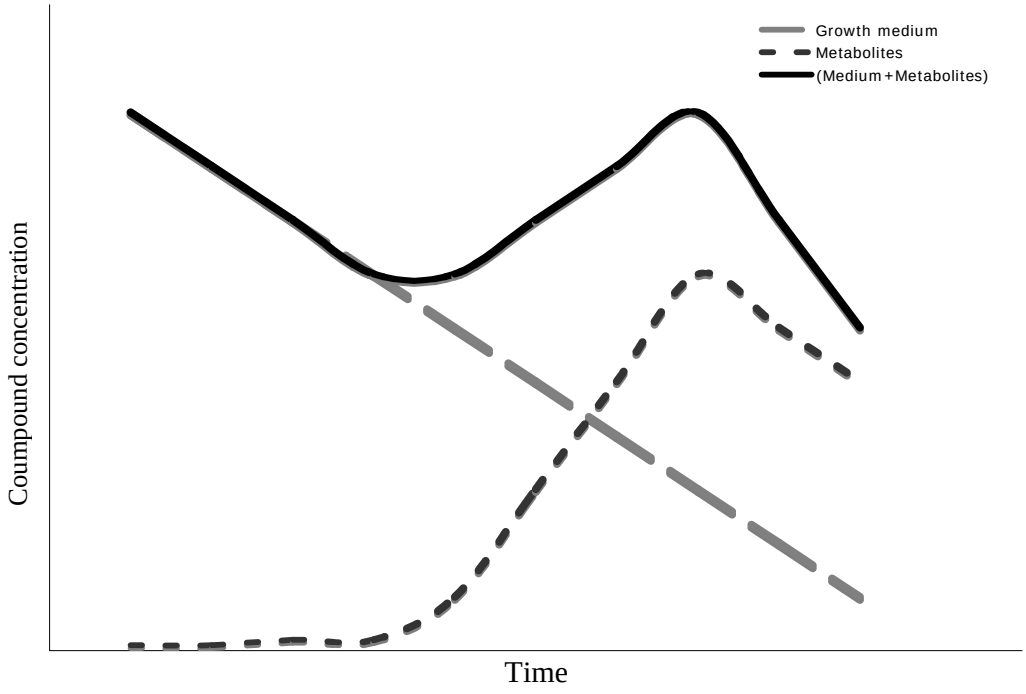

Figure 5.5. Theoretical model of medium concentration (light gray) and metabolites (dark gray, dashed) with time. If the medium decreases linearly and the metabolites increase with time, the sum of the two might result in a cycling effect.

The cells might produce these metabolites in a cyclical manner as it has been reported for AI-2 in *E. coli* (48). It could also be that the metabolites are being produced over the whole period of time, with the concentration increasing with increasing cell density. The time course experiments were conducted with *E. coli* and *P. aeruginosa* and

in both cases, the cycling events were very similar. This leads to the conclusion that AI-2 is not involved in this cycling effect, as *P. aeruginosa* does not possess the *luxS* gene and therefore is unable to produce AI-2. Future research should be conducted to assess whether the iron depletion effect is stronger than the metabolite effect occurring later on during growth. This could be accomplished by adding iron at later in the growth cycle. and checking whether the cycling effect still occurs. Presumably, under natural, steady-state conditions, an equilibrium would be reached between nutrient concentration and metabolite production.

It has been hypothesized that iron is an important factor involved in *M. avium* biofilm formation, as iron has been shown to be important in biofilm formation for a number of other bacteria. In *P. aeruginosa*, intracellular iron levels serve as a cue for biofilm formation, and FurA is at least one factor involved in the process (4). Low iron concentration induces biofilm formation in *Staphylococcus aureus* Newman. Again, Fur is involved, however not as a negative transcriptional regulator but as an activator at low iron levels (17). Iron has been shown to be very important in *M. smegmatis* biofilm formation. Ohja and co-workers demonstrated that iron is necessary for biofilm formation, but not for the growth of planktonic cultures (26).

In order to study whether similar effects are true for *M. avium*, planktonic cultures and biofilms were grown in the absence and presence of iron and the transcriptional response was studied using qRT-PCR.

The genes were chosen according to the following criteria. *ahpC*, *ahpD*, and *treS* are under the transcriptional control of *oxyR*. These genes are involved in the oxidative stress response and have been linked to biofilm formation under oxidative stress in

Chapter 3. *katG* is involved in oxidative stress, but also in the iron response in mycobacteria and is under the control of *furA* (49). *ideR* is also a global regulator of the iron response in mycobacteria and *groEL* is a heat shock protein involved in biofilm formation in *M. smegmatis* (25).

The results showed that no obvious change in gene regulation was observed in the biofilm cells, whereas all genes tested were upregulated in response to iron in the planktonic cells. These results suggest that the iron requirements are different in biofilm and planktonic cells of *M. avium.* The close link between iron and oxidative stress has been shown in a number of studies (13, 34, 35). Ferrous iron is reactive and in the presence of oxygen generates reactive oxygen species (ROS). Therefore, the gene responses to iron and oxidative stress may be linked. It has been shown that both the *furA* and *katG* genes are upregulated in response to oxidative stress in *M. smegmatis* although FurA acts as a negative regulator for *katG* (22).

The iron dependent regulator IdeR has been identified as a repressor of iron acquisition, an activator of iron storage and a positive regulator of the oxidative stress response in mycobacteria (30). *ideR* as well as the oxidative stress response genes were upregulated in the planktonic culture, indicating that the intracellular iron levels were high and needed regulation. The same iron concentration in biofilm cells did not trigger upregulation of any of the above-mentioned genes indicating that there is a higher tolerance and/or a higher need for iron in biofilm cells.

Oxidative stress has been shown to favor biofilm formation in a number of bacteria before (13, 15, 34, 35), and the oxidative stress in the planktonic culture caused by high iron concentrations could be the cue for biofilm formation.

Many questions remain unanswered and lead to several directions for future research. For example, in order to study the influence of iron on biofilm formation in more detail, different concentrations of iron should be added to *M. avium* cultures to check for a possible concentration-dependent effect and also for the tolerance level in both biofilms and planktonic cultures.

Microarray studies in both planktonic and biofilm cultures in the absence and presence of iron would give a more complete picture of the genes affected by iron.

References

1. Auger, S., E. Krin, S. Aymerich, and M. Gohar. 2006. Autoinducer 2 affects biofilm formation by Bacillus cereus. Appl Environ Microbiol 72:937-41.

2. Angenent, L. T., S. T. Kelley, A. St Amand, N. R. Pace, and M. T. Hernandez. 2005. Molecular identification of potential pathogens in water and air of a hospital therapy pool. Proc Natl Acad Sci U S A 102:4860-5.

3. Bassler, B. L., M. Wright, R. E. Showalter, and M. R. Silverman. 1993. Intercellular signalling in Vibrio harveyi: sequence and function of genes regulating expression of luminescence. Mol Microbiol 9:773-86.

4. Banin, E., K. M. Brady, and E. P. Greenberg. 2006. Chelator-induced dispersal and killing of Pseudomonas aeruginosa cells in a biofilm. Appl Environ Microbiol 72:2064-9.

5. Belisle, J. T., M. R. McNeil, D. Chatterjee, J. M. Inamine, and P. J. Brennan. 1993. Expression of the core lipopeptide of the glycopeptidolipid surface antigens in rough mutants of Mycobacterium avium. J Biol Chem 268:10510-6.

6. Belisle, J. T., L. Pascopella, J. M. Inamine, P. J. Brennan, and W. R. Jacobs, Jr. 1991. Isolation and expression of a gene cluster responsible for biosynthesis of the glycopeptidolipid antigens of Mycobacterium avium. J Bacteriol 173:6991-7.

7. Burgess, N. A., D. F. Kirke, P. Williams, K. Winzer, K. R. Hardie, N. L. Meyers, J. Aduse-Opoku, M. A. Curtis, and M. Camara. 2002. LuxS-dependent quorum sensing in Porphyromonas gingivalis modulates protease and haemagglutinin activities but is not essential for virulence. Microbiology 148:763-72.

8. Chen, X., S. Schauder, N. Potier, A. Van Dorsselaer, I. Pelczer, B. L. Bassler, and F. M. Hughson. 2002. Structural identification of a bacterial quorum-sensing signal containing boron. Nature 415:545-9.

9. DeLisa, M. P., C. F. Wu, L. Wang, J. J. Valdes, and W. E. Bentley. 2001. DNA microarray-based identification of genes controlled by autoinducer 2-stimulated quorum sensing in Escherichia coli. J Bacteriol 183:5239-47.

10. Diggle, S. P., A. Gardner, S. A. West, and A. S. Griffin. 2007. Evolutionary theory of bacterial quorum sensing: when is a signal not a signal? Philos Trans R Soc Lond B Biol Sci 362:1241-9.

11. Falkinham, J. O., 3rd. 2003. Mycobacterial aerosols and respiratory disease. Emerg Infect Dis 9:763-7.

12. Falkinham, J. O., 3rd, C. D. Norton, and M. W. LeChevallier. 2001. Factors influencing numbers of Mycobacterium avium, Mycobacterium intracellulare, and other Mycobacteria in drinking water distribution systems. Appl Environ Microbiol 67:1225-31.

13. Fields, J. A., and S. A. Thompson. 2008. Campylobacter jejuni CsrA mediates oxidative stress responses, biofilm formation, and host cell invasion. J Bacteriol.

14. Fuqua, W. C., S. C. Winans, and E. P. Greenberg. 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J Bacteriol 176:269-75.

15. Geier, H., S. Mostowy, G. A. Cangelosi, M. A. Behr, and T. E. Ford. 2008. Autoinducer-2 triggers the oxidative stress response in Mycobacterium avium, leading to biofilm formation. Appl Environ Microbiol **74:**1798-804.

16. Higgins, D. A., M. E. Pomianek, C. M. Kraml, R. K. Taylor, M. F. Semmelhack, and B. L. Bassler. 2007. The major Vibrio cholerae autoinducer and its role in virulence factor production. Nature 450:883-6.

17. Johnson, M., A. Cockayne, and J. A. Morrissey. 2008. Iron-regulated biofilm formation in Staphylococcus aureus Newman requires ica and the secreted protein Emp. Infect Immun 76:1756-65.

18. Kleerebezem, M., L. E. Quadri, O. P. Kuipers, and W. M. de Vos. 1997. Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. Mol Microbiol 24:895-904.

19. Le Dantec, C., J. P. Duguet, A. Montiel, N. Dumoutier, S. Dubrou, and V. Vincent. 2002. Occurrence of mycobacteria in water treatment lines and in water distribution systems. Appl Environ Microbiol 68:5318-25.

20. McNab, R., and R. J. Lamont. 2003. Microbial dinner-party conversations: the role of LuxS in interspecies communication. J Med Microbiol 52:541-5.

21. Meissner, G., and W. Anz. 1977. Sources of Mycobacterium avium complex infection resulting in human diseases. Am Rev Respir Dis 116:1057-64.

22. Milano, A., F. Forti, C. Sala, G. Riccardi, and D. Ghisotti. 2001. Transcriptional regulation of furA and katG upon oxidative stress in Mycobacterium smegmatis. J Bacteriol 183:6801-6.

23. Miller, S. T., K. B. Xavier, S. R. Campagna, M. E. Taga, M. F. Semmelhack, B. L. Bassler, and F. M. Hughson. 2004. Salmonella typhimurium recognizes a chemically distinct form of the bacterial quorum-sensing signal AI-2. Mol Cell 15:677-87.

24. Moore, J. S., M. Christensen, R. W. Wilson, R. J. Wallace, Jr., Y. Zhang, D. R. Nash, and B. Shelton. 2000. Mycobacterial contamination of metalworking fluids: involvement of a possible new taxon of rapidly growing mycobacteria. Aihaj 61:205-13.

25. Ojha, A., M. Anand, A. Bhatt, L. Kremer, W. R. Jacobs, Jr., and G. F. Hatfull. 2005. GroEL1: a dedicated chaperone involved in mycolic acid biosynthesis during biofilm formation in mycobacteria. Cell 123:861-73.

26. Ojha, A., and G. F. Hatfull. 2007. The role of iron in Mycobacterium smegmatis biofilm formation: the exochelin siderophore is essential in limiting iron conditions for biofilm formation but not for planktonic growth. Mol Microbiol 66:468-83.

27. O'Toole, G. A., L. A. Pratt, P. I. Watnick, D. K. Newman, V. B. Weaver, and R. Kolter. 1999. Genetic approaches to study of biofilms. Methods Enzymol 310:91-109.

28. Penny, M. E., R. B. Cole, and J. Gray. 1982. Two cases of Mycobacterium kansasii infection occurring in the same household. Tubercle 63:129-31.

29. Primm, T. P., C. A. Lucero, and J. O. Falkinham, 3rd. 2004. Health impacts of environmental mycobacteria. Clin Microbiol Rev 17:98-106.

30. Rodriguez, G. M., and I. Smith. 2003. Mechanisms of iron regulation in mycobacteria: role in physiology and virulence. Mol Microbiol 47:1485-94.

31. Rickard, A. H., R. J. Palmer, Jr., D. S. Blehert, S. R. Campagna, M. F. Semmelhack, P. G. Egland, B. L. Bassler, and P. E. Kolenbrander. 2006. Autoinducer 2: a concentration-dependent signal for mutualistic bacterial biofilm growth. Mol Microbiol 60:1446-56.

32. Rozen, S., and H. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132:365-86.

33. Schauder, S., K. Shokat, M. G. Surette, and B. L. Bassler. 2001. The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. Mol Microbiol 41:463-76

34. Seib, K. L., H. J. Wu, Y. N. Srikhanta, J. L. Edwards, M. L. Falsetta, A. J. Hamilton, T. L. Maguire, S. M. Grimmond, M. A. Apicella, A. G. McEwan, and M. P. Jennings. 2007. Characterization of the OxyR regulon of Neisseria gonorrhoeae. Mol Microbiol 63:54-68.

35. Shanks, R. M., N. A. Stella, E. J. Kalivoda, M. R. Doe, D. M. O'Dee, K. L. Lathrop, F. L. Guo, and G. J. Nau. 2007. A Serratia marcescens OxyR homolog mediates surface attachment and biofilm formation. J Bacteriol 189:7262-72.

36. Shelton, B. G., W. D. Flanders, and G. K. Morris. 1999. Mycobacterium sp. as a possible cause of hypersensitivity pneumonitis in machine workers. Emerg Infect Dis 5:270-3.

37. Sperandio, V., A. G. Torres, J. A. Giron, and J. B. Kaper. 2001. Quorum sensing is a global regulatory mechanism in enterohemorrhagic Escherichia coli O157:H7. J Bacteriol 183:5187-97.

38. Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrener, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrock-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. Hancock, S. Lory, and M. V. Olson. 2000. Complete genome sequence of Pseudomonas aeruginosa PA01, an opportunistic pathogen. Nature 406:959-64.

39. Surette, M. G., M. B. Miller, and B. L. Bassler. 1999. Quorum sensing in Escherichia coli, Salmonella typhimurium, and Vibrio harveyi: a new family of genes responsible for autoinducer production. Proc Natl Acad Sci U S A 96:1639-44.

40. Taylor, R. H., J. O. Falkinham, 3rd, C. D. Norton, and M. W. LeChevallier. 2000. Chlorine, chloramine, chlorine dioxide, and ozone susceptibility of Mycobacterium avium. Appl Environ Microbiol 66:1702-5.

41. Touati, D. 2000. Iron and oxidative stress in bacteria. Arch Biochem Biophys 373:1-6.

42. Turenne, C. Y., M. Semret, D. V. Cousins, D. M. Collins, and M. A. Behr. 2006. Sequencing of hsp65 distinguishes among subsets of the Mycobacterium avium complex. J Clin Microbiol 44:433-40.

43. Turenne, C. Y., R. Wallace, Jr., and M. A. Behr. 2007. Mycobacterium avium in the postgenomic era. Clin Microbiol Rev 20:205-29.

44. von Reyn, C. F., J. N. Maslow, T. W. Barber, J. O. Falkinham, 3rd, and R. D. Arbeit. 1994. Persistent colonisation of potable water as a source of Mycobacterium avium infection in AIDS. Lancet 343:1137-41.

45. Watson, W. T., T. D. Minogue, D. L. Val, S. B. von Bodman, and M. E. Churchill. 2002. Structural basis and specificity of acyl-homoserine lactone signal production in bacterial quorum sensing. Mol Cell 9:685-94.

46. West, S. A., A. S. Griffin, A. Gardner, and S. P. Diggle. 2006. Social evolution theory for microorganisms. Nat Rev Microbiol 4:597-607.

47. Xavier, K. B., and B. L. Bassler. 2003. LuxS quorum sensing: more than just a numbers game. Curr Opin Microbiol 6:191-7.

48. Xavier, K. B., and B. L. Bassler. 2005. Regulation of uptake and processing of the quorum-sensing autoinducer AI-2 in Escherichia coli. J Bacteriol 187:238-48.

49. Yamazaki, Y., L. Danelishvili, M. Wu, E. Hidaka, T. Katsuyama, B. Stang, M. Petrofsky, R. Bildfell, and L. E. Bermudez. 2006. The ability to form biofilm influences Mycobacterium avium invasion and translocation of bronchial epithelial cells. Cell Microbiol 8:806-14.

50. Zahrt, T. C., J. Song, J. Siple, and V. Deretic. 2001. Mycobacterial FurA is a negative regulator of catalase-peroxidase gene katG. Mol Microbiol 39:1174-85.

CHAPTER 6

THESIS SYNTHESIS AND FUTURE DIRECTIONS

This dissertation demonstrates the importance of a number of factors that contribute to *Mycobacterium avium* biofilm formation. *M. avium* is an important environmental bacterium that thrives under various different environmental conditions. It causes respiratory disease predominantly in immunocompromised patients, such as the elderly or AIDS patients. In addition, otherwise healthy people who are frequently exposed to *M. avium* have been reported to develop acute pneumonitis and disseminated disease. The infection is usually acquired from environmental sources, such as potted plants, hot tubs, swimming pools and drinking water. *M. avium* is able to form biofilms in drinking water systems (3) and therefore can lead to exposure risk, even if the source water is not contaminated with large numbers of the bacterium. These biofilms represent a potential health problem and therefore, research is necessary to investigate both mycobacterial biofilm formation and its prevention. In this work, three different approaches were used to study different factors and their importance in biofilm formation.

Cell-to-cell signaling has been shown to be important in biofilm formation for a number of bacterial species (1, 2, 4, 5, 6). Since a myriad of different bacteria are encountered in the environment, the influence of the universal signaling molecule autoinducer-2 (AI-2) on *M. avium* biofilm was investigated in Chapter 2. The addition of AI-2 to planktonic *M. avium* cultures leads to increased biofilm formation as

demonstrated using crystal violet staining. In order to reveal the molecular mechanisms behind that process, a whole-genome transcriptome study using microarrays was performed. The transcriptional profiles of AI-2 treated versus non-treated *M. avium* cells were compared. This experiment revealed a set of 5 genes to be significantly upregulated. Four of the five genes are involved in the oxidative stress response of *M. avium*, indicating that AI-2 does not act as a classic signaling molecule in *M. avium* but rather as an unspecific reactive oxygen species. Experiments with hydrogen peroxide showed that the compound triggered the same transcriptional response and also led to biofilm formation. These experiments suggest that oxidative stress promotes biofilm formation in *M. avium.*

Future experiments should investigate the effect of other signaling molecules, such as AHLs, on *M. avium* cultures in order to determine whether the bacterium is able to monitor its environment for other bacterial species and whether this would also influence biofilm formation. Quorum sensing circuits have been identified in a large number of environmental bacteria and would be plausible if *M. avium* used cell-to-cell communication to regulate the behavior of the population.

Further research should be directed at resolving the structure and function of the hypothetical protein that was upregulated in response to AI-2. This would be a first step in determining the link between oxidative stress and biofilm formation on a molecular level. Since oxidative stress has been shown to promote biofilm formation, it would be very interesting to examine the role of chlorine in this process. *M. avium* is a frequent inhabitant of drinking water pipes and has been recovered from the drinking water even though mycobacteria in the source water were below detection (3). It has been suggested

that the bacterium proliferates attached to the plumbing. Chlorination of the drinking water may cause oxidative stress in *M. avium* and could therefore be one factor that leads to biofilm formation in this environment.

The research described in Chapter 4 was focused on the role of the mycobacterial cell envelope in biofilm formation. The cell envelope mediates the direct physical contact between the cell and an underlying surface, and it therefore may be an important factor in the process of biofilm formation. It was shown that serovar-specific glycopeptidolipids (ssGPLs) are involved in biofilm formation. This research was part of a collaboration with the Cangelosi lab at the Seattle Biomedical Research Institute. The goal was to assess the role of the GPL synthases PstAB in biofilm formation. Using three different model systems and different mutations in the *pstAB* genes, it could be shown that GPLs are required for the colonization of some, but not all of the surfaces selected for testing. This suite of experiments demonstrated the importance of different test conditions. Since the cell envelope mediates the direct physical contact of the cells with the surface, it was important to test different surfaces. It was shown that PstAB is necessary for the attachment of the cells to PVC microtiter plates under static conditions. When the Permanox and glass surfaces of chamber slides were tested, no difference in the number of attached cells was found between the parent and the mutant strains. However, the morphology of the biofilms was altered. The third system was a recirculating water system that contained stainless steel coupons. No statistical difference was found between attached mutant cell numbers and attached parent cells. However, the number of accumulated mutant cells in the planktonic phase was much lower than for the parent strain.

These results show that many factors play a role in the attachment behavior of bacterial cells. The complex interplay of different factors can alter the outcome of an experiment in a significant manner.

Future research should be directed towards further improvements in the test systems. The recirculating system could be run at different temperatures to compare the bacterial behavior in hot and cold water. Furthermore, in a realistic system, the water is not continuously running, or recirculating. More surfaces should be tested in order to get an insight into the material properties that influence biofilm formation. Further biofilm mutant screening should be carried out under different conditions in order to get a more complete picture of the bacterial surface properties and their involvement in biofilm formation.

The research conducted in Chapter 5 provided an insight in the complexity of the interplay of different factors that are involved in biofilm formation. The cell-free supernatants of a number of bacterial species were prepared and their influence on *M. avium* biofilm formation was tested. The supernatants were isolated hourly in order to examine the effect of the growth stage of the bacterial species on *M. avium* biofilm formation. A number of factors appeared to influence biofilm formation as cyclical effects were observed. i.e., different metabolites produced at different growth stages may either stimulate or inhibit biofilm formation.

One effect in particular was identified to be iron. When iron was added to *M. avium* cultures, the biofilm formation was almost doubled compared to the negative control. In conclusion, different environmental factors influence mycobacterial biofilm formation to different extents.

Chapter 3 demonstrated that AI-2 induces biofilm formation when added to *M. avium* cultures. However, this experiment was conducted under very stringent conditions. The compound was dissolved in sterile purified water and added at relatively high concentrations. In Chapter 5, supernatants from two bacterial cultures, *P. aeruginosa* and *E. coli*, were used from different growth stages to investigate their effect on biofilm formation.

Although *E. coli* produces AI-2 and *P. aeruginosa* does not, the effect of their cell-free supernatants was almost identical, indicating that AI-2 did not play a significant role in this process. In this case, the effect of AI-2 may have been mitigated by interaction with other compounds in the medium, while in Chapter 3 only water was present. Alternatively, the concentration of the added AI-2 was significantly different from the concentration of the naturally produced AI-2 in the medium.

The interaction of multiple factors on biofilm formation should be the focus of future research. In addition, future research should more closely simulate natural conditions. Most of the research reported in this dissertation focused on *M. avium* in drinking water systems, and therefore future experiments should involve tap water as a medium rather than the rich LB medium. Research should also be directed to analyzing the composition of microbial communities within water distribution systems. Natural biofilms can therefore be more closely modeled in future research.

Overall, this dissertation demonstrated that *M. avium* readily forms biofilms in response to a number of cues relevant to the drinking water environment. It forms biofilms in reaction to oxidative stress, and it reacts to the presence of iron and other bacterial species. The cell envelope is an important factor involved in the attachment

process, but ultimately, the interaction of all factors must be considered in order to obtain a realistic understanding of the process of *M. avium* biofilm formation, which can ultimately lead to biofilm control.

References

1. Auger, S., E. Krin, S. Aymerich, and M. Gohar. 2006. Autoinducer 2 affects biofilm formation by Bacillus cereus. Appl Environ Microbiol 72:937-41.

2. Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280:295-8.

3. Falkinham, J. O., 3rd, C. D. Norton, and M. W. LeChevallier. 2001. Factors influencing numbers of Mycobacterium avium, Mycobacterium intracellulare, and other Mycobacteria in drinking water distribution systems. Appl Environ Microbiol 67:1225-31.

4. Merritt, J., F. Qi, S. D. Goodman, M. H. Anderson, and W. Shi. 2003. Mutation of luxS affects biofilm formation in Streptococcus mutans. Infect Immun 71:1972-9.

5. Shao, H., R. J. Lamont, and D. R. Demuth. 2007. Autoinducer 2 Is Required for Biofilm Growth of Aggregatibacter (Actinobacillus) actinomycetemcomitans. Infect Immun 75:4211-8.

6. Waters, C. M., W. Lu, J. D. Rabinowitz, and B. L. Bassler. 2008. Quorum sensing controls biofilm formation in Vibrio cholerae through modulation of cyclic di-GMP levels and repression of vpsT. J Bacteriol 190:2527-36.

APPENDIX A

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