Molecular and Ecological Mechanisms of Bacterial Response to the Drinking Water Disinfectant Monochloramine

by

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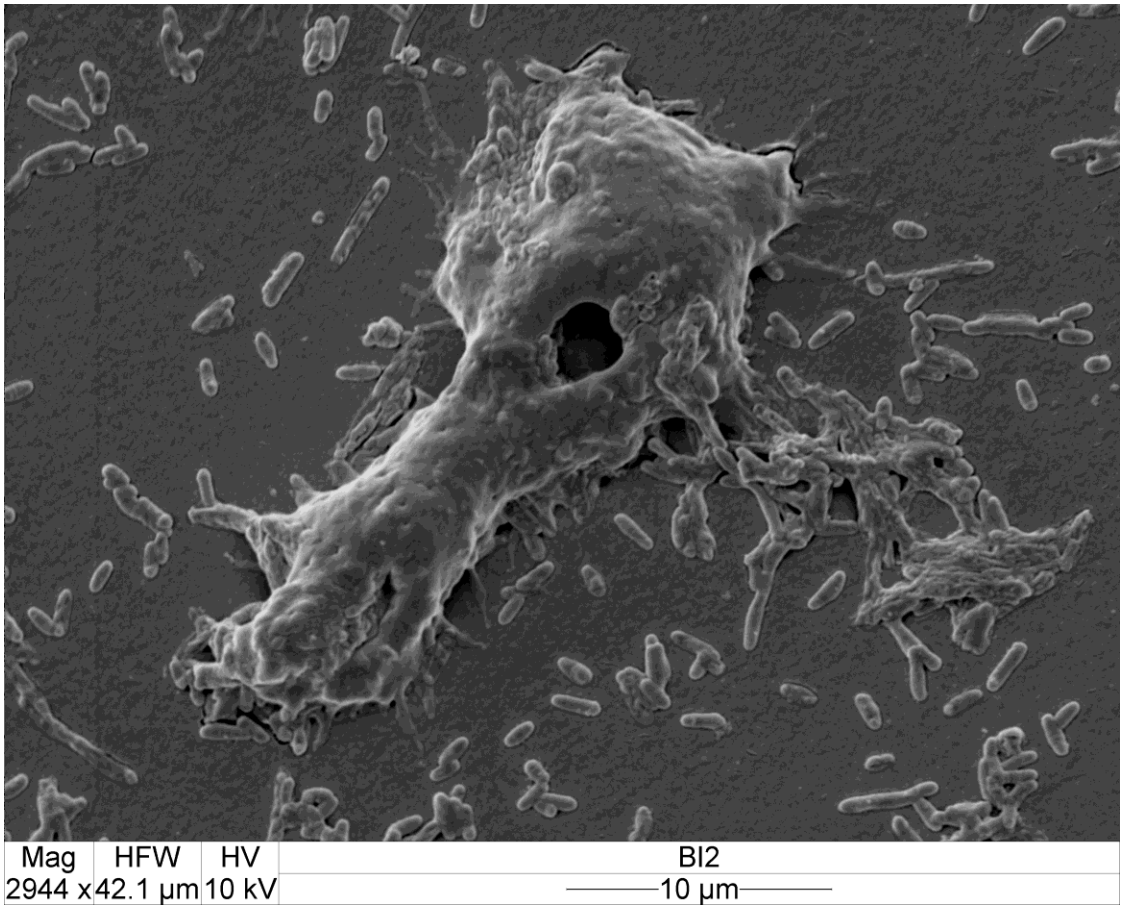

Scanning electron micrograph of *Acanthamoeba polyphaga* and *Mycobacterium avium*

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Nothing is more consonant with Nature than that she puts into operation in the smallest detail that which she intends as a whole.

- Johann Wolfgang von Goethe

This work is a dedication to Nature, whose mysteries are one of the great joys of life.

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Two chapters of this dissertation have been published at the time of writing and I would like to acknowledge the co-authors of this work. Chapter 2 was published under the title "Microbial Ecology of Drinking Water Distribution Systems" (Berry, D., C. Xi, and L. Raskin (2006).*Current Opinion in Biotechnology*, 17(3): 297-302) and chapter 4 is published under the title "Effect of Growth Conditions on *Escherichia coli* Inactivation with Monochloramine" (Berry, D., C. Xi, and L. Raskin (2009). *Environmental Science and Technology*, 43(3): 884-889). I appreciate the support of my co-chairs Lutgarde Raskin and Chuanwu Xi both as co-authors on these publications and more generally for their guidance, time, and financial support.

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Abstract

This work explores the molecular, cellular and ecological intricacies of the bacterial response to the drinking water disinfectant monochloramine using *Escherichia coli* and *Mycobacterium avium* as model bacteria. It was found that exposure of *M. avium* to a sub-lethal dose of monochloramine resulted in rapid cell wall permeabilization and intracellular thiol oxidation. The oxidative stress (OxyR) response was induced very strongly and rapidly and many virulence-associated genes also were upregulated, though whether this response increases *M. avium* virulence to humans must be further studied. The role of environmental conditions in inducing monochloramine resistance of *E. coli* also was explored. Growth of *E. coli* in either biofilm mode or at a suboptimal temperature of 20 °C increased its resistance to monochloramine. Comparative transcriptional profiling of cells grown in biofilm mode, at 20 °C, or after monochloramine exposure was performed in order to define a "drinking water stressome", which was characterized by widespread metabolic inhibition, regulation of redox-active genes, and induction of osmotic and cell envelope stress responses. Overall, there appears to be extensive overlap between response to monochloramine and to other stresses, such as general oxidative stress and osmotic stress. Finally, the relevance of the interaction of *M. avium* and *Acanthamoeba* was explored as a possible survival mechanism in drinking water treatment and distribution. *M. avium* formed stable infections within a range of *Acanthamoeba* strains, maintaining its viability for at least 28 days. *Acanthamoeba*-associated *M. avium* was much more resistant to monochloramine than *M. avium* alone and inactivation kinetics of intracellular *M. avium* exposed to monochloramine closely matched the inactivation kinetics of *Acanthamoeba castellanii* Neff, suggesting that acanthamoebal inactivation may be a useful surrogate for intracellular *M. avium* inactivation. Overall, this research underscores the importance of

biological processes in drinking water treatment and distribution, characterizing the biological complexity of bacterial response to monochloramine, the complexities emerging from response to conditions typically found in drinking water distribution, and the interactions of bacterial pathogens with free-living amoebae.

Chapter 1
Introduction

The introduction of chlorination and filtration processes into drinking water treatment during the early twentieth century is a major public health landmark that resulted in a significant reduction in both infant and total mortality rates (Cutler and Miller, 2005). However, survival and growth of pathogenic and non-pathogenic bacteria is still observed during distribution of treated drinking water (LeChevallier et al., 1996; Emtiazi et al., 2004) and it is estimated that in the US approximately 12% of acute gastroenterititis occurring among immunocompenent individuals is caused by drinking water contaminated with protozoan, viral, or bacterial pathogens (Colford et al., 2006). A survey of disease outbreaks associated with drinking water in the US found that bacterial pathogens were responsible for approximately 47% of outbreaks with a known etiology (Blackburn et al., 2004). The mechanisms behind the survival of bacteria during distribution of treated drinking water are not well-understood, though hypotheses include: adaptive gene response and induction of a resistant phenotype (Sanderson and Stewart, 1997; Szomolay et al., 2005), protection within biofilms (Srinivasan et al., 1995; Mah and O'Toole, 2001), and protection within microbial eukaryotes (Molmeret et al., 2005).

The primary goal of this body of research is to make contributions to the field of drinking water disinfection and more specifically to help characterize the response and molecular and ecological resistance mechanisms of bacteria to drinking water disinfection. Bacterial response to disinfection is a complex phenomenon that can be viewed at many levels. Figure 1.1 illustrates the three main areas of research presented in this dissertation and the relevant chapters for each area. Bacterial response to disinfection is defined at the cellular level in terms of immediate physiological effects of the disinfectant, direct and indirect sensing of the disinfectant, and the subsequent cellular response to the presence of the disinfectant. The profiling of the transcriptional response of organisms

exposed to different stressors has recently been developed as an approach to identify a core set of genes responsible for increased resistance to stresses, termed the "stressome" (Wu et al., 2007). The stressome is the shared commonalities between responses to environmental factors and to disinfectant exposure and it is an indirect mechanism of resistance to disinfection also considered in this research. Research is conducted at the ecological level in terms of protection of bacteria from disinfection when within biofilms and free-living amoebae. These research areas are further expanded upon in the discussion of each chapter below.

The research approaches employed in this dissertation draw upon the fields of engineering, microbiology and ecology, and molecular biology (Figure 1.2). This cross-disciplinary strategy was used to create synergy between fields and to facilitate the application of cutting-edge tools from other disciplines into engineering research.

Monochloramine was chosen as the disinfectant for this study. It is currently the second most commonly used disinfectant in US drinking water treatment facilities and its use is becoming more widespread because of concerns about formation of regulated disinfection-by-products during disinfection with free chlorine (Rose et al., 2007). There are, however, emerging concerns about the use of monochloramine and the formation of non-regulated but carcinogenic disinfection-by-products, which is currently an active area of research (Richardson et al., 2008).

Two model bacterial strains were selected for this work, *Escherichia coli* K-12 MG1665 and *Mycobacterium avium* 104. *E. coli* is a member of the EPA-regulated "Total Coliforms" contaminant and some *E. coli* strains cause acute gastrointestinal illness and urinary tract and bowel infections (http://www.epa.gov/safewater/contaminants/ecoli.html). *M. avium* is a facultative human pathogen that can persist and grow in surface waters and in treated drinking water (Vaerewijck et al., 2005). It has been implicated in a variety of human and animal diseases including hypersensitivity pneumonitis, respiratory problems, cervical lymphadenitis (Falkinham III, 2003), chronic bowl disease, allergies, and pulmonary infections (Primm et al., 2004). Both organisms also were selected in part because of the

availability of their sequenced genomes and existing knowledge about their fundamental biology.

This dissertation consists of seven chapters. Chapters 2 – 6 are written as stand-alone-chapters and have been already published or are prepared for publication as peer-reviewed journal publications. In addition to the short introduction presented in this first chapter, each of these chapters provides an introduction with literature review relevant for the topics covered in the respective chapters. Overall conclusions and future research directions are presented in Chapter 7. In addition to the full chapters, a preliminary characterization of the microbial communities present in the Ann Arbor drinking water distribution system is presented in Appendix A.

Chapter 2 provides a review of the literature with a focus on the microbiology of drinking water distribution systems. This literature review was conducted in 2005 and published in 2006 in the journal *Current Opinion in Biotechnology* (Berry et al., 2006) and focuses on recent findings related to how growth state and microbial interactions affect pathogen survival. Incorporation of microbial ecology considerations into process optimization and modeling of drinking water treatment and distribution is also discussed. More recent and more specific literature on relevant topics is reviewed and discussed in other chapters.

Chapters 3-6 examine different aspects of the bacterial response to monochloramine, focusing initially on the direct relationship between the bacterial cell and monochloramine (Chapter 3), then expanding the scope to account for the effect of environmentally-relevant conditions (Chapters 4 and 5), and finally exploring the role of bacterial interactions with environmental acanthamoebae (Chapter 6).

Chapter 3 is a characterization of the cellular and genetic response of *M. avium* soon after exposure to a low dose of monochloramine. This chapter examines the time scales of physiological changes upon exposure to monochloramine, including changes in cell wall permeability and in the level of intracellular thiols as a marker of intracellular redox state. The induction of oxidative stress response and the overall transcriptional profile are also studied.

Chapter 4 explores the effect of different growth conditions on *E. coli* resistance to monochloramine. Treated drinking water is an unfavorable environment for bacterial growth and exposes bacteria to multiple stresses, this work was undertaken to explore whether growth conditions encountered in treated drinking water and in drinking water distributions systems could induce a resistant phenotype to monochloramine inactivation. The experimental approach utilized cultivation under defined, controlled conditions and inactivation kinetics assays to determine sensitivity to monochloramine. This chapter has been published in the journal *Environmental Science and Technology* (Berry et al., 2009a).

Chapter 5 builds from the work of Chapter 4 by exploring the common transcriptional response of *E. coli* grown under conditions that increase resistance to monochloramine and *E. coli* that are exposed to monochloramine. The overall goal of this analysis is to begin to understand if there is a genetic basis for increased resistance of bacteria in drinking water, or in other words to evaluate if a "drinking water stressome" can be determined.

Finally, Chapter 6 explores the interaction of *M. avium* with free-living amoebae to determine the importance of association with acanthamoebae on bacterial fate during drinking water treatment and distribution. The chapter examines the infectivity, stability, and viability of intracellular *M. avium* in *Acanthamoeba* hosts for a variety of conditions and explores inactivation kinetics of *M. avium*, acanthamoebae, and acanthamoebae-associated *M. avium*. This chapter was submitted for consideration of publication by the journal *Environmental Microbiology* (Berry et al., 2009b).

1. 1 Figures

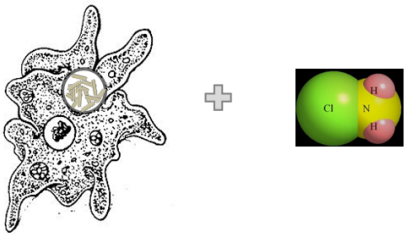
Area of Research	Chapter
Physiological response Response of bacteria to monochloramine 	2,3
The Stressome Interactions between monochloramine response and growth conditions 	2,4,5
Ecological sheltering Protection within amoebae and response to monochloramine 	2,6

Figure 1.1 Primary areas of research presented in this dissertation. The chapter numbers indicate where each chapter fits within this framework.

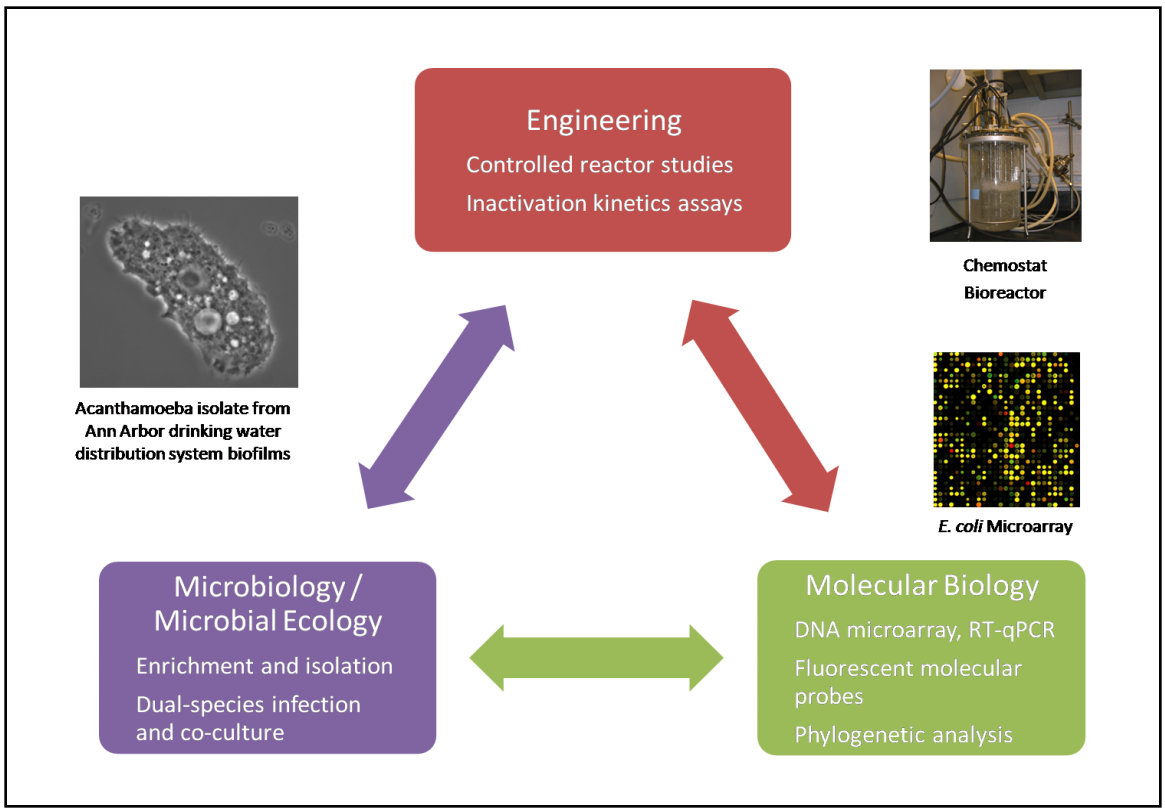


Figure 1.2 Example of methods employed in dissertation research.

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Chapter 2
Microbial Ecology of Drinking Water Distribution Systems

2.1 Abstract

The supply of clean drinking water is a major, and relatively recent, public health milestone. Control of microbial growth in drinking water distribution systems, often achieved through the addition of disinfectants, is essential to limiting waterborne illness, particularly in immunocompromised subpopulations. Recent inquiries into the microbial ecology of distribution systems have found that pathogen resistance to chlorination is affected by microbial community diversity and interspecies relationships. Research indicates that multispecies biofilms are generally more resistant to disinfection than single-species biofilms. Other recent findings are the increased survival of the bacterial pathogen *Legionella pneumophila* when present inside its protozoan host *Hartmannella vermiformis* and the depletion of chloramine disinfectant residuals by nitrifying bacteria, leading to increased overall microbial growth. Interactions such as these are unaccounted for in current disinfection models. An understanding of the microbial ecology of distribution systems is necessary to design innovative and effective control strategies that will ensure safe and high-quality drinking water.

2.2 Introduction

Many problems in drinking water distribution systems (DSs) are microbial in nature, including biofilm growth (Camper 2004; Primm et al. 2004), nitrification (Regan et al. 2003), microbially mediated corrosion (Beech and Sunner 2004), and persistence of frank and opportunistic pathogens (Emtiazi et al. 2004). The conventional approach to biological control in distribution systems, i.e., maintaining a disinfectant residual, is often ineffective at controlling microbial growth (LeChevallier et al. 1996). Our understanding of the mechanisms of microbial growth in the presence of disinfectants is superficial and studying the microbial ecology of DSs will continue to provide needed insights to help

resolve public health concerns associated with microbial growth in these engineered systems.

In this paper, we describe the importance of biofilm processes in DSs. We then review the state of knowledge of microbial community diversity in DSs, with a focus on nitrifiers, bacterial pathogens, and relationships between bacterial pathogens and protozoa. We review complexities associated with controlling microbial growth and also discuss monitoring and modeling strategies used to improve our understanding of biological processes in DSs. Due to the abundance of literature on DSs and the availability of relevant reviews (e.g. (Szewzyk et al. 2000)), we have narrowed the scope of this review to studies on microbial ecology and microorganisms in real and model DSs published primarily during the last three years.

2.3 The importance of biofilms

Biofilms are suspected to be the primary source of microorganisms in DSs that are fed adequately treated water and have no pipeline breaches (LeChevallier et al. 1996), and are particularly of concern in older DSs (Geldreich 1996). In a recent study of DSs in Parisian suburbs, it was found that biofilms attached to the pipe wall contained 25 times more bacterial cells than the bulk water (Servais 2004). Biofilms predominate because attached cells have certain advantages over planktonic cells, such as the ability to metabolize recalcitrant organics (Camper 2004) and increased resistance to chlorine and other biocides (Emtiazi et al. 2004; Tachikawa et al. 2005). Disinfection with chlorine dioxide and chlorite, for example, can reduce the concentration of planktonic bacteria, while having little to no effect on the concentration of biofilm bacteria (Gagnon 2005). The mechanism behind the observed resistance of biofilm cells to disinfection is unknown, though hypotheses include mass transfer resistance (Stewart et al. 1996), formation of persister cells (Roberts and Stewart 2005), and protection due to production of extracellular polymeric substances (Allesen-Holm et al. 2006). The history of disinfection in DSs can also influence biofilm growth. Lapses in chlorination can lead to regrowth of biofilm communities and increased resistance of biofilm bacteria to chlorine (Codony et al. 2005). Such findings implicate the importance of maintaining a continuous disinfectant residual in DSs.

2.4 Microbial community diversity

Information on the microbial community diversity of DSs is scant because molecular microbial ecology tools have not yet been used widely in this field. Moreover, opportunities to sample biofilms from real DSs are limited. Therefore, many studies have used surrogates such as model DSs and removable coupons for biofilm attachment inserted (for short times) in real DSs. Limitations with such studies are illustrated in a long-term (three year) study of a model DSs (Martiny et al. 2003). In this study, it was found by 16S rRNA gene sequence analysis that biofilm species richness was comparable to the species richness in the bulk water during the initial stages of biofilm formation due to attachment of bulk water cells, and then decreased as a dominant bacterium related to *Nitrospira* colonized the surfaces, comprising 78% of the biofilm cells. Biofilm species richness increased again as a stable biofilm community composition was achieved after almost two years (Martiny et al. 2003). This work suggests that biofilm development may require several years before steady-state is achieved, which limits the relevance of short-term model studies (Martiny et al. 2003). Consistent with this observation, other studies with model DSs suggest that as biofilms age, cell density stabilizes and species diversity increases (Rogers et al. 1994).

Researchers who have started the process of characterizing microbial diversity in DSs have isolated a number of novel bacterial strains from municipal DSs (Kalmbach et al. 1997; Rickard et al. 2005). In most cases, a rigorous characterization of these strains is still incomplete. A recent analysis of the bulk water of a chlorinated DS found that gram-positive bacteria and *Alpha-*, *Beta-*, and *Gammaproteobacteria* constituted the major groups among heterotrophic isolates (Tokajian 2005). *Alphaproteobacteria* were the dominant isolates in both chloraminated and chlorinated water from model DSs, whereas *Betaproteobacteria* were found to be more abundant in chloraminated water than in chlorinated water (Williams et al. 2004). 16S rRNA gene-directed PCR and denaturing gradient gel electrophoresis (DGGE) revealed that *Betaproteobacteria* were also abundant in biofilms of non-chlorinated DSs (Emtiazi et al. 2004). These studies indicate that microbial community diversity is impacted by the disinfection strategy. There is also evidence that diversity can affect disinfection efficacy and pathogen survival. For example, recent work with a flow cell system showed that mixed species biofilms were

more resistant to biocides than single species biofilms (Elvers et al. 2002). The specific mechanism for this is unknown, but a more complete picture of microbial community diversity and inter-species relationships should facilitate a better understanding of disinfection resistance phenomena.

2.5 Nitrifiers

Nitrifying organisms, belonging primarily to the *Alpha-*, *Beta-*, and *Gammaproteobacteria*, have been the subject of several DS studies because nitrification can contribute to depletion of monochloramine and results in the formation of nitrate (Regan et al. 2002). *Nitrosomonas* spp., members of the *Betaproteobacteria*, were identified using 16S rRNA gene-targeted T-RFLP and sequencing of ammonia monooxygenase genes as dominant ammonia-oxidizing bacteria (AOB) in biofilm and bulk water samples from pilot- and full-scale DS studies (Regan et al. 2002; Regan et al. 2003; Lipponen et al. 2004). Another subgroup of *Betaproteobacteria*, *Nitrosospira* spp., was found to constitute a small fraction of the AOB in these systems (Regan et al. 2002; Regan et al. 2003; Lipponen et al. 2004). Use of 16S rRNA gene-directed PCR and DGGE also confirmed the presence of both *Nitrosomonas* spp. and *Nitrosospira* spp. in DS bulk water and biofilms (Hoefel et al. 2005). *Nitrospira* spp. were identified in several studies as the dominant nitrite oxidizing bacteria (NOB) in bulk water and biofilms using 16S rRNA gene clone libraries (Martiny et al. 2005) and 16S rRNA gene-targeted T-RFLP (Regan et al. 2002; Regan et al. 2003). *Nitrobacter* spp., NOB belonging to the *Alphaproteobacteria*, were also detected in biofilms of chloraminated drinking water (Regan et al. 2003).

Nitrification processes can be very important to distribution system management strategies because they affect chloramine residual. In a comparison of chlorinated and chloraminated distribution systems, losses in chloramine level due to nitrification (measured by the increase in nitrate) led to increased overall microbial growth, as determined by heterotrophic plate counts (Wen and Burne 2002). It appears that AOB are present in chloraminated systems irrespective of temperature fluctuations, and that they can be controlled only through very high chloramine levels or very low chloramine levels, because of the scarcity of ammonia (Pintar and Slawson 2003).

2.6 Pathogens

Persistence and growth of pathogens is a central concern in DSs. Field surveys using PCR and southern blot hybridization reported regular detection of pathogens, including *Legionella* spp. and atypical mycobacteria (Emtiazi et al. 2004). *Cryptosporidium* spp. oocysts were detected in bulk water samples (Nichols et al. 2003) and *Helicobacter* spp. were identified in biofilms (Park et al. 2001) in DSs using nested PCR methods. Multiplex PCR analysis was used to detect *Mycobacterium avium* and *Mycobacterium intracellulare* as well as several other *Mycobacterium* spp. in water column and biofilm samples (Falkinham et al. 2001). *Aeromonas* spp. also have been found in DSs, and PCR-based methods were used to quantify the abundance of specific virulence factor genes in isolated *Aeromonas* strains in drinking water (Allesen-Holm et al. 2006). In addition to the detection of specific pathogens and virulence factors, one study monitored antibiotic resistance genes in DS biofilms. Using PCR-based methods, resistance genes responsible for vancomycin-resistance (*van*A) and for [beta]-lactamase activities (*amp*C) were detected in DS biofilms (Emtiazi et al. 2004).

Besides bacterial and protozoan pathogens, viral pathogens also persist in DSs. For example, enteroviruses and adenoviruses have been found in distribution systems (reviewed in (Skraber 2005)). Since many pathogenic viruses are known to be stable in the environment and are resistant to conventional inactivation methods (Nwachcuku and Gerba 2004), it is clear that more research is necessary to understand the role of pathogenic viruses in DS related waterborne illnesses.

The use of molecular tools to detect pathogens in drinking water systems, including PCR-based methods, DNA- and RNA-targeted hybridizations, and microarray based methods, allows for a much more sensitive detection of pathogens than was previously possible with culture-based methods (reviewed in (Call 2005)). While several studies have begun to apply these tools to study DS management strategies, a substantial part of the studies in this area are still performed with conventional culture based techniques. Two examples of application of molecular techniques in DS management research are noteworthy. A flow chamber study verified that the presence of high concentrations of disinfectants was not sufficient to eliminate survival of pathogens, including *L. pneumophila* and

Escherichia coli (Williams and Braun-Howland 2003). Similarly, another study found that application of two common disinfectants, monochloramine and UV, did not deter *L. pneumophila* from accumulating in biofilms in a pilot-scale DS (Langmark et al. 2005). More conventional studies in this area, for example, found that biofilms exposed to strains of *E. coli* and *Klebsiella pneumoniae* developed stable populations of both opportunistic pathogens proportional to the biofilm density of heterotrophic bacteria (Pozos et al. 2004). Likewise, *Mycobacterium xenopi* was found to colonize drinking water biofilm (Dailloux et al. 2003). It was further determined that *M. xenopi* exhibited long term persistence and that a steady concentration of *M. xenopi* cells was returned to the water column from biofilms (Dailloux et al. 2003). Whether the studies employed molecular or conventional techniques, they highlight the danger of pathogen survival in biofilms: Pathogens in biofilms are protected from disinfection and are being released to the bulk water used for human consumption.

2.7 Bacterial pathogen–protozoon interactions

Studying the ecology of bacterial pathogen-protozoon interaction may help to improve our understanding of the persistence of bacterial pathogens in drinking water. For example, it has been estimated that the amoeba *Acanthamoeba polyphaga* can contain between 1 to 120 *M. avium* cells and can host even higher levels of *L. pneumophila* (Steinert et al. 1998). An inactivation study for the bacterial pathogen *Burkholderia pseudomallei* found that co-culture with the amoeba *Acanthamoeba astronyxis* increased the resistance of *B. pseudomallei* to disinfection, requiring 100 times more monochloramine to achieve similar disinfectant efficacy than when cultured alone (Harrison et al. 2005). Additionally, depletion of disinfectant may result in a re-colonization of biofilms by bacterial pathogens, such as *L. pneumophila*, protected in amoeba (Emtiazi et al. 2004). *Legionella pneumophila* has been found to proliferate in drinking water biofilms in the presence of the protozoon *Hartmannella vermiformis*, and after 14 days of co-culture intracellular growth was found in 90% of the protozoa (Kuiper et al. 2004). There is also evidence that intracellular growth selects for virulence factors that affect pathogenesis in protozoon hosts (reviewed in (Molmeret et al. 2005)).

2.8 Complexities associated with controlling microbial growth

Optimizing the management of DSs and controlling microbial growth is difficult due to the complexity of these systems. Survival of microorganisms is based upon interactions of many variables, including temperature (Ndiongue 2005), pipe surface (Lehtola 2005), nutrient levels (Butterfield and Wattie 1946; Wijeyekoon et al. 2004), and type and concentration of disinfectants (Norton et al. 2004). Microbial growth can be controlled to some extent through providing a disinfectant residual (Chu et al. 2003) and reduction in biodegradable organic matter (Gagnon et al. 2000). Uncontrollable events, such as seasonal fluctuations of precipitation, can lead to even greater complexity. For example, a study of Mexico City's chlorinated DS found that levels of fecal streptococci were significantly higher in the dry season than in the wet season, while *Helicobacter pylori* levels remained fairly constant through the seasons (Mazari-Hiriart et al. 2005). This illustrates that uncontrollable complexity such as seasonal precipitation can lead to species-specific responses.

Another, often uncontrollable, complexity is the type of pipe material used in the DS. Certain pipe materials can stimulate growth by releasing iron and phosphorus in their bioavailable forms (Lehtola et al. 2004; Patten et al. 2004) and by neutralizing the disinfectant residual (Hallam et al. 2002; Lehtola 2005). Soft deposits that settle to the pipe floor can be a major source of available nutrients, and removal of such deposits has been associated with reduced microbial growth (Lehtola et al. 2004). It should also be noted that release of some compounds, such as copper from copper pipes, slows biofilm development, presumably because they are toxic or inhibitory to microorganisms (Lehtola et al. 2004; van der Kooij et al. 2005).

2.9 Integrating system knowledge: monitoring and modeling

The fundamental biological concern in drinking water supply is to minimize contamination with pathogens. As discussed above, pathogen survival in DSs is based upon complex interactions between physical, chemical, and operational factors, and microbial ecology. An important initial step to controlling pathogens is to develop effective monitoring strategies that take the microbial ecology of DSs into account. Culture-based methods often underestimate or distort the community profile because

many microorganisms are in a viable but non-culturable state, prompting interest in alternative monitoring methods (Hoefel et al. 2005). Pathogen-specific monitoring may take the form of PCR-based methods, nucleic acid hybridizations, or immunological based methods (Call 2005; Rudi et al. 2005; Tallon et al. 2005). Metagenomic analyses can be used to determine the metabolic and functional potential of entire microbial communities. A metagenomic approach already has proved useful in determining phylogenetic and functional gene diversity (Schmeisser et al. 2003), and could be used to further determine the presence of genes conferring virulence (Allesen-Holm et al. 0) and antibiotic resistance (Emtiazi et al. 2004). Linking metagenomic approaches with quantitative molecular tools will make it possible to integrate effective monitoring with control of microbial growth.

The complexity of controlling microbial growth in DSs calls for the use of mathematical models. However, it is challenging to accurately model the processes and interactions in DSs. Multi-species biofilm models are becoming increasingly complex (Gheewala et al. 2004; Picioreanu et al. 2004; Rittmann et al. 2004; Xavier et al. 2005), as are models describing biofilm disinfection (Roberts and Stewart 2004; Roberts and Stewart 2005) and bacterial regrowth in DSs (DiGiano and Zhang 2004). A limitation of current disinfection models is that they almost exclusively include single-species models, with some exceptions (Piriou et al. 1998; Woolschlager et al. 2001). The development of multi-species models of DS biofilms that take into account the effects of disinfectants on microbial ecology will help to determine optimal operational parameters and lead to knowledgeable decisions regarding management of drinking water supply. The complexity of the DS then must be accounted for by incorporating the multi-species disinfection model into a large-scale, spatially-distributed hydraulic model that integrates knowledge about the layout of the pressurized pipe system, such as the mechanistic model recently developed by DiGiano and Zhang (DiGiano and Zhang 2004).

2.10 Conclusion

It is clear that standard chlorination strategies are sometimes inadequate for controlling regrowth in the DS, and can be improved upon with a better understanding of microbial ecology. Bacterial, protozoon and viral pathogens can resist disinfection through

protection within biofilms and resistant host cells. From the viewpoint of environmental biotechnology, this complexity presents a great challenge to providing safe, clean drinking water to the public. Future research will utilize advanced, non-culture based monitoring techniques to more completely describe pathogen presence in DSs. The elucidation of resistance mechanisms will allow the DS to be modeled accurately and will provide insights into novel control strategies.

2.11 References

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Chapter 3
Cellular and Genetic Response of *Mycobacterium avium* to Monochloramine

3.1 Abstract

Mycobacterium avium is an opportunistic bacterial pathogen that is very resistant to disinfection and is known to survive in treated drinking water. This work demonstrates that *M. avium* undergoes rapid cellular changes upon exposure to a sub-inhibitory dose (0.5 mg/L as Cl_2) of the drinking water disinfectant monochloramine, including an increase in cell permeability and oxidation of low molecular weight thiols. Rapid and strong induction of the oxidative stress response was observed using qRT-PCR. Transcriptional profiling of *M. avium* exposed to monochloramine for 10 min identified 34 additional monochloramine-responsive genes, including upregulation of many virulence-associated genes. Expression levels of selected virulence-associated genes were confirmed using qRT-PCR and generally were found to be time-sensitive, with reduced expression at 20 and 40 min. These results demonstrate that *M. avium* senses and responds to the presence of a sub-inhibitory dose of monochloramine and also suggests that oxidative stress may trigger virulence responses in *M. avium*.

3.2 Introduction

Mycobacterium avium is an opportunistic bacterial pathogen that can persist and grow in surface waters and in treated drinking water (Vaerewijck et al. 2005). *M. avium* has been implicated or is suspected to be a causative agent in a variety of human and animal diseases, including hypersensitivity pneumonitis, respiratory problems, cervical lymphadenitis (Falkinham III 2003), chronic bowl disease, allergies, and pulmonary infections (Primm et al. 2004). It is of particular concern for immuno-compromised subpopulations such as AIDS patients (Falkinham III 2003). *M. avium* can be transmitted to humans directly from the environment via inhalation or ingestion pathways (Vaerewijck et al. 2005). The ingestion pathway occurs primarily through the use and consumption of contaminated drinking water, while inhalation can occur from exposure to aerosols generated from a variety of sources, including from metal-working fluids in

industry, indoor swimming pools, hot tubs, showers, and water-damaged buildings (Falkinham III 2003).

M. avium is resistant to inactivation with a broad array of disinfectants, including free chlorine, chloramines, chlorine dioxide, ozone, and UV (Taylor et al. 2000; Luh et al. 2008; Shin et al. 2008; Vicuña-Reyes et al. 2008). In addition, *M. avium* can survive within acanthamoebae, in which they are afforded even greater protection from disinfection (Berry et al. 2009b), which is a concern for effective drinking water treatment and distribution (Berry et al. 2006). While the resistance of *M. avium* to disinfection is well-studied (Taylor et al. 2000; Luh et al. 2008; Shin et al. 2008; Vicuña-Reyes et al. 2008), its response mechanisms are not. The goal of this study was to characterize the response of *M. avium* to a sub-lethal dose of the commonly-used drinking water disinfectant monochloramine. Changes in cell wall permeability, intracellular thiol concentration, and transcriptional profiling of differential gene expression were measured using a monochloramine dose of 0.5 mg/L (as Cl_2) for exposure times between 0 and 90 min. Previous work has demonstrated that *M. avium* is still viable after being challenged by this monochloramine dose and exposure times (Luh et al. 2008), so the responses observed are expected to be due to reversible stress and cell injury.

3.3 Results and discussion

3.3.1 Impact of monochloramine on cell membrane permeability

It has previously been demonstrated that free chlorine exposure causes extensive permeabilization of the cytoplasmic membrane of *Escherichia coli* and *Listeria monocytogenes* (Virto et al. 2005). Additionally, free chlorine and monochloramine rapidly increases cell permeability in endothelial cells (Tatsumi and Fliss 1994). Propidium iodide (PI), an intercalating molecule that is fluorescent when bound to nucleic acids, was used to determine cell membrane permeability of *M. avium* exposed to a sub-lethal dose of monochloramine (0.5 mg/L as Cl_2). PI has been used as an indicator of cell membrane integrity for *M. avium* (Steinert et al. 1998), and to measure inactivation kinetics of *E. coli* exposed to free chlorine (Cunningham et al. 2008). Though PI is preferentially taken up by cells with compromised membranes, it is also taken up to a lesser extent by live cells (Shi et al. 2007). Therefore, cell membrane permeability was quantified as PI fluorescence of treated cells normalized to PI fluorescence of non-treated cells. Enhanced PI uptake was observed within 5 min of *M. avium* exposure to 0.5

mg/L (as Cl_2) monochloramine and increased until approximately 40 min, when it reached a plateau (Figure 3.1). *M. avium* is still viable after being challenged by this monochloramine dose and exposure time (Luh et al. 2008), suggesting that the increase in cell permeability is a reversible process that does not necessarily result in cell death.

3.3.2 Impact of monochloramine on level of intracellular low molecular weight thiols

Intracellular thiol levels in *M. avium* were measured using monochlorobimane (MCB), a molecular probe that fluoresces when conjugated with low molecular weight thiols such as reduced glutathione (Fernández-Checa and Kaplowitz 1990), and a range of intracellular thiols (Vanderven et al. 1994). Mycothiol is the major thiol found in *Actinobacteria* and has many of the functions of glutathione, which is the dominant thiol in other *Bacteria* and *Eukarya* but is absent in *Actinobacteria* (Newton et al. 2008). In the present study, exposure to 0.5 mg/L (as Cl_2) monochloramine induced a rapid oxidation of intracellular thiols, with a marked decrease in thiol levels by 5 min and continued decrease to 60 min, when the detection limit of the assay was reached (Figure 3.2). Free chlorine and monochloramine have been observed to rapidly deplete intracellular thiol levels in endothelial cells (Tatsumi and Fliss 1994), and intracellular thiol depletion has been observed for sub-inhibitory doses of free chlorine (Pullar et al. 1999). In *E. coli*, inactivation with monochloramine is known to be accompanied by thiol oxidation, although complete oxidation of intracellular thiols is not believed to be necessary for inactivation (Jacangelo et al. 1987). *E. coli* proteins have been shown to have varying reactivity to reactive oxidant species (Leichert et al. 2008), and chloramines are known to be more selective in oxidizing thiol groups than free chlorine (Peskin and Winterbourn 2001; Peskin and Winterbourn 2003), suggesting that monochloramine oxidation of protein thiols may selectively trigger different sensing systems than other oxidants such as free chlorine.

3.3.3 Measuring the oxidative stress response using RT-qPCR

Unlike frank pathogenic mycobacteria, many opportunistic pathogenic mycobacteria such as *M. avium* have a functional OxyR-regulated oxidative stress response (Sherman et al. 1995). The expression of *oxyR* and aconitases (*ahpCD*) believed to be regulated by OxyR and important in detoxification was measured during exposure of *M. avium* to monochloramine. Expression of *oxyR* and *ahpCD* was induced rapidly and strongly upon exposure of *M. avium* to monochloramine, with maximum expression measured at 10 min exposure time for all three

genes (Figure 3.3). *oxyR* and *ahpCD* are conserved and upregulated during oxidative stress in other mycobacteria (den Hengst and Buttner 2008). *M. avium* is known to express *ahpC* after hydrogen peroxide treatment (Sherman et al. 1995) and the levels of AhpC in *Mycobacterium marinum* increase after hydrogen peroxide treatment (Pagan-Ramos et al. 1998). Additionally, inactivation of *oxyR* in *M. marinum* decreases *ahpC* levels and increases sensitivity to the antibiotic isoniazid (Pagan-Ramos et al. 2006). The results with *M. avium* presented in the current study provide the first evidence that the OxyR regulon is responsive to monochloramine treatment and suggest that AhpCD may play an important detoxification role during monochloramine stress.

3.3.4 Identification of monochloramine-responsive genes in *M. avium* using microarray

Global transcriptional profiling of *M. avium* exposed to 0.5 mg/L (as Cl_2) monochloramine for 10 min was determined using microarray technology in order to identify monochloramine-responsive genes. The microarray study identified 34 genes with at least two-fold differential expression (all upregulated) (Fig 4). Several of the upregulated genes, or homologues in other mycobacteria, have been associated with oxidative stress response in other studies (listed in Table 3.1). This is generally consistent with the observation of oxidative stress response induction using qRT-PCR, although increased expression of *oxyR* and *ahpCD* were not detected in the microarray data. Many of the oxidative stress-responsive genes are also associated with virulence or expressed during infection. Additionally, several upregulated genes that are not implicated in oxidative stress response are also virulence-associated. Table 3.1 demonstrates that many upregulated genes are virulence-associated genes or have homologues in other mycobacteria that are virulence-associated (Table 3.1). A recent microarray study found that exposure of *Staphylococcus aureus* to free chlorine induces virulence genes (Chang et al. 2007). The results in the present study add weight to the possibility that disinfection processes may generally induce virulence responses in bacterial pathogens.

3.3.5 Expression of virulence-associated genes using qRT-PCR

The expression levels after exposure for 10, 20, and 40 min to 0.5 mg/L (as Cl_2) monochloramine of four upregulated genes that are virulence-associated were confirmed with qRT-PCR: *rpfA* (MAV_0996), which encodes an autocrine growth factor important in recovery from dormancy in *M. avium* (Kell and Young 2000); Elongation factor TU (MAV_0417), which

is expressed during macrophage infection of *M. avium* (Brunori et al. 2004) and *M. bovis* (Monahan et al., 2001); *mce1c* (MAV_5013), which is part of the *mce1* operon expressed by *M. tuberculosis* upon phagocytosis by human macrophages (Graham and Clark-Curtiss 1999); and MAV_4349, a member of the PPE protein family, which is believed to be an important part of mycobacterial virulence (Mackenzie et al. 2009). These genes were selected because they were the most highly induced virulence-associated genes in the microarray data for which primers could be successfully designed. All four genes were induced at 10 min exposure to monochloramine, and had reduced expression at 20 and 40 min exposure times (Fig 3.5). The similar trend observed with these four genes suggests that virulence-associated genes may be induced most significantly in the early stages of exposure to monochloramine and that expression of virulence genes may be reduced as the cell is damaged to a greater extent.

3.4 Conclusion

Exposure of *M. avium* to a sub-lethal dose of monochloramine resulted in rapid changes to cell wall permeability, intracellular thiol concentration, and gene expression. The OxyR response was induced very strongly within 10 min of exposure to monochloramine. Additionally, many virulence-associated genes were upregulated within 10 min of exposure to monochloramine, though expression levels appeared to decline when exposure times were extended to 20 and 40 min. These results suggest that *M. avium* exposure to monochloramine may increase the expression of virulence genes, though whether this increases *M. avium* virulence to humans must be further studied.

3.5 Materials and methods

3.5.1 Strain and culture conditions

Mycobacterium avium 104, an isolate from an AIDS patient (Bermudez et al. 1997), was provided by Gerard Cangelosi (Seattle Biomedical Research Institute, Seattle, Washington). *M. avium* was cultured on Middlebrook 7H9 broth supplemented with 10% albumin-dextrose-catalase (ADC) enrichment and 0.2% glycerol (Sigma-Aldrich, St. Louis, MO) and cells were harvested at an OD_{600} of 0.4-0.5 for all experiments.

3.5.2 Monochloramine preparation and challenge

Monochloramine was prepared and quantified as described previously (Berry et al. 2009a). *M. avium* was exposed to a dose of 0.5 mg/L (as Cl_2) monochloramine for several time durations at

a temperature of 20 °C. All exposure conditions tested were previously determined to be sub-inhibitory (Luh et al. 2008). Monochloramine was quenched by the addition of 0.12% sodium thiosulfate (Sigma-Aldrich, St. Louis, MO).

3.5.3 Fluorescent probes

A bacterial culture of 10^7 cells/mL suspended in PBS was exposed to 0.5 mg/L (as Cl_2) monochloramine. Samples were harvested at several time points and the disinfectant was quenched by the addition of 0.12% sodium thiosulfate. Either propidium iodide or monochlorobimane was then added to cell suspensions. Propidium iodide (LIVE/DEAD *Bac*Light Bacterial Viability Kit, Molecular Probes, Carlsbad, CA) was added to cell suspensions at a final concentration of 30 µM and incubated for 30 min at room temperature (RT) in the dark. Monochlorobimane (Molecular Probes, Carlsbad, CA) was added at a final concentration of 100 µM and was incubated for 50 min at RT in the dark. The same procedure was carried through for non-treated control cells suspended in PBS without monochloramine. After incubation, cell suspension fluorescence was monitored for monochloramine-treated and non-treated cells in a microplate reader (Synergy HT, BioTek Instruments, Inc., Winooski, VT) using excitation/emission wavelengths of 480 ± 20 nm / 645 ± 40 nm for propidium iodide and 360 ± 40 nm / 460 ± 40 nm for monochlorobimane. Fluorescence measurements of treated cells at each time point were normalized by dividing by the fluorescence of non-treated cells. Experiments were conducted using four biological replicates.

3.5.4 RNA extraction and cDNA preparation

RNA was extracted using a low-pH, hot-phenol chloroform extraction method as follows. Low-pH, hot-phenol:chloroform (65 ºC, pH 4.5, with IAA 125:24:1) (Ambion, Austin, TX) was added to mycobacterial cells suspended in PBS in RNase-free polypropylene tubes containing 0.5 g of 0.1 mm diameter zirconia/silica beads (BioSpec Products, Inc., Bartlesville, OK) and incubated for 5 min at 65 ºC with periodic mixing. Cells were homogenized for 2 min (Mini-Beadbeater-96, BioSpec Products, Inc., Bartlesville, OK) and incubated for 5 min at 65 ºC with periodic mixing. The tubes were centrifuged at 16,000 x *g* for 20 min at 4 ºC and the aqueous phase was transferred to a 2 ml phase-lock-gel tube (PLG Heavy, 5 PRIME Inc., Gaithersburg, MD). An equal part of the phenol:chloroform mixture was added, mixed, and centrifuged at 14,000 x *g* for 5 min at RT. The aqueous phase was transferred to another phase lock gel tube and an equal part

of a chloroform:isoamyl alcohol (24:1) mixture was added, mixed, and centrifuged at 14,000 x *g* for 5 min at RT. The aqueous phase was transferred to a new tube and nucleic acids were precipitated by addition of an equal part isopropanol and a 2 h incubation at -20 ºC. Nucleic acids were pelleted (16,000 x *g* for 30 min at 4 ºC), the supernatant was decanted, and nucleic acids were re-suspended in 90 µl RNase-free water. DNA was digested using 8 U DNase (TURBO DNA-*free* Kit, Ambion, Austin, TX) and a 30 min incubation at 37 ºC. Following DNase inactivation, RNA solution was transferred to a fresh tube and purity was spectrophotometrically determined using 260 nm/280 nm absorbance ratios (Nanodrop ND-1000, Nanodrop Technologies, Wilmington, DE) and using a PCR control to ensure no residual DNA. RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

For qRT-PCR analyses, cDNA was synthesized from purified RNA using the Verso 2-Step QRT-PCR kit (Thermo Fisher Scientific Inc., Waltham, MA) according to the manufacturer's instructions. For microarray analyses, 10 µg RNA was reverse transcribed to double stranded cDNA using 1 µl random hexamer primers using the Superscript Double-Stranded cDNA Synthesis Kit (Invitrogen, Carlesbad, CA) according to the manufacturer's instructions (http://tools.invitrogen.com/content/sfs/manuals/superscript_doublestrand_man.pdf). Purified cDNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) and was shipped on dry ice to Roche Nimblegen (Madison, WI) for labeling and microarray hybridizations.

3.5.5 Microarray studies

An expression array for *Mycobacterium avium* 104 was designed by Roche Nimblegen, Inc. using the completed genome in the NCBI database (NC_008595). The array design featured 19 different 60-mer oligonucleotide probes targeting each gene, with an overall coverage of 5,103 out of 5,120 coding sequence (CDS) regions in the genome. Four replicates of each probe were spotted on each array. cDNA was labeled with Cy-3 and hybridized to microarrays using a Nimblegen Hybridization system according to the Nimblegen protocol (http://www.nimblegen.com/products/lit/hybe_system_userguide_v1p2.pdf) and scanned with a GenePix 4000B scanner. Fluorescence intensity data was extracted using NimbleScan software (Nimblegen).

Microarray data were processed using SNOMAD tools and executed in R (Colantuoni et al. 2002). Briefly, data were normalized using global mean normalization and local mean normalization to account for spatial heterogeneity on the microarray slide. Probe-level signals were combined to give a log-transformed fold-change followed by a local mean normalization to account for signal intensity bias. Corrected fold-changes for each probe were combined at the gene target level to yield fold-change, standard deviation, and p-value for each gene. Statistical significance of p-values was corrected for multiple comparisons by implementing a false discovery rate (FDR) correction (Benjamini and Hochberg 1995). Genes were considered differentially expressed if they had an absolute fold-change of >=2 and were significant at the FDR = 0.01 level.

3.5.6 qRT-PCR

SYBR green-based qRT-PCR was performed using 10 ng cDNA template and 200 nM specific primers (Table 3.2) and the Verso 2-Step QRT-PCR kit (Thermo Fisher Scientific Inc., Waltham, MA) according to the manufacturer's instructions. The thermocycler program comprised an initial enzyme activation step of 95°C for 15 min followed by 40 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s. Reactions were performed in technical duplicate and biological triplicate with a Mastercycler ep *realplex*^{2} (Eppendorf AG, Hamburg, Germany). The threshold cycle values were normalized to levels of *rplL* (ribosomal protein L7/L12, 50S rRNA) and *rpsE* (30S rRNA protein 5) in each sample and then expression fold-changes between treated and control cells were calculated using the $\Delta\Delta CT$ method (Nolan et al. 2006). PCR primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and checked for specificity for the target gene in the *M. avium* genome using the BLAST algorithm (NCBI). All primers used in this study are listed in Table 3.2.

3.6 Figures and tables

Table 3.1 *M. avium* genes upregulated after 10 min exposure to 0.5 mg/L (as Cl_2) monochloramine, detected with microarray and/or qRT-PCR. Evidence that the gene or a homologous protein in another *Mycobacterium* strain is involved in oxidative stress or virulence is also indicated. If no gene annotation was available in NCBI, the annotation of a homologous protein was substituted when possible. N.S. indicates non-significant differential gene expression.

Locus	Microarray fold-change	qRT-PCR fold-change	Oxidative Stress	Virulence	Annotation	References
MAV_0019	2.2 ± 1.2				Serine/threonine protein kinases Drp72	
MAV_0053	2.0 ± 1.1				Hypothetical protein	
MAV_0054	2.0 ± 1.1			60% similar to Mtc28 (MtubT9_010100002141) of *M. tuberculosis* T92	Mtc28 (proline rich 28 kDa antigen) (*M. tuberculosis* T92)	(Manca et al., 1997)
MAV_0177	2.7 ± 1.4				Copper resistance protein CopC	
MAV_0417	2.2 ± 1.3	2.8 ± 0.2	30% similar to EF-Tu (Rv0685) of *M. tuberculosis* H37Rv	30% similar to EF-Tu (Rv0685) of *M. tuberculosis* H37Rv 30% similar to EF-Tu (Mb0704) of *M. bovis* AF2122/97	Elongation factor Tu GTP binding domain-containing protein	(Manganelli et al., 2001) (Monahan et al., 2001)
MAV_0510	2.5 ± 1.4			69% similar to Rv3657c of *M. tuberculosis* H37Rv	Flp pilus assembly protein TadC (54% similar to *Gordonia bronchialis* DSM	(Tomich et al., 2007)

					43247)	
MAV_0511	2.0 ± 1.1			79% similar to Rv3656c of *M. tuberculosis* H37Rv	Hypothetical protein	(Tomich et al., 2007)
MAV_0512	2.0 ± 1.2			70% similar to Rv3655c of *M. tuberculosis* H37Rv	TadE family protein (57% similar to *Geobacter bemidjiensis* Bem)	(Tomich et al., 2007)
MAV_0578	2.2 ± 1.1			99% similar to MAP_0482 of *M. avium* subsp. *paratuberculosis* K-10	Transcriptional regulator, LuxR family protein	(Patel et al., 2006)
MAV_0995	2.0 ± 1.1				Molybdopterin converting factor, subunit 2	
MAV_0996	2.2 ± 1.1	2.7 ± 0.6	71% similar to RpfA (Rv0867c) of *M. tuberculosis* H37Rv	71% similar to RpfA (Rv0867c) of *M. tuberculosis* H37Rv	Resuscitation-promoting factor RpfA	(Kana et al., 2008) (Kendall et al., 2004)
MAV_1257	2.1 ± 1.2				Hypothetical protein	
MAV_1346	2.1 ± 1.1				PE family protein	
MAV_1722	2.0 ± 1.1			67% similar to RpfE (Rv2450c) of *M. tuberculosis* H37Rv	Resuscitation-promoting factor RpfE (*M. tuberculosis* H37Rv)	(Fisher et al., 2002; Kana et al., 2008)
MAV_2328	2.5 ± 1.5				S-adenosyl-methyltransferase MraW	
MAV_2329	3.5 ± 1.3				Hypothetical protein	

MAV_2429	2.0 ± 1.2				PPE family protein	
MAV_2838	N.S.	15.6 ± 0.5	75% similar to OxyR (AF034861) of *M. marinum* ATCC 15069 *M. avium* 104		Hydrogen peroxide-inducible genes activator, OxyR (*M.* *marinum* ATCC 15069)	(Pagan-Ramos et al., 2006; Geier et al., 2008)
MAV_2839	N.S.	237.2 ± 0.6	90% similar to AhpC (Rv2428) of *M. tuberculosis* H37Rv *M. avium* 104	90% similar to AhpC (Rv2428) of *M. tuberculosis* H37Rv	Alkylhydroperoxide reductase, AhpC	(Hillas et al., 2000; Fisher et al., 2002; Rohde et al., 2007; Fontan et al., 2008; Geier et al., 2008)
MAV_2840	N.S.	8.5 ± 0.2	74% similar to AhpD (Rv2429) of *M. tuberculosis* H37Rv *M. avium* 104	74% similar to AhpD (Rv2429) of *M. tuberculosis* H37Rv	Alkylhydroperoxidase, AhpD	(Hillas et al., 2000; Fisher et al., 2002; Rohde et al., 2007; Fontan et al., 2008; Geier et al., 2008)
MAV_2956	2.1 ± 1.2				ATP-dependent RNA helicase	
MAV_3189	2.0 ± 1.2				nicotinate-nucleotide pyrophosphorylase, NadC	
MAV_3280	2.1 ± 1.2				Hypothetical protein	
MAV_3281	3.1 ± 1.1				Hypothetical protein	

MAV_3282	2.0 ± 1.2			34% similar to PhoR (Rv0758) of *M. tuberculosis* H37Rv	Two-component regulator - sensor kinase (74% similar to HisKA (MMAR_2299)of *Mycobacterium marinum* M)	(Asensio et al., 2006; Gonzalo-Asensio et al., 2008)
MAV_3640	2.6 ± 1.1				Antibiotic biosynthesis monooxygenase domain-containing protein	
MAV_3979	2.0 ± 1.1				Hypothetical protein	
MAV_4087	2.0 ± 1.2				Transferase	
MAV_4088	2.3 ± 1.1			47% similar to GmhA (MT0122) of *M. tuberculosis CDC1551*	Phosphoheptose isomerase, GmhA	(Rohde et al., 2007)
MAV_4349	2.9 ± 1.3	2.2 ± 0.4		98% similar to PPE36 of *M. avium* subsp. *avium*	PPE family protein	(Mackenzie et al., 2009)
MAV_4381	2.0 ± 1.1				Oxidoreductase	
MAV_4464	2.3 ± 1.1		94% similar to rplP (Rv0708) *M. tuberculosis* H37Rv	94% similar to RplP (Rv0708) *M. tuberculosis* H37Rv	50S ribosomal protein L16	(Manganelli et al., 2001)
MAV_4774	2.1 ± 1.1				Hypothetical protein	
MAV_4792	2.0 ± 1.1				Hypothetical protein	
MAV_4872	2.0 ± 1.3				PPE family protein	

MAV_4986	2.1 ± 1.1				ErfK/YbiS/YcfS/YnhG family protein	
MAV_5013	2.2 ± 1.2	6.8 ± 0.4	80% similar to Mce1C (Rv0171) of *M. tuberculosis* H37Rv	80% similar to Mce1C (Rv0171) of *M. tuberculosis* H37Rv	Mammalian cell entry protein Mce1C	(Gioffré et al., 2005) (Mehra and Kaushal, 2009)

Table 3.2 Primers used for qRT-PCR.

Gene	Primer	Oligonucleotide Sequence (5'-3')	Reference
EF-Tu	MAV_0417 forward MAV_0417 reverse	GATCACGGCAAATCGACTC AGCCCAAATCGATGGTCAG	This study
rpfA	MAV_0996 forward MAV_0996 reverse	GGCGAATGGGATCAGGTAG GTGTTGATGCCCCAGTTG	This study
oxyR	MAV_2838 forward MAV_2838 reverse	GGATGGCACTGGGTGACTAC CCGTAGGTGTTGAGGGACAG	(Geier et al., 2008)
ahpC	MAV_2839 forward MAV_2839 reverse	AGCACGAGGACCTCAAGAAC GTGACCGAGACGAACTGGAT	(Geier et al., 2008)
ahpD	MAV_2840 forward MAV_2840 reverse	GTACGCCAAGGATCTCAAGC GTACTTGCCGTCCAAGAAGC	(Geier et al., 2008)
MAV_4349 (PPE family)	MAV_4349 forward MAV_4349 reverse	GTTGGGTTCGGTTCGAAAG GTTGGGGTGACTTGCTTTTC	This study
rpsE	MAV_4448 forward MAV_4448 reverse	GATGGCGACAAGAGCAACTAC CTTGGAGACTCGGTTGATGG	This study
rplL	MAV_4507 forward MAV_4507 reverse	CAAAGATGTCCACCGACGAC AGCAGGGTCATCTCCTTGAAC	This study
mce1C	MAV_5013 forward MAV_5013 reverse	GATCAAGACCGACACCATCC AGAACGCGTCGTAAATCTGG	This study

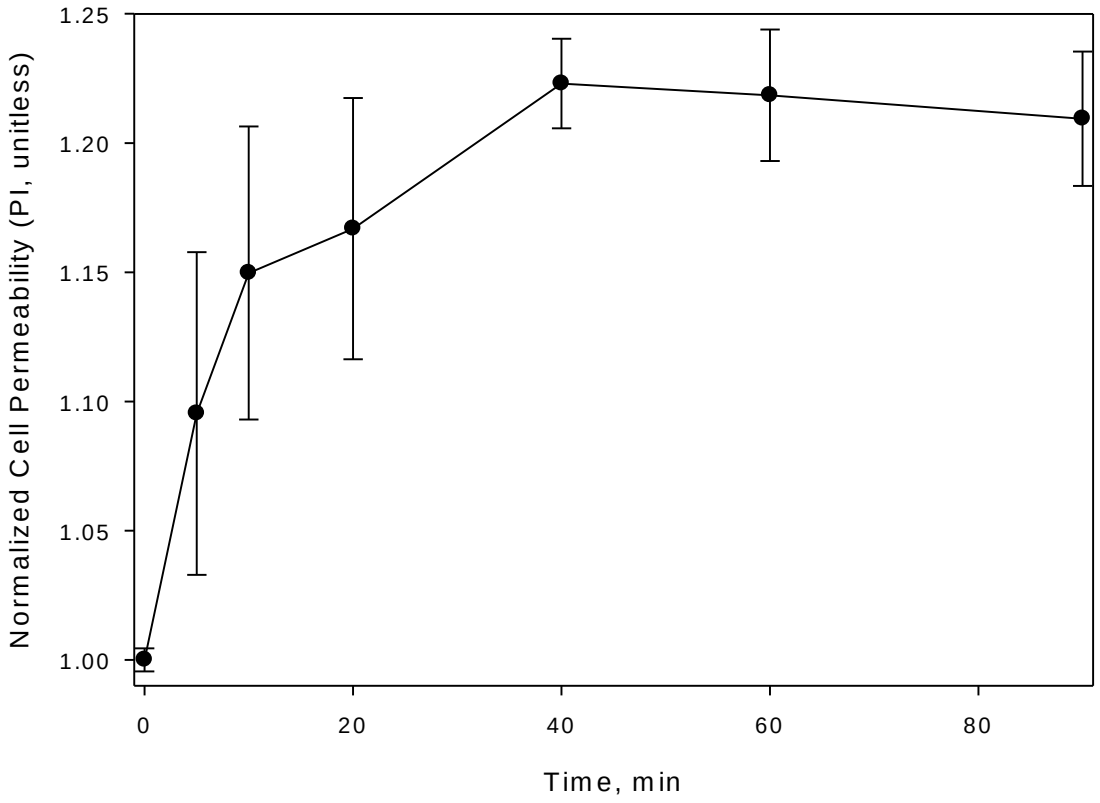

Figure 3.1 Permeability of *M. avium* exposed to 0.5 mg/L (as Cl_2) monochloramine for different times, as measured by propidium iodide (PI) fluorescence. Error bars indicate standard deviations of four biological replicates.

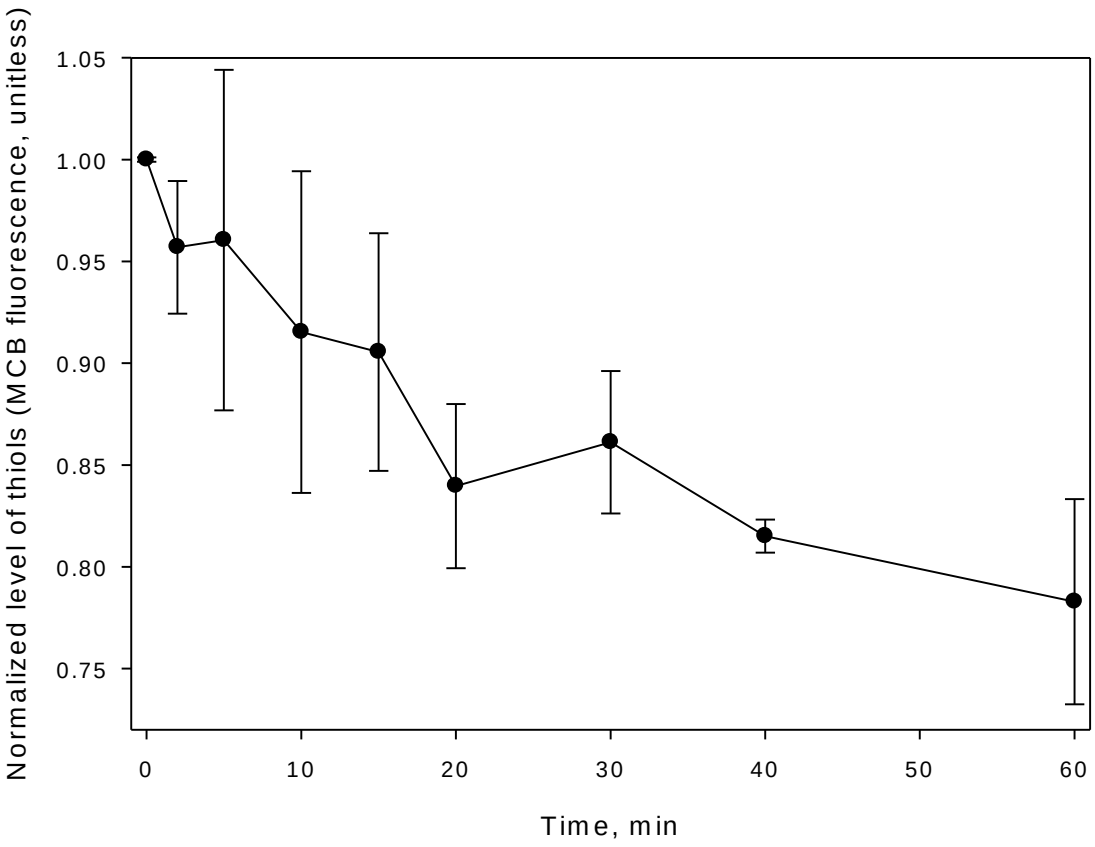

Figure 3.2 Intracellular thiol level *M. avium* exposed to 0.5 mg/L (as Cl_2) monochloramine for different times, as measured by monochlorobimane (MCB) fluorescence. Error bars indicate standard deviations of four biological replicates.

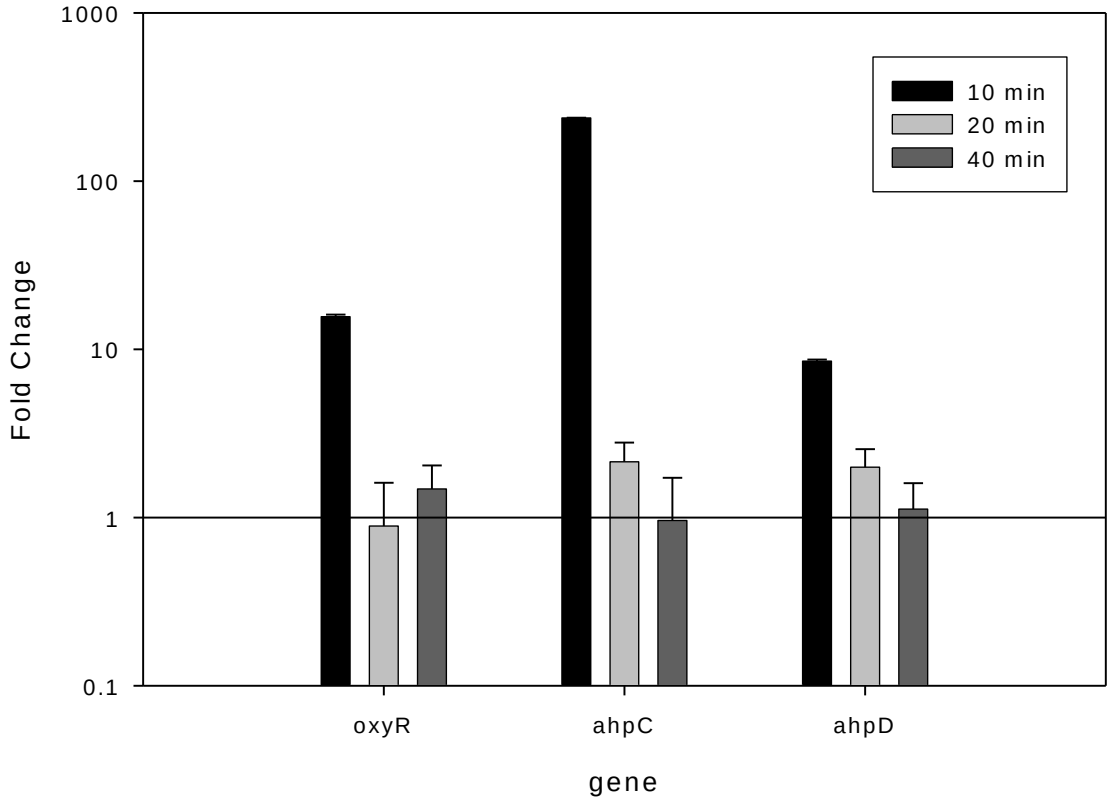

Figure 3.3 Expression levels of oxidative stress response-related genes at different exposure times to 0.5 mg/L (as Cl_2) monochloramine. Error bars indicate standard deviations of replicate measurements calculated using the ΔΔCT method.

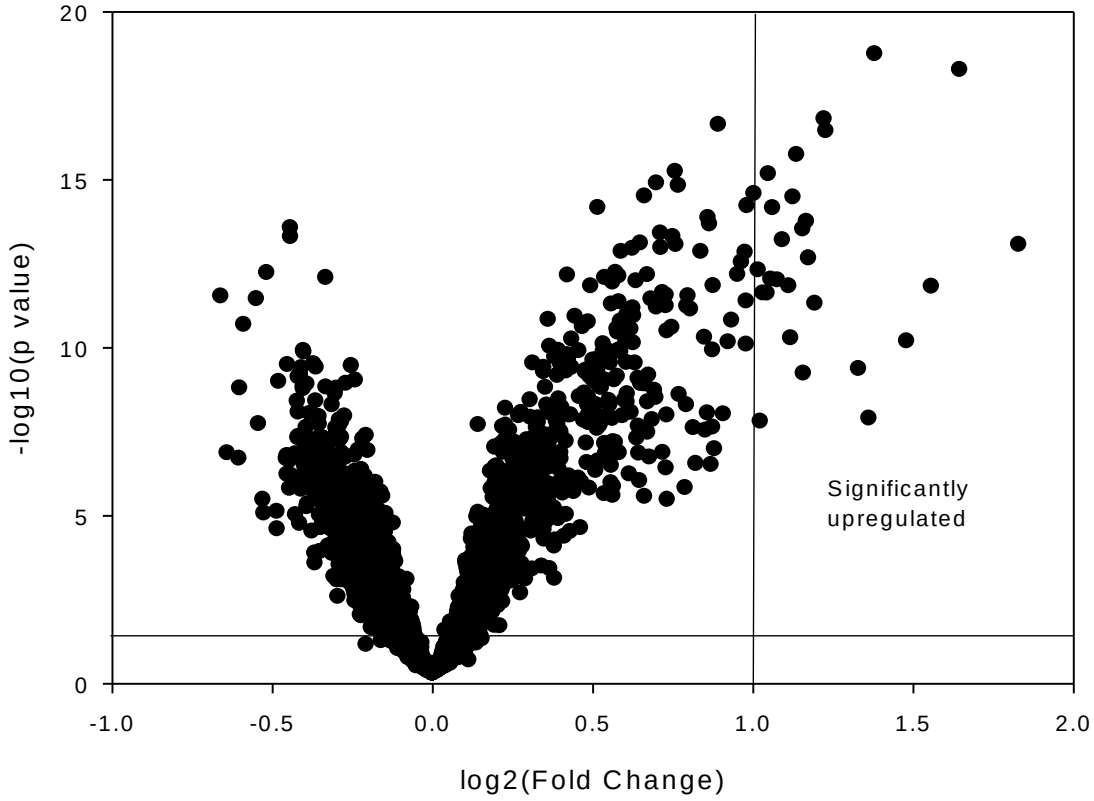

Figure 3.4 "Volcano plot" of microarray data obtained by exposing *M. avium* cells for 10 min to 0.5 mg/L (as Cl_2) monochloramine. Data show the relationship between differential gene expression level and statistical confidence (as negative log-transformed p value) for each gene. Genes of interest are those most highly upregulated(to the right of the vertical line) and with high statistical significance (above the horizontal line), which are located on the upper-right side of the plot.

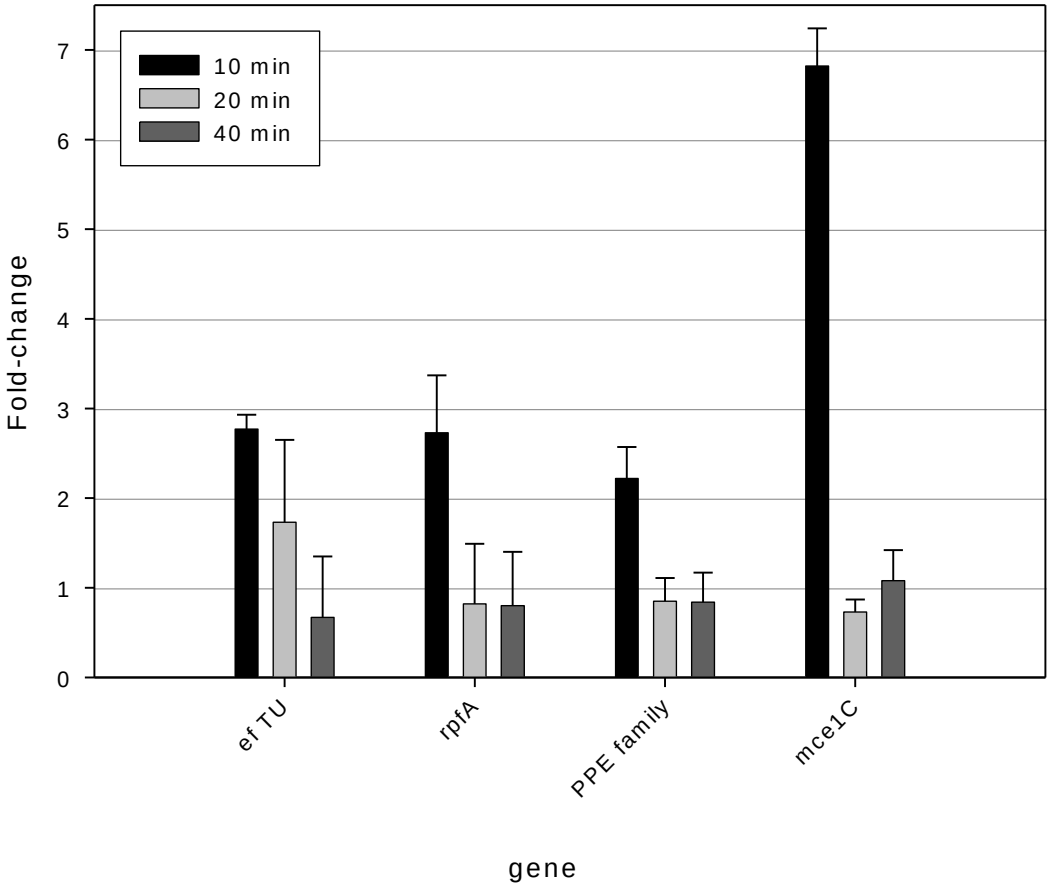

Figure 3.5 Expression levels of monochloramine-sensitive virulence-associated genes at different exposure times to 0.5 mg/L (as Cl_2) monochloramine. Error bars indicate standard deviations of replicate measurements calculated using the ΔΔCT method.

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Chapter 4
Effect of Growth Conditions on *Escherichia coli* Inactivation with Monochloramine

4.1 Abstract

Reduced susceptibility of bacteria to disinfection is a serious concern in drinking water distribution systems (DWDS), yet the mechanisms and conditions governing reduced susceptibility are not well characterized. The effects of growth temperature, growth rate, and growth mode (suspended growth versus growth in biofilms) on inactivation kinetics of *Escherichia coli* exposed to monochloramine were studied in order to understand growth conditions that may reduce susceptibility of bacteria to disinfectants in DWDS.

Cells grown at a suboptimal temperature (20 ºC) were significantly less sensitive to monochloramine inactivation (using 0.5 and 5.0 mg/L monochloramine (as Cl_2)) than cells grown at an optimal temperature (37 ºC). Cells grown in biofilms were also significantly less sensitive than cells grown in suspension. No difference in inactivation kinetics was observed for cells grown in monolayer versus multilayer biofilms and between cells grown at different growth rates in chemostat bioreactors. Biofilm cells were estimated to grow at specific growth rates (μ) averaging between μ=0.08-0.13 h^{-1}, which were approximately within the range of tested suspended growth conditions (μ=0.04-0.10 h^{-1}) using fluorescence in situ hybridizations targeting 16S rRNA. This result indicates that the reduced susceptibility of biofilm cells to monochloramine inactivation is not related to their specific growth rate within the range tested in this study. This work suggests that growth at suboptimal temperatures and growth in biofilms are important factors contributing to reduced susceptibility of bacteria to inactivation with monochloramine.

4.2 Introduction

Growth of bacteria is commonly observed in drinking water distribution systems (DWDS) despite the presence of disinfectant residual (LeChevallier et al. 1996; Falkinham et al. 2001), and is undesirable for a number of reasons. For example, the survival and growth of waterborne pathogens in DWDS constitutes a public health risk (Craun et al. 2006) and bacteria growing in biofilms can increase the rate of pipe corrosion (Beech and Sunner 2004). Several possible mechanisms governing decreased bacterial susceptibility to disinfection have been suggested, including mass transfer resistance and disinfectant consumption in biofilms (Stewart et al. 1996), protective ecological relationships (Berry et al. 2006), slow growth rate (Mah and O'Toole 2001), persister cell formation (Harrison et al. 2005), nutrient limitation (Roberts and Stewart 2004), and specific adaptive responses (Szomolay et al. 2005). It is also possible and likely that several mechanisms are acting in concert to confer decreased susceptibility to inactivation with disinfectants (Chambless et al. 2006).

Bacteria are subject to a wide range of growth conditions in DWDS because of heterogeneous microenvironments, the possibilities of suspended and biofilm growth modes, and varying levels of disinfectants and nutrients at different points in the pipe network. These different growth conditions influence the physiological state of microorganisms and may alter the level of bacterial susceptibility to disinfection. Monochloramine is increasingly being used by drinking water utilities as a disinfectant residual to prevent microbial growth in DWDS (Richardson 2003). However, the effect of physiological state on the inactivation kinetics of bacteria exposed to monochloramine only has been studied to a limited extent. For example, the role of specific growth rate in monochloramine inactivation is unknown, but specific growth rate has been shown to affect susceptibility of microorganisms to chlorine dioxide (Berg et al. 1982), thermal energy, UVA radiation, and solar radiation (Berney et al. 2006). Growth in biofilms may also be important to decreased susceptibility of bacteria to disinfection. Tachikawa et al. (Tachikawa et al. 2005) reported that *Pseudomonas aeruginosa* biofilm cells exposed to monochloramine are less sensitive to inactivation than suspended cells. The decreased susceptibility to monochloramine observed by Tachikawa et al. (Tachikawa et al. 2005)

may have been caused either by the shielding of attached cells from monochloramine by a protective biofilm matrix, or by the induction of a less susceptible phenotype within the biofilm. Therefore, it remains unclear whether cells grown in biofilms are less sensitive to inactivation to monochloramine because they exhibit a physiological profile different from that of suspended cells or because of other mechanisms.

The objective of the current study was to determine how diverse growth conditions affect inactivation of bacteria with monochloramine. Specifically, this study examined the effects of growth temperature, specific growth rate, and biofilm formation on *E. coli* inactivation with monochloramine.

4.3 Results and discussion

4.3.1 Selection of bacterial strain and design of culture medium

Escherichia coli K-12 MG 1655 was chosen as a model bacterium because its genome is well-studied, which facilitates further work to investigate the molecular mechanisms that decrease the organism's sensitivity to monochloramine under different growth conditions. The disadvantage of using this strain, as with all model organisms, is that it is not a drinking water isolate and thus may not be representative of the behavior of indigenous drinking water bacteria. The culture medium was designed to include commonly found organic compounds in water, and therefore included non-preferred and non-utilizable electron donors for *E. coli*. The chemical composition of the medium was based on studies of the organic chemical composition of natural surface and ground waters (Routh et al. 2001; Rosenstock and Simon 2003; Langwaldt et al. 2005) and drinking waters (Volk et al. 2005). The concentrations of organic substrates added were several orders of magnitude greater than levels in drinking water sources reported in the literature, which was necessary to stimulate sufficient microbial growth and obtain adequate biomass for inactivation experiments. Trace elements and macronutrients were added at concentrations determined to facilitate carbon-limited growth of *E. coli* under similar conditions (Egli 2000; Ihssen and Egli 2004).

4.3.2 Applicability of Delayed Chick-Watson model

The Delayed Chick-Watson model provided a good fit for the data from all tested conditions (Table 4.1). All tested conditions resulted in optimal *n* values between 1.15

and 1.22. When n was set at 1.2, R^2 values for each condition individually varied between 0.89 and 0.97. Values for n vary widely in the literature, but other researchers have found similar n values when testing *E. coli* inactivation with monochloramine at pH 8, including n = 1.19 (Butterfield and Wattie 1946) and 1.23 (Kaymak 2003). Variation in n values has been reported for monochloramine inactivation of different organisms and at different pH values (Haas and Karma 1984; Kaymak 2003). A rigorous validation of the applicability of a single n value to model bacterial inactivation with monochloramine would require testing of a wide range of disinfectant concentrations and exposure times, which was not undertaken because the goal of this study was not to validate a single n value, but to compare inactivation kinetics across growth states. The Delayed Chick-Watson model, optimized for the conditions tested in this study, proved to be an effective model for comparison of the inactivation kinetics for the experiments presented. Variation in n may be related to a combination of factors, including organism-specific physiology, inactivation conditions, pH, and nutrient availability in the growth media, so it is anticipated that any study testing a unique combination of these factors would produce a different optimized n value.

4.3.3 Effect of growth temperature for chemostat and biofilm cultures

Temperatures in DWDS are often suboptimal for growth of mesophilic microbes, with a typical range between 10-20 ºC (Geldreich 1996). However, inactivation kinetics studies often culture bacteria at optimal temperature conditions, for example 37 ºC for *E. coli*. Therefore, we examined the effect of growth temperature on inactivation kinetics (Figure 4.1). It is important to note that all inactivation experiments were conducted identically at 20 ºC, so the only difference in the experiments was the growth temperature preceding inactivation. The results showed that *E. coli* cells cultured at the optimal growth temperature of 37 ºC were significantly more sensitive to inactivation than when grown at a suboptimal growth temperature of 20 ºC, both in chemostat culture ($F(1,29) = 4.66$, $p = 0.04$ for $\mu=0.04$ $h^{-1}$and $F(1,25) = 4.63$, $p = 0.04$ for $\mu=0.10$ h^{-1}) and when grown in multilayer biofilms ($F(1,33) = 7.30$, $p = 0.01$). This suggests that bacteria in most DWDS are less sensitive to inactivation than expected based on inactivation experiments conducted at optimal temperature.

4.3.4 Effect of growth rate of suspended cultures for optimal and suboptimal temperatures

Specific growth rate has been suggested to be important to induction of stress response systems (Ihssen and Egli 2004) and in the susceptibility of microorganisms to inactivation with chlorine dioxide (Berg et al. 1982), thermal, UVA, and solar radiation (Berney et al. 2006). However, in the current study, specific growth rate was not observed to be related to inactivation kinetics for *E. coli* grown in chemostat reactors at μ=0.04 h^{-1} and μ=0.1 h^{-1}, at both optimal ($F(1,325) = 0.20$, $p = 0.66$) and suboptimal ($F(1,29) = 2.66$, $p = 0.11$) temperatures (Figure 4.2). Only a limited range of growth rates was evaluated because the maximum specific growth rate of suspended cells using the growth medium designed in this study was relatively low (μ= 0.15 h^{-1}). This is a low growth rate compared with the growth rates used in studies that observed inactivation kinetics to vary with specific growth rate (Berg et al. 1982; Berney et al. 2006). It is unlikely, however, that bacteria in DWDS would be able to achieve specific growth rates as high as those used in previous studies (up to μ= 1.0 h^{-1} (Berg et al. 1982; Berney et al. 2006)) because of the oligotrophic conditions and low temperatures in DWDS, so results from the current study may provide more practical implications for growth in DWDS.

4.3.5 Effect of growth mode: suspended vs. biofilm

Biofilm growth is commonly observed in DWDS and has been implicated as the primary source of microorganisms found in distributed drinking water (Berry et al. 2006). Tachikawa *et al.* (Tachikawa et al. 2005) found that biofilm cells were less sensitive to monochloramine inactivation than suspended cells. They performed their inactivation experiments with attached biofilm cells sheltered in an intact biofilm, leaving the possibility that the biofilm matrix could play a role in shielding the cells. While decreased susceptibility of biofilm cells due to inherent physiological differences is consistent with the theory of adaptive response of biofilm cells to antibiotics (Szomolay et al. 2005), we set out to verify this using monochloramine as the disinfectant in the current study. In our experiments, cells that had been grown in biofilm mode were detached and dispersed into single-cell suspensions before inactivation studies. The dispersion of biofilm cells was verified by microscopy for every experiment. The advantage of this method is that biofilm cells are exposed to inactivation conditions

similar to conditions experienced by suspended cells, minimizing the possibility of mass transfer resistance or chemical interactions with an intact biofilm matrix. We observed that biofilm-grown cells were significantly less sensitive to inactivation than cells grown in suspended cultures in chemostat (μ= 0.04 h^{-1}) at both optimal ($F(1,25) = 15.01$, $p < 0.0001$) and suboptimal ($F(1,37) = 96.62$, $p < 0.0001$) temperatures (Figure 4.3). This result suggests that growth in biofilm mode may induce a physiological state that is less sensitive to monochloramine inactivation regardless of whether the biofilm is intact at the time of inactivation. In addition, cells grown in developing biofilms, defined as biofilms with only a monolayer of cells, did not exhibit significantly different inactivation kinetics than cells grown in multilayer biofilms ($F(1,41) = 0.98$, $p = 0.33$). This result suggests that the decreased susceptibility of cells to monochloramine occurs in the initial stages of biofilm formation, and that the physiologically-based disinfectant susceptibility does not decrease during subsequent biofilm development. These results do not rule out the possibility that cellular production of extracellular polymeric substances (EPS) creates a protective barrier that remains intact after dispersion of the biofilm matrix. EPS has been observed to decrease susceptibility of *E. coli* to disinfection with chlorine (Ryu and Beuchat 2005) and solar-induced nanocatalysts (Liu et al. 2007), although it also has been observed to have no effect on *Klebsiella pneumonia* disinfection with free chlorine and monochloramine (LeChevallier et al. 1996). Experimental and theoretical evidence also suggest that monochloramine is not limited by mass transfer resistance through EPS in biofilms (Cochran et al. 2000). Further studies of the molecular mechanisms responsible for the decrease in susceptibility when biofilm growth is initiated are necessary.

4.3.6 FISH analysis of growth rate in biofilms

The specific growth rate of cells grown in biofilm mode was estimated by measuring the abundance of 16S rRNA molecules per cell with fluorescently-labeled oligonucleotide hybridization probes. Modulation of the cellular content of 16S rRNA with respect to growth rate is a well-documented phenomenon (Bremer and Dennis 1996), and some studies have used FISH to monitor cellular growth rate (Cangelosi and Brabant 1997; Licht et al. 1999; Oerther et al. 2000). Using 16S rRNA-targeted probes, we observed an increase in fluorescence intensity per pixel with respect to specific growth rate in all

chemostat-grown and batch-grown (log-phase) cultures of *E. coli*, indicating that the rRNA content per cell was higher for higher specific growth rates (Figure 4.4). This relationship was best modeled with an exponential regression (R^2=0.99). A linear regression did not explain as much variance (R^2=0.73). Bacterial rRNA content has been variously reported to increase both proportionally (Churchward et al. 1982; Poulsen et al. 1993) and exponentially (Schaechter et al. 1958) with respect to increasing growth rate. Differences in the shape of rRNA increase may arise from differences in organisms, culture conditions, growth rates tested, and methods used to measure and quantify rRNA.

Monolayer biofilms had slightly higher average fluorescence intensities than multilayer biofilm cells (9.7 ± 0.8 and 6.3 ± 0.1 arbitrary units per pixel (a.u./pixel), respectively). The total range of cellular fluorescence intensities for both conditions was between 3.3 and 11.8 a.u./pixel, which is within the range of the suspended growth rates tested and is approximately analogous to a range of cellular growth rates between μ= 0.08-0.13 h^{-1}. These data indicate that biofilm cells were growing at specific growth rates comparable to those of the suspended cells tested. This observation, coupled with our finding that the variation in specific growth rate in suspended cultures did not affect inactivation kinetics for the range of growth rates evaluated, suggests that growth in biofilm mode may induce a less sensitive physiological state that is not associated with growth rate-related changes.

4.4 Conclusion

In conclusion, monochloramine inactivation kinetics of *E. coli* cells grown at a variety of conditions could be described using the Delayed Chick-Watson model with n=1.2. The results demonstrated a decreased susceptibility to inactivation with monochloramine when grown in biofilm mode and at low temperatures. Unexpectedly, the specific growth rate within the range of growth rates evaluated was not found to be a parameter that influenced inactivation kinetics for conditions typical for DWDS.

4.5 Materials and methods

4.5.1 Bacterial strains and growth media

Escherichia coli K-12 MG 1655, obtained from the American Type Culture Collection (ATCC, Manassas, VA) (ATCC 700926) was used for all experiments. Stock cultures were preserved at -80 ºC in 15% glycerol and maintained on Luria Bertani (LB) agar

slants at 4 ºC. A carbon-limited synthetic medium was used for all experiments (2.88 g/L oxalic acid, 3.78 g/L succinic acid, 3.52 g/L pyruvic acid, 5.89 g/L glucose, 1.70 g/L L-(+)-arabinose, 1.70 g/L D-(+)-xylose, 2.05 g/L D-(+)-galactose, 1.25 g/L glycine, 1.75 g/L L-serine, 2.45 g/L L-glutamic acid, 14.33 g/L NH_4Cl, 2.49 g/L NaH_2PO_4, 4.02 g/L Na_2HPO_4, 1.52 g/L KBr, 2.22 g/L Na_2SO_4, 0.98 g/L $MgCl_2$, 2.77 g/L $CaCl_2$, 2.42 g/L $FeCl_3$*6(H_2O), 61 mg/L $MnCl_2$*4(H_20), 42 mg/L $ZnCl_2$, 5 mg/L $CuSO_4$, and 8 mg/L $CoCl_2$*6(H_20)). The medium design is discussed below (in "Selection of bacterial strain and design of culture medium").

4.5.2 Bioreactors

Chemostat bioreactors (two-liter glass reactors (Applikon, Schiedam, The Netherlands)) were used to culture organisms at a fixed specific growth rate by controlling the hydraulic residence time of the reactor. Bioreactor pH was maintained at 8.0 ± 0.1 via automated addition of 0.1 M NaOH and 0.1 M HCl. Continuous sparging with sterile air resulted in an oxygen saturation of greater than 90%. The stirrer speed was set at 800 r.p.m. and the temperature was controlled at 20 ºC or 37 ºC. Biomass was harvested after 8-10 volume changes to ensure that steady state conditions had been achieved (Sternberg et al. 1999; Ihssen and Egli 2004). Cell density was measured to determine steady-state and was measured by direct counts using cell counting chambers (Improved Neubauer, Hawksley, Lancing, England). Annular bioreactors (BioSurfaces Technologies, Bozeman, MT) were used to grow biofilms on removable glass coupons. Annular bioreactors were operated at 90 r.p.m. to simulate the shear force that is created in a pipeline by water flowing at a velocity of 0.3 m/s (Camper 2004). Monolayer and multilayer biofilms were harvested after 2 d and 14 d culturing, respectively, at 20 ºC. Using phase-contrast microscopy, it was determined that monolayer biofilms consisted of attached cells that had not yet formed micro-colonies, whereas multilayer biofilms exhibited complete surface coverage and contained multilayer biofilm structures. Cells were harvested from the glass coupons by scraping the coupons three times with a pre-sterilized 0.45-µm nitrocellulose membrane filter (Millipore Corp., Bedford, MA), followed by vortexing the filter in 15 ml of sterile phosphate-buffered saline (PBS). By quantifying the recovered cells and the cells remaining on the coupons using phase-contrast microscopy, this method was found to consistently recover over 99% of cells from the coupons.

Dispersion of biofilm cells into single-cell suspensions was verified with phase-contrast microscopy.

4.5.3 Inactivation conditions

All inactivation experiments were performed in 500-mL batch reactors at pH 8 ± 0.1, 20 ºC, and using 0.5 and 5.0 mg/L (as Cl_2) monochloramine. Monochloramine was prepared by adding sodium hypochlorite to a well-mixed buffered solution of excess ammonium chloride and was used immediately after preparation (Driedger et al. 2001). Glassware was washed with monochloramine prior to the experiments and a control reactor of monochloramine was used to ensure that the concentrations of monochloramine, determined using the DPD titrimetric method (Eaton et al. 1995), did not change significantly from the start to the end of each experiment. Cells from the bioreactors were harvested, pelleted, washed, and re-suspended in 0.01 M PBS (pH 8). Cells were then added to an inactivation reactor to yield a final concentration of approximately 10^8 colony forming units (CFU)/L. Samples were taken from the reactor at several time points between one and 200 min, and immediately transferred to a dilution bottle with 0.01 M PBS (pH 7.2) and 0.12% sodium thiosulfate pentahydrate to neutralize the monochloramine. All inactivation experiments were conducted in duplicate. A control reactor without any monochloramine was also operated for each experiment. The viability of the cells in the control reactors remained constant throughout the experiments (95% CI [0.975, 1.025]). Cells were recovered by filtering samples through pre-sterilized 0.45 µm nitrocellulose membrane filters (Millipore Corp., Bedford, MA). Filters were incubated at 37 ºC on LB plates in duplicate for 48 h and then enumerated. Membrane filtration was conducted in duplicate and the arithmetic average of the filtration replicates was used to calculate the viable cell number for each condition.

4.5.4 Fluorescence *in situ* hybridization (FISH) analysis

Cells for FISH analysis were cultivated in continuous culture in chemostat bioreactors, as described above. A fluorescently-labeled oligonucleotide hybridization probe (S-D-Bact-0338-a-A-18) was used to target the *E. coli* 16S rRNA, as described earlier (Amann et al. 1990; Oerther et al. 2000). The optimal fixation and hybridization conditions for this probe were previously determined (Amann et al. 1990). Images were taken using an epifluorescence microscope (Axioplan, Zeiss, Thornwood, NY) with a 100-W mercury

lamp, a 100× Plan Neofluar objective, and a digital camera (AxioCam, Zeiss, Thornwood, NY). An automated image analysis program was designed (Zhou et al. 2007), using Visilog 6 image analysis software (Noesis, Paris, France). The mean pixel intensity per cell was quantified and the mean background intensity was subtracted, resulting in a normalized intensity value. The average and standard error of the mean pixel intensity was determined for sample populations of at least 500 cells for each condition. The limit of detection was calculated as the mean background plus three times the standard deviation of the background.

4.5.5 Inactivation kinetic modeling and statistical analysis

The Delayed Chick-Watson inactivation kinetics model (Watson 1908; Driedger et al. 2001) (Equation 4.1) was applied to all inactivation data.

$$\ln\left(\frac{N}{N_0}\right) = \begin{cases} 1 & \text{if} \quad C^n t \le C^n t_{LAG} \\ -k(C^n t - C^n t_{LAG}) & \text{if} \quad C^n t > C^n t_{LAG} \end{cases} \tag{4.1}$$

In Equation (4.1), N_0 is the initial number of microorganisms, determined by sampling control inactivation reactors without monochloramine, N is the number of microorganisms surviving at time t (min), C is the concentration of monochloramine (mg/L as Cl_2), k is the inactivation rate constant, n is an empirical parameter that describes the relative importance of the concentration of disinfectant, and $C^n t_{LAG}$ accounts for the lag-phase before pseudo-first order inactivation begins. The parameter n was optimized to maximize the amount of variation in the data accounted for by the model. The Delayed Chick-Watson model was used because a lag-phase was observable for cells grown under some conditions tested. Analysis of covariance (ANCOVA) was performed with SPSS software (SPSS Version 16, SPSS Inc., Chicago, IL) to determine if there was a statistical difference in inactivation kinetics between different conditions.

4.5 Tables and figures

Table 4.1 Rate constants (k), lag constant ($C^{1.2}t_{LAG}$) and coefficient of determination (R^2) of the Delayed Chick-Watson inactivation model for each growth condition and with the fitting value, *n*=1.2, for every growth condition.

Growth Conditions			Delayed Chick-Watson Model Parameters		
Mode	**Specific Growth Rate, h^{-1}**	**Temperature, ºC**	**k, $(mg/L)^{-1.2}$- min^{-1}**	**$C^{1.2}t_{LAG}$, $(mg/L)^{1.2}$-min**	**R^2**
Suspended	0.04	20	0.157	0	0.94
Suspended	0.04	37	0.392	5.96	0.97
Suspended	0.10	20	0.204	4.52	0.92
Suspended	0.10	37	0.373	5.63	0.96
Monolayer Biofilm	Heterogeneous	20	0.077	0	0.89
Multilayer Biofilm	Heterogeneous	20	0.067	0	0.93
Multilayer Biofilm	Heterogeneous	37	0.147	3.21	0.94

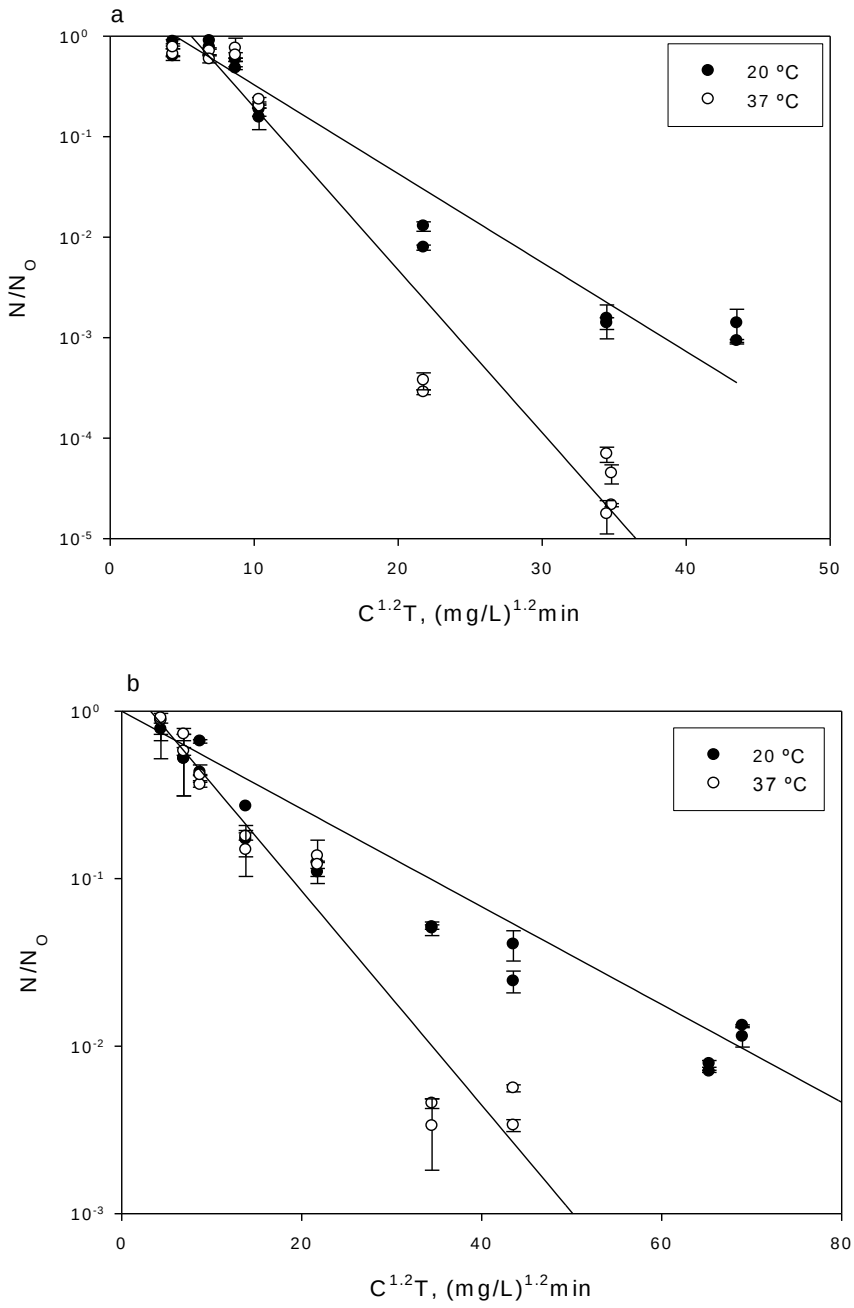

Figure 4.1 Effect of growth at 20 ºC and 37 ºC on inactivation kinetics of (a) *E. coli* cells grown in suspension at the same specific growth rate (0.10 h^{-1}), and (b) cells grown in a multilayer biofilm. Regression lines of each data series are fitted with the Delayed Chick-Watson inactivation model and error bars represent standard error of the mean measurement.

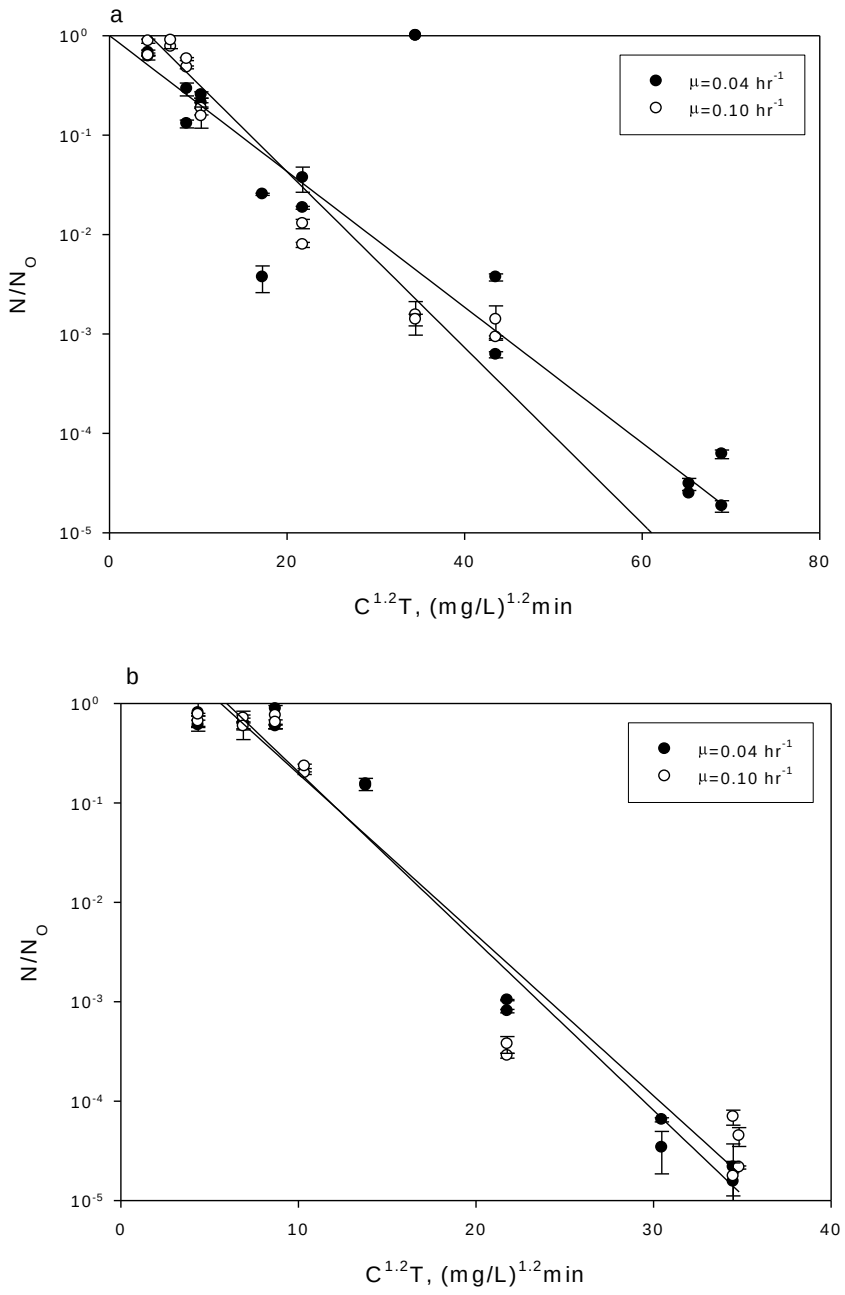

Figure 4.2 Inactivation kinetics of *E. coli* cells grown in suspension at specific growth rates of 0.10 h^{-1} and 0.04 h^{-1} (a) at suboptimal (20 ºC) ,and (b) optimal (37 ºC) temperatures. Regression lines of each data series are fitted with the Delayed Chick-Watson inactivation model and error bars represent standard error of the mean measurement.

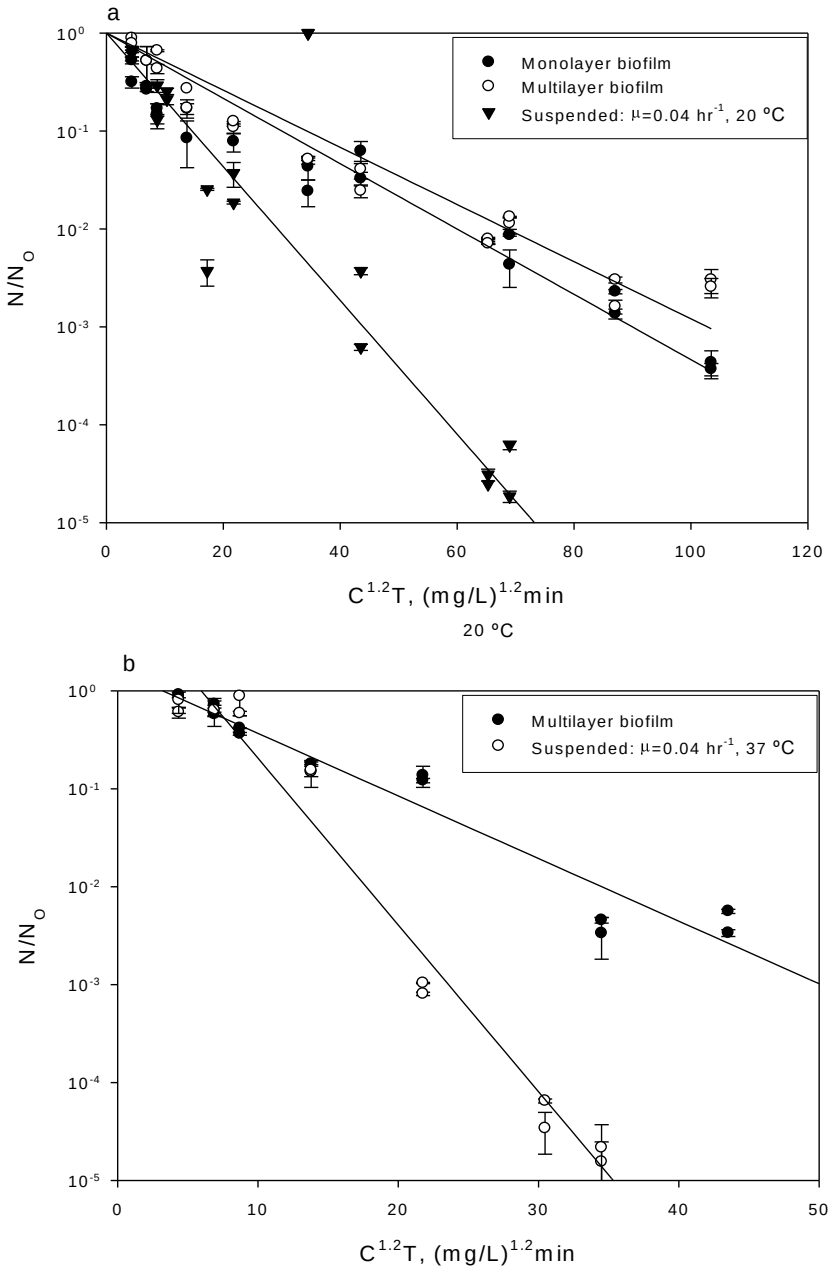

Figure 4.3 Inactivation kinetics of *E. coli* cells grown at (a) 20 ºC in monolayer and multilayer biofilms and suspended culture at a specific growth rate of 0.04 h^{-1}, and (b) 37 ºC in multilayer biofilm and suspended culture at a specific growth rate of 0.04 h^{-1}. Regression lines of each data series are fitted with the Delayed Chick-Watson inactivation model and error bars represent standard error of the mean measurement.

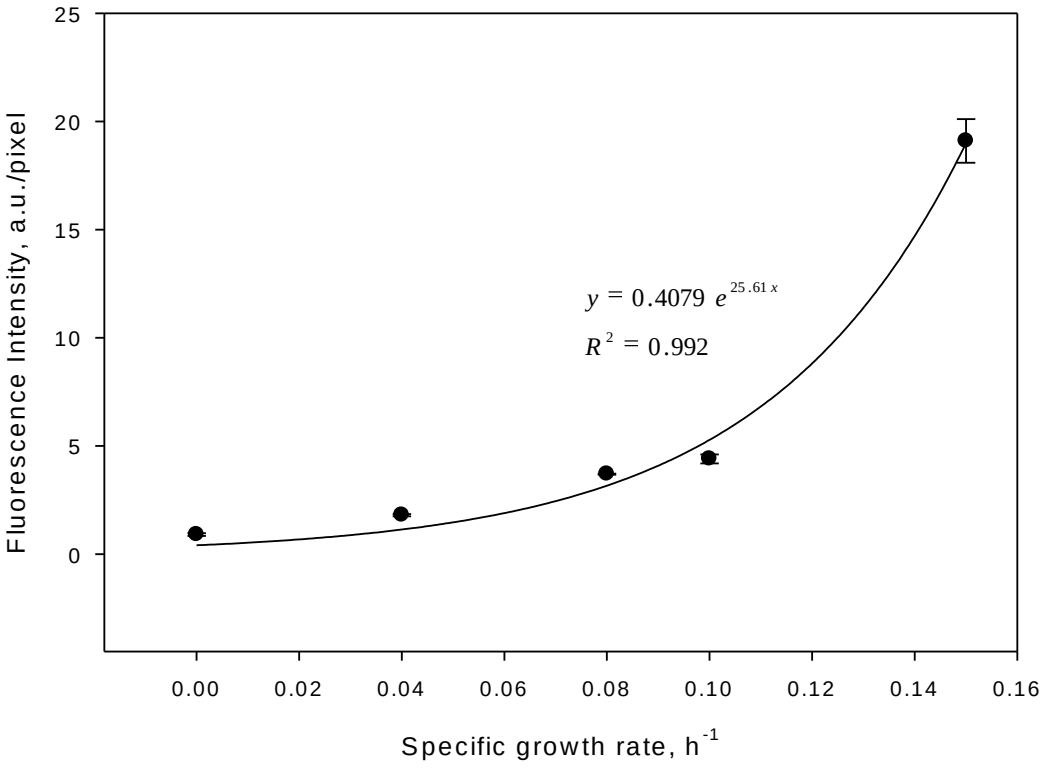

Figure 4.4 Relationship between fluorescence intensity (arbitrary units, a.u.) per pixel determined after FISH and specific growth rate of *E. coli* cells grown in continuous culture. The regression line indicates the fitted exponential equation and error bars represent standard error from at least 500 measurements.

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Chapter 5
Comparative transcriptomics of *Escherichia coli* exposed to the disinfectant monochloramine, cultured at a suboptimal growth temperature, and grown in biofilms

5.1 Abstract

Escherichia coli growth in biofilms and growth at a suboptimal temperature of 20 °C have been shown to increase resistance to inactivation with monochloramine (Berry, D., C. Xi, and L. Raskin. 2009. *Environ Sci Technol* 43:884–889). A comparative transcriptomic approach was used to identify genes that are differentially expressed under these growth conditions inducing monochloramine resistance and also during exposure to monochloramine. Microarray analysis revealed a common set of differentially-expressed genes. Specifically, the numbers of differentially expressed genes were 48 for monochloramine exposure and biofilm growth, 58 for monochloramine exposure and growth at 20 °C, and nine for all three conditions. Functional gene categories found to be important in response to tested growth conditions and exposure to monochloramine included: general metabolic inhibition, redox and oxidoreductase response, cell envelope integrity response, control of iron and sulfur transport metabolism and several genes of unknown function. Single gene deletion mutant analysis verified that loss of many of the upregulated genes increases *E. coli* sensitivity to monochloramine. Constitutive expression of downregulated genes in single gene mutants yielded mixed results for sensitivity to monochloramine, suggesting that stress responses might not be finely-tuned to optimal survival during monochloramine disinfection.

5.2 Introduction

Bacteria have developed a number of strategies for adapting to different environmental conditions. Knowledge about transcriptional activity is essential to developing a better

picture of cell physiology for environmentally relevant conditions (Martínez et al. 2003). Coliform survival in drinking water supply systems is a commonly observed problem despite the widespread practice of drinking water disinfection using oxidative disinfectants (e.g., free chlorine, chloramine) (Berry et al. 2006). Free chlorine (hypochlorous acid) is the best studied drinking water disinfectant (Small et al. 2006; Winter et al. 2008), but a comparative study of three broad-acting oxidants (peracetic acid, hypochlorous acid and hydrogen peroxide) suggest that that bacteria respond on a transcriptional level differently to different oxidants (Small et al. 2007).

Monochloramine is the second most commonly used disinfectant in US drinking water treatment facilities, after free chlorine (Rose et al. 2007). Despite the widespread use of monochloramine, its mode of action is not completely elucidated. Exposure of *E. coli* to levels of monochloramine typically used in drinking water inhibits bacterial transport, respiration, and substrate dehydrogenation, but does not severely damage the cell envelope or nucleic acid functioning (Jacangelo et al. 1991). To the best of our knowledge, only two other studies have examined global gene expression of bacteria to monochloramine exposure over time (Holder et al. 2009; Berry et al. In preparation). Holder et al. (2009) observed monochloramine exposure of *E. coli* induced genes involved in responses to other oxidants, biofilm formation genes, and genes important to survival within host cells (Holder et al. 2009). Berry et al. (2009) found that exposing *Mycobacterium avium* to monochloramine resulted in upregulation of oxidative stress (oxyR) response and virulence-associated genes (Berry et al. In preparation).

It is clear that the sensitivity of bacteria to inactivation with disinfectants is greatly influenced by growth conditions. This is particularly important in drinking water, which provides an oligotrophic, low temperature environment. For example, (Stewart and Olson 1992) observed that growth under reduced nutrient conditions reduced the effectiveness of monochloramine in oxidizing sulfhydryl groups in *Klebsiella pneumonia*. Growth of *E. coli* at suboptimal temperatures has been observed to decrease its sensitivity to chlorine dioxide (at 15 °C) (Berg et al. 1982) and monochloramine (at 20 °C) (Berry et al. 2009). Additionally, biofilms have been implicated as one of the primary sources of bacteria in drinking water distribution systems (Berry et al. 2006), and biofilm growth

decreases monochloramine sensitivity of both *E. coli* (Berry et al. 2009) and P. aeruginosa (Tachikawa et al. 2005) compared to planktonic growth.

Since widely varying stress factors appear to result in a substantial amount of common differential gene expression, non-specific transcriptional responses have been suggested to be important (López-Maury et al. 2008). The present study examines the global gene expression of *E. coli* during exposure to a low dose of monochloramine to determine if such general response systems are expressed during disinfection with monochloramine. Furthermore, this study evaluates the commonalities between the stress responses of *E. coli* exposed to monochloramine, cultured at a suboptimal temperature, and in biofilms. This was achieved by comparing the transcriptional profiles obtained for growth under these three conditions. Genes that were differentially-expressed during monochloramine exposure and during either one or both of the other growth conditions were further examined for their importance in monochloramine sensitivity and analyzed using functional annotation clustering as well as broad functionality. The goal of this analysis is to identify and characterize a set of genes may allow cells grown at suboptimal temperatures and in biofilms to be less sensitive to inactivation with monochloramine.

5.3 Results

5.3.1 Growth conditions and gene expression profiles for individual conditions

A chemostat culture of *E. coli* K-12 grown at 37 °C at a specific growth rate, μ, of 0.1 h^{-1} was used as a control for all microarray experiments. The global gene expression of control cells was compared to gene expression profiles of cells for three different treatment conditions: (i) cells grown at 37 ^{o}C were exposed to monochloramine, (ii) cells were grown in continuous culture at a suboptimal temperature (20 °C), and (iii) cells were grown in biofilms at 37 °C (Table 5.1). Differential-expression was defined as greater than twofold difference in expression levels and $P<0.05$. A chemostat culture of *E. coli* grown at $\mu = 0.1$ h^{-1} and 37 °C was exposed to a 1.0 mg/L (as Cl_2) dose of the disinfectant monochloramine for 15 minutes at 20 °C. Control cells were exposed to PBS under the same conditions. Monochloramine exposure resulted in at least twofold differential expression of 364 genes (88 upregulated and 276 downregulated) (Table 5.S1, Figure 5.1). Microarray analysis of a chemostat culture of *E. coli* grown at $\mu = 0.1$

h^{-1} and 20 °C resulted in differential expression of 214 genes (79 upregulated and 135 downregulated) in comparison to growth at 37 °C (Table 5.S2). Growth of cells in biofilms formed on glass slides and exposed to a continuous shear force (equivalent to a 1 ft/s water velocity through a 6" diameter pipe) for 14 days yielded 982 differentially-expressed genes (320 upregulated and 662 downregulated) as compared to the suspended chemostat culture (Table 5.S3).

5.3.2 Common sets of differentially-expressed genes

Gene expression profiles from the individual conditions were screened for a common set of differentially-expressed genes. The criterion for inclusion of a gene in a common gene set was differential expression in the same direction (i.e. upregulated or downregulated) for compared conditions. This approach was used in order to identify a common gene expression profile for cells exposed to monochloramine and cells grown either at suboptimal temperature (20 °C) or in biofilms, since cells grown under these conditions have previously been observed to show decreased sensitivity to monochloramine (Berry et al., 2009).

Cells grown in biofilms and cells exposed to monochloramine shared 48 expressed genes (22 upregulated and 26 downregulated) (Figure 5.1 and Table 5.2). Of these, 16 genes were related to metabolism (10 upregulated and 6 downregulated) and 18 were either poorly characterized or of unknown function. Cells exposed to monochloramine additionally showed downregulation of eight motility genes.

The comparison of gene expression profiles for cells exposed to monochloramine and cells grown in suspension at 20 °C without monochloramine exposure resulted in a common set of 58 genes (7 upregulated and 51 downregulated). Of these 58 genes, 22 were related to metabolism (2 upregulated and 20 downregulated). Genes that can be assigned to information storage and processing (transcription and translation) were downregulated (7 genes). Cellular processes and signaling genes were almost all downregulated (5 of 6 genes), with the exception of *ampE*. Additionally, 23 of these genes were poorly characterized or of unknown function (4 upregulated and 19 downregulated).

When comparing gene expression profiles of all three conditions, a common expression profile of only nine genes (4 upregulated and 5 downregulated) was observed. Interestingly, all nine genes are either known or putative membrane proteins, according to NCBI COG annotations. Of these nine, two genes encoding cell wall permeability enzymes were down-regulated (*acrE*, a multi-drug efflux system gene, and *mtr*, which codes for an amino acid permease), while two membrane proteins were upregulated (*hemD*, which produces a heme biosysnthesis protein, and *ampE*, which is involved in defense mechanisms). The other five genes are not adequately characterized for functional importance.

5.3.3 Monochloramine sensitivity of *E. coli* lacking genes upregulated in common gene expression profiles

Of the 25 genes that were upregulated both during monochloramine exposure and either biofilm growth or growth at 20 °C, single gene deletion mutants of 15 of these genes produced a statistically significant increase in monochloramine sensitivity (Table 5.3). The remaining gene deletion mutants showed no statistically difference in monochloramine sensitivity as compared to wild-type cells.

5.3.4 Monochloramine sensitivity of *E. coli* constitutively expressing genes downregulated in common gene expression profiles

Of the 72 genes that were downregulated during monochloramine exposure and either biofilm growth or growth at 20 °C, the effect of constitutive expression of the downregulated gene on monochloramine sensitivity of the *E. coli* mutant was tested for all but two (*yicM* and *yihA*). Constitutive expression of 30 of these genes increased monochloramine sensitivity and 22 did not significantly affect sensitivity. Surprisingly, constitutive expression of 19 of the genes decreased *E. coli* sensitivity to monochloramine.

5.3.4 GFP-promoter confirmation of expression of selected genes

The expression of selected genes upregulated in the microarray data was confirmed using *E. coli* strains with GFP-promoter fusion of *cysQ*, *ampDE*, or *hemCD*, and exposed to a dose of 0.5 mg/L monochloramine for 30 min. All three strains had elevated signals by

30 min exposure, and the relative increase in expression was statistically significant ($P<0.05$) for *cysQ* at 5.6 min and *ampDE* and *hemCD* at 11.1 min (Figure 5.2).

5.4 Discussion

5.4.1 Metabolic inhibition

During monochloramine exposure, *E. coli* drastically downregulated general metabolic pathways, including downregulation of genes related to carbohydrate transport and metabolism (19 out of 20 differentially-expressed), energy conversion and production (17 out of 21 differentially-expressed), nucleotide transport and metabolism (9 out of 9 differentially-expressed), and cell motility (8 out of 8 differentially-expressed). Decreased respiration has been observed for *E. coli* exposed both to monochloramine (Jacangelo et al., 1991) and hypochlorous acid (Albrich and Hurst, 1982). Downregulation of metabolic genes, and in particular permeases and transport genes, has also been observed during acid shock in *E. coli* (Maurer et al., 2005) and *Lactobacillus* (Azcarate-Peril et al., 2005) and during hypochlorous acid exposure of *Pseudomonas aeruginosa* (Small et al., 2007). When exposed to hypochlorous acid, *P. aeruginosa* downregulated genes encoding membrane proteins, particularly genes related to permeases and transporters of carbohydrates and catabolites (Small et al., 2006). In this study, a significant downregulation of several classes of permeases was observed both during monochloramine exposure and during either low temperature or biofilm growth. Constitutive expression of many of these permeases increased susceptibility of the cells, including permeases involved in carbohydrate transport (*yegB, proP*), amino acid transport (*mtr*, and ABC-type transporters *gltL* and *potF*), and nucleotide transport (*yicE*) (Table 5.3).

Post-transcriptional inhibition of metabolic systems may also be an important mechanism in monochloramine resistance of cells cultured under environmentally-relevant conditions. Cold shock genes *cspCG* were up-regulated under biofilm growth. CspC is believed to be an RNA chaperone that acts as a transcription antiterminator (Weonhye et al., 2000) and CspG is known to block protein synthesis (Etchegaray and Inouye, 1999) that had previously been observed to be induced during biofilm growth (Domka et al., 2007). The response of *E. coli* to low-temperature growth previously identified *cspCG*

induction (White-Ziegler et al., 2008), though this was not observed in the current study. While cold shock genes were not upregulated during low temperature growth, the gene *yfiA* was upregulated, which is a translation inhibitor and ribosome stability factor observed in stationary-phase cultures that binds to *rpoH* (Agafonov et al., 2001; Vila-Sanjurjo et al., 2004). Another mRNA stabilizing gene, *chpR*, which stabilizes mRNA transcripts by counteracting *chpF* (Aizenman et al., 1996) was also upregulated during low temperature growth and monochloramine exposure.

5.4.2 Role of cellular redox couples

Maintaining the redox state of the cell is an important challenge for microorganisms facing oxidative stress. The major redox couples in proteobacterial cell are the glutathione disulfide/glutathione couple, the $NADP^+$/NADPH couple, and the thioredoxin system (Schafer and Buettner, 2001). Thioredoxin reductase (*trxB*) was up-regulated during monochloramine exposure and during biofilm growth in this study and gene deletion mutants in *trxB* had increased sensitivity to monochloramine.

Several studies have demonstrated the importance of *trxB* in response oxidative stress, such as to hydrogen peroxide (Takemoto et al., 1998), and thioredoxin reductase has been considered as an important drug target (Becker et al., 2000) because of its protective role during infection. A gene expression study of *Lactobacillus* over-expressing thioredoxin reductase found upregulation of purine and pyrimidine biosynthesis, stress-response (including groEL), cysteine amino acid metabolism, and genes involved in the cellular envelope (Serrano et al., 2007). In the current study, we observed similar results during monochloramine exposure as well as during growth conditions that decrease sensitivity to monochloramine, meaning upregulation and increased monochloramine sensitivity of single gene deletion mutants in genes related to purine ribonucleoside transport (*yicM*), iron acquisition (*fhuD*) (data from monochloramine exposure alone showed up-regulation of *fhuBCD*), sulfur metabolism (*ybbC*), iron-sulfur assembly (*ynhA*), cysteine metabolism (*cysQ*), general stress (*htpG*), and cell envelope stress (*ybgF*). Also, downregulation of *sugE*, a suppressor of groEL, was observed. Constitutive expression of *sugE* increased monochloramine sensitivity (Table 5.3). Downregulation of *yheM*, which is responsible for oxidation of sulfur (Ikeuchi et al., 2006), was observed and constitutive expression of

yheM also increased *E. coli* sensitivity to monochloramine. The flavodoxin *fldB* was upregulated under all conditions, and while its role in oxidative stress response is not completely elucidated (Gaudu and Weiss, 2000) it is likely that it is involved in maintaining the reduced state of iron-sulfur clusters (Storz and Zheng, 2000).

The gene *kdpE* was upregulated during monochloramine exposure and biofilm growth. *kdpE* is part of the *kdpDE* 2-component regulatory system, which has been identified as important for potassium regulation as well as response to osmotic stress (Heermann et al., 2003). A mutation in *trxB* has also been shown to reduce *kdp* expression (Sardesai and Gowrishankar, 2001), which may indicate overlap in response systems. In *S. aureus*, intracellular microbiocides (H_2O_2 and HOCl) induced *kdpDE* and iron uptake, and elimination of iron uptake systems increased sensitivity of mutants (Palazzolo-Ballance et al., 2008).

5.4.3 Upregulated genes with differential sensitivity to monochloramine

Of the 15 genes that were upregulated during monochloramine exposure and either during growth in biofilms or at 20 °C and whose deletion led to increased cell sensitivity to monochloramine, most are known or suspected to be involved in iron and sulfur regulation, membrane integrity, or redox control. Several genes are involved in sulfur and iron regulation and Fe-S biosynthesis: *cysQ* is involved in sulfate assimilation (Neuwald et al., 1992); *ybbC* is a predicted protein that is believed to be part of a transcriptional unit with *rhsD*, which is induced during sulfate starvation (van der Ploeg et al., 1996); *fhuD* is involved in iron transport (Burkhardt and Braun, 1987); and *ynhA* (*sufE*) encodes a sulfur acceptor protein involved in Fe-S cluster assembly (Loiseau et al., 2003). *ybgF* is a predicted protein that may be in a transcriptional unit with *tolB* and *pal* (Vianney et al., 1996), so could be involved in membrane integrity via the Tol-Pal Cell Envelope Complex (Walburger et al., 2002). Mutants in *tol-pal* system have increased sensitivity to drugs and detergents (Davies and Reeves, 1975; Cascales et al., 2000). *htpG* is part of the HSP90 protein family and is a chaperone in protein refolding (Bardwell and Craig, 1987; Thomas and Baneyx, 2000). It is involved in response to heat shock (Heitzer et al., 1992) and low pH stress (Heyde and Portalier, 1990). Two of the genes (*trxB* and *fldB*) have oxidoreductase activity, as discussed above. *kdpE* is a DNA-

binding transcriptional regulator, and was also discussed above. *livK* is involved in leucine transport (Adams et al., 1990). *ampE* encodes a membrane-bound protein that may play a role as a sensor in beta-lactamase induction, although its role is not well-understood (Bennett and Chopra, 1993). Three genes have unknown function (*yigG, yjgL, yjhB*).

5.4.4 Downregulated genes with differential sensitivity to monochloramine

The effect of constitutive expression of genes that were downregulated during monochloramine exposure and either biofilm growth or growth at 20 °C were to determine whether down-regulation was important for reducing monochloramine sensitivity. Constitutive expression of about 40% of these genes did increase monochloramine sensitivity and about 30% did not significantly affect sensitivity. Interestingly, constitutive expression of about 30% of these genes actually decreased sensitivity to monochloramine, suggesting that the transcriptional response to monochloramine involves stress protection mechanisms that are not specific to survival during monochloramine stress. Different stresses can lead to induction of a conserved set of genes (Small et al., 2007) and proteins (Blom et al., 1992) and gene expression networks are complicated by cross-talk in signal transduction pathways (Cooper et al., 2005) and stochastic noise in gene regulation (Elowitz et al., 2002; Raser and O'Shea, 2005). Given the complexity of gene regulation, it is not surprising that the overexpression of some genes that are downregulated during monochloramine exposure may confer an advantage to the cell.

5.5 Conclusion

Comparative transcriptomic analysis revealed that *E. coli* exposed to monochloramine and *E. coli* grown in conditions that decrease monochloramine sensitivity share a transcriptional fingerprint characterized by general metabolic inhibition, redox and oxidoreductase response, cell envelope integrity response, control of iron and sulfur transport metabolism and several genes of unknown function. There is extensive overlap between differential gene expression observed in the current study and response to other stress factors, such as other broad-acting oxidants, heat shock, cold shock, acid shock, and osmotic shock. The role of some genes identified in this study in conferring

resistance to monochloramine is not well understood, and therefore this research is a useful step for further elucidation of molecular mechanisms of resistance to monochloramine inactivation.

5.6 Materials and methods

5.6.1 Bacterial strains and culture conditions

Escherichia coli K-12 MG 1655 (American Type Culture Collection, ATCC 700926) was maintained on Luria-Bertani (LB) agar and was used for all microarray experiments. Chemostat bioreactors (two-liter glass reactors (Applikon, Schiedam, The Netherlands)) were used to culture organisms at 37 ºC in 1:10 LB broth at a specific growth rate (μ) of 0.1 h^{-1} using a stirrer speed of 200 rpm. Cell concentrations in the chemostat bioreactors were monitored via total cell counts using a cell counting chamber (Improved Neubauer, Hawksley, Lancing, England) and biomass was harvested after steady state was achieved, typically after 8-10 volume changes. Annular bioreactors (BioSurfaces Technologies, Bozeman, MT) were used to grow biofilms on removable glass coupons. Annular bioreactors were operated at 90 rpm at 37 ºC and biofilms were harvested after 14 days. Cells were harvested from the glass coupons by scraping the coupons three times with a pre-sterilized 0.45-μm nitrocellulose membrane filter (Millipore Corp., Bedford, MA), followed by vortexing the filter in 15 ml of sterile phosphate-buffered saline (PBS), as described previously (Berry et al., 2009). *E. coli* K-12 single gene deletion mutants from the Keio Collection (Baba et al., 2006) were grown in LB amended with 25 μg/ml of kanamycin. Strains carrying plasmids with IPTG-inducible constitutive expression of single genes from of the AKSA library (Kitagawa et al., 2005) were grown in LB with 25 μg/ml chloramphenicol, and 0.1 mM isopropyl-ß-D-thiogalactoside (IPTG) (Sigma-Aldrich, St. Louis, Mo) was added to induce over-expression of the single cloned gene. The mutants and the wild-type strain BW25113 were kindly provided from National Institute of Genetics, Mishima.

5.6.2 Monochloramine preparation and exposure

Monochloramine was prepared by adding sodium hypochlorite to a well-mixed buffered solution of excess ammonium chloride and was used immediately after preparation (Driedger et al., 2001). The concentration of monochloramine was determined using the

DPD titrimetric method (Eaton et al., 1995). Monochloramine exposure experiments for microarray analysis were performed at pH 8 ± 0.1, 20 ºC, using 1.0 mg/L (as Cl_2) monochloramine. Cells from the bioreactors were harvested, pelleted, washed, and re-suspended in 0.01 M phosphate buffered saline (PBS) (pH 8). Cells were added to a monochloramine exposure reactor to yield a final concentration of approximately 10^9 colony forming units (CFU)/mL and exposed to monochloramine for 15 min, which has previously been shown to be a sub-lethal exposure time for this monochloramine dose (Berry et al., 2009). A 0.12% solution of sodium thiosulfate pentahydrate was added to neutralize the monochloramine and RNAlater (Ambion, Austin, TX) was added to stop mRNA generation and decay. Control experiments followed the same procedure using PBS in lieu of monochloramine.

5.6.3 RNA isolation and purification

Cells preserved in RNAlater were pelleted and re-suspended in 0.75 ml boiling lysis solution (2% SDS, 16mM EDTA, 200mM NaCl). Lysis solution was transferred to 2 ml RNase-free polypropylene tubes containing 0.5 g of 0.1 mm diameter zirconia/silica beads (BioSpec Products, Inc., Bartlesville, OK) and incubated for 5 min at 100 ºC with periodic mixing. 0.75 ml of 65 ºC phenol:chloroform, pH 4.5 (with IAA 125:24:1) (Ambion, Austin, TX), was added and tubes were incubated for 5 min at 65 ºC with periodic mixing. Cells were then homogenized for 2 min (Mini-Beadbeater-96, BioSpec Products, Inc., Bartlesville, OK) and incubated for 5 min at 65 ºC with periodic mixing. The tubes were centrifuged at 16,000 x *g* for 20 min at 4 ºC and the upper aqueous phase was transferred to a 2 ml phase-lock-gel tube (PLG Heavy, 5 PRIME Inc., Gaithersburg, MD). 0.75 ml of phenol:chloroform was added and tubes were mixed by inversion and centrifuged at 14,000 x *g* for 5 min at room temperature (RT) to separate the phases. The aqueous phase was decanted into another phase lock gel tube and 0.75 ml chloroform:isoamyl alcohol (24:1) was added and tubes were mixed by inversion and centrifuged at 14,000 x *g* for 5 min at RT. The aqueous phase was transferred to a new tube and 0.75 ml isopropanol was added. Tubes were mixed and incubated at -20 ºC for 2 h. Next, tubes were centrifuged at 16,000 x *g* for 30 min at 4 ºC. The supernatant was decanted and nucleic acids were re-suspended in 90 µl RNase-free water. DNA was digested using 8 U TurboDNase (Ambion, Austin, TX) and a 30 min incubation at 37 ºC.

RNA was purified using the RNAeasy kit (Qiagen, Valencia, CA), followed by addition of 0.3 M sodium acetate and 2.5 volumes ethanol and incubation at -20 ºC for 2 h. RNA was re-suspended in RNase-free water and purity was determined spectrophotometrically using 260 nm/280 nm absorbance ratios (Nanodrop ND-1000, Nanodrop Technologies, Wilmington, DE) and lack of degradation was confirmed by visualization of intact rRNA via polyacrylamide gel electrophoresis.

5.6.4 cDNA synthesis and labeling

cDNA was synthesized from 20 µg of total RNA using random hexamer primers and was subsequently labeled with Cy3 or Cy5 dyes (Amersham Biosciences, Piscataway, NJ) with the Amino-Allyl indirect labeling kit according to the manufacturer's instructions (Ambion, Austin, TX). The efficiency of the labeling procedure was assessed with a Nanodrop ND-1000 spectrophotometer. The labeled cDNA was used immediately for microarray hybridizations.

5.6.5 Microarray hybridization

E. coli K-12 whole genome expression microarray slides were produced by the University of Alberta, Department of Biological Sciences Microarray and Proteomics facility. Each microarray targets 4,289 ORFs in *E. coli* K-12 (as well as additional ORFs in *E. coli* O157:H7 EDL933 and Sakai strains) using 70mer probes spotted in triplicate. Slides were pre-hybridized at 42 ºC for 4 h in pre-hybridization solution (5X SSC, 0.1% (w/v) SDS, 1 mg/ml BSA) followed by three 5 min washes in 0.1X SSC and one 30 s wash in MilliQ water and drying by centrifugation at 1600 x *g* for 2 min. Hybridization and post-hybridization procedures were carried out according to the slide manufacturer's instructions (Corning Epoxide Coated Slides, Corning Inc., Acton, MA). Biological replicates and technical triplicates were performed for each condition and dye-swap controls were conducted to minimize dye bias.

5.6.6 DNA microarray data analysis

Arrays were scanned using an Axon model 4000 scanner (Molecular Devices Corporation, Union City, CA) and images were processed using SpotFinder software (TIGR, Boston, MA). Spots with integrated signal intensities in both channels greater than the mean background intensity and one standard deviation of background intensity

were included in downstream analysis. Locally-weighted polynomial regression (LOWESS) normalization, centering, and scaling to control for biases in inter-slide variance was performed using Acuity 3.1 software (MDS Analytical Technologies, Sunnyvale, California). Genes that had at least twofold change and with P-values < 0.05 were considered differentially expressed. Genes were annotated according to functional class using the National Center for Biotechnology (NCBI) Cluster of Orthologous Groups of proteins (COG) database (Tatusov et al., 2000; Tatusov et al., 2001).

5.6.7 Monochloramine sensitivity assays

Monochloramine sensitivities of single gene mutants and wild-type *E. coli* were determined by exposing exponentially grown cells at a dilution 10^3 cells per well suspended in pH 8.0 PBS in a multi-well plate to varying concentrations of monochloramine (between 0.2 and 5 mg/L as Cl_2) for 30 minutes. At the end of the monochloramine exposure, an equal volume of 2X LB amended with appropriate antibiotics was added to quench the monochloramine and provide medium for recovery. IPTG was also added to the recovery medium to induce over-expression in single gene mutant strains. Cells were incubated for 24 hours at 37 °C and 100 rpm shaking. After the 24 hour recovery period, optical absorbance was measured with a microplate spectrophotometer (Synergy HT Multi-Mode Microplate Reader, BioTek Instruments, Inc., Winooski, VT) and an optical density of less than 0.1 at 600 nm was used as a threshold to indicate growth inhibition. Data from sensitivity assays are presented as a fold-change in sensitivity between single-gene mutants and wild-type cells to normalize for variations in wild-type sensitivity of different *E. coli* strains. Experiments were conducted in triplicate and standard deviations of measurements were determined by evaluating the uncertainty generated by the resolution of the assay, as described previously (Cordero et al., 2006). The statistical significance of the results was evaluated using the two-sample T test and is presented as 95% CI.

5.6.8 Confirmation of gene expression during monochloramine exposure with GFP-promoter fusions

E. coli strains with GFP-promoter fusions (Zaslaver et al., 2006) were tested for promoter activity during monochloramine exposure as described previously (Holder et al., 2009). Briefly, cells were washed and re-suspended in PBS and exposed to 0.5 mg/L (as Cl_2)

monochloramine. Fluorescence (at 530nm) was monitored for monochloramine-treated and non-treated cells (Synergy HT, BioTek Instruments Inc., Winooski, VT) to determine the changes in fluorescence due to treatment with monochloramine. Fluorescence measurements were normalized by cell concentration (OD630) and then normalized to the baseline fluorescence readings before the start of the experiment.

5.7 Tables and figures

Table 5.1 Treatment conditions and growth properties.

Treatment	Treatment Conditions	Control Conditions
Monochloramine exposure of continuous culture ($\mu = 0.1\ h^{-1}$, 37°C)	1 mg/L (as Cl_2) NH_2Cl for 15 min at 20 °C	PBS for 15 min at 20 °C
20 °C growth	Continuous culture ($\mu = 0.1\ h^{-1}$, 20 °C)	Continuous culture ($\mu = 0.1\ h^{-1}$, 37 °C)
Biofilm growth	14 d biofilm on glass slides at 37 °C	Continuous culture ($\mu = 0.1\ h^{-1}$, 37 °C)

Table 5.2 Numbers of differentially-expressed genes per NCBI COG functional category for each condition (≥ 2.0 fold or ≤ 0.5 fold, P < 0.05). The numbers of differentially-expressed genes are expressed as: (# upregulated / # downregulated). Abbreviations are used for monochloramine exposure (Mono), biofilm growth (BF), and growth at 20 °C (20 °C). Common sets of differentially-expressed genes are listed according to the expression profiles compared, as indicated by a "+".

COG Category	COG Group	Mono	BF	20 °C	Mono + BF	Mono +20°C	Mono +BF +20°C
Cellular Processes and Signalling	Cell motility	0 / 8	6 / 12	1 / 0	0 / 2	0 / 0	0 / 0
	Cell wall/membrane/envelope biogenesis	6 / 14	21 / 33	4 / 9	0 / 1	0 / 4	0 / 1
	Defense mechanisms	1 / 1	4 / 8	2 / 2	1 / 0	1 / 0	1 / 0
	Intracellular trafficking and secretion	1 / 0	1 / 2	0 / 1	0 / 1	0 / 0	0 / 0
	Posttranslational modification, protein turnover, chaperones	5 / 15	7 / 25	3 / 1	2 / 2	0 / 0	0 / 0
	Signal transduction mechanisms	3 / 5	15 / 17	3 / 3	2 / 0	0 / 1	0 / 0
	Cell division and chromosome partitioning	0 / 0	1 / 7	0 / 0	0 / 0	0 / 0	0 / 0
Information Storage and Processing	Replication, recombination and repair	1 / 8	11 / 24	2 / 3	1 / 1	0 / 1	0 / 0
	Transcription	6 / 9	20 / 30	3 / 6	0 / 1	0 / 4	0 / 0
	Translation, ribosomal structure and biogenesis	3 / 13	14 / 22	2 / 3	0 / 0	0 / 2	0 / 0
Metabolism	Amino acid transport and metabolism	10 / 21	18 / 48	5 / 8	1 / 2	0 / 6	0 / 1
	Carbohydrate	1 / 19	11 / 40	5 / 7	1 / 2	0 / 2	0 / 0

	transport and metabolism						
	Coenzyme transport and metabolism	4 / 6	10 / 21	2 / 3	2 / 0	1 / 2	1 / 0
	Energy production and conversion	4 / 17	18 / 44	3 / 10	2 / 1	1 / 3	0 / 0
	Inorganic ion transport and metabolism	7 / 15	16 / 53	2 / 10	3 / 0	0 / 4	0 / 0
	Lipid transport and metabolism	2 / 5	7 / 8	0 / 1	1 / 1	0 / 0	0 / 0
	Nucleotide transport and metabolism	0 / 9	9 / 8	2 / 3	0 / 0	0 / 2	0 / 0
	Secondary metabolites biosynthesis, transport and catabolism	2 / 3	3 / 6	1 / 4	0 / 0	0 / 1	0 / 0
Poorly Characterized	Function unknown	1 / 14	18 / 41	5 / 12	0 / 2	2 / 3	0 / 0
	General function prediction only	7 / 19	23 / 64	5 / 10	0 / 3	0 / 0	0 / 0
Not in COGs	Not in COGs	22 / 75	85 / 157	25 / 38	6 / 7	2 / 16	2 / 3

Table 5.3 Genes expressed in more than one condition and sensitivity of mutant strains to monochloramine. Conditions tested were monochloramine exposure (mono), biofilm growth (BF) and growth at 20 °C (20 °C). Sensitivity to monochloramine is presented as 95% CI of fold-change between sensitivity of mutants and wild-type strains. The two bracketed numbers indicate the lower and upper bounds of the estimated change in sensitivity with 95% confidence. Statistically significant differences in sensitivity are denoted in bold.

Gene Name	Blattner No.	Differential expression (log2) under each condition			95% Confidence Interval for Fold-change in Sensitivity	
		Mono	BF	20 °C	Single Gene Deletion	Single Gene Over-expression
acrE	b3265	0.42	0.44	0.46		[0.54 , 1.46]
ais	b2252	2.36	4.33	2.64	[0.54 , 1.46]	
ampE	b0111	2.54	25.33	2.95	**[1.04 , 1.96]**	
brnQ	b0401	0.46	<2 fold	0.44		[0.54 , 1.46]
cchA	b2457	0.39	<2 fold	0.41		[1.54 , 2.46]
ccmD	b2198	0.32	0.18	<2 fold		[1.54 , 2.46]
chpR	b2783	0.19	<2 fold	0.50		**[1.54 , 2.46]**
cusS	b0570	2.24	2.60	<2 fold	**[1.04 , 1.96]**	
cyoE	b0428	0.42	<2 fold	0.48		[0.54 , 1.46]
cysG	b3368	0.42	<2 fold	0.48		[1.54 , 2.46]
cysQ	b4214	1.94	3.64	<2 fold	**[1.04 , 1.96]**	
cysU	b2424	0.32	0.31	<2 fold		**[0 , 0.56]**
degQ	b3234	0.50	0.33	<2 fold		[0.54 , 1.46]
fhuD	b0152	2.03	4.17	<2 fold	**[1.04 , 1.96]**	
fldB	b2895	2.55	8.26	<2 fold	**[1.04 , 1.96]**	
flgH	b1079	0.49	0.48	<2 fold		**[0 , 0.86]**
glgS	b3049	0.45	<2 fold	0.44		[0.54 , 1.46]

gltL	b0652	0.34	<2 fold	0.48		**[1.54 , 2.46]**
hcaC	b2540	0.29	<2 fold	0.32		**[1.54 , 2.46]**
hemD	b3804	1.95	3.70	2.04		**[1.54 , 2.46]**
htpG	b0473	2.22	2.57	<2 fold	**[1.04 , 1.96]**	
hycC	b2723	0.01	<2 fold	0.24		[0.54 , 1.46]
hycF	b2720	0.50	<2 fold	0.48		**[0.04 , 0.96]**
kdpE	b0694	2.39	2.71	<2 fold	**[1.04 , 1.96]**	
livK	b3458	2.12	2.43	<2 fold	**[1.04 , 1.96]**	
lspA	b0027	0.43	<2 fold	0.51		[0.54 , 1.46]
manY	b1818	2.74	5.06	<2 fold	[0.54 , 1.46]	
mtr	b3161	0.51	0.44	0.46		**[1.54 , 2.46]**
murB	b3972	0.20	<2 fold	0.48		[0.54 , 1.46]
narH	b1225	0.42	0.41	<2 fold		[0.54 , 1.46]
nuoB	b2287	2.17	5.01	<2 fold	[0.54 , 1.46]	
panB	b0134	2.03	2.85	<2 fold	[0.54 , 1.46]	
pgpB	b1278	2.13	2.78	<2 fold	[0.54 , 1.46]	
potF	b0854	0.31	<2 fold	0.35		**[1.54 , 2.46]**
ppc	b3956	0.20	<2 fold	0.49		**[2.04 , 2.96]**
proP	b4111	0.50	0.48	<2 fold		**[1.54 , 2.46]**
proY	b0402	0.34	0.39	<2 fold		**[0 , 0.66]**
pspA	b1304	0.30	0.43	<2 fold		[0.54 , 1.46]
pspD	b1307	0.37	<2 fold	0.44		**[0.04 , 0.96]**
purB	b1131	0.19	<2 fold	0.45		**[2.04 , 2.96]**

putA	b1014	0.30	<2 fold	0.40		[0.54 , 1.46]
rplF	b3305	0.36	<2 fold	0.48		[0.54 , 1.46]
rplK	b3983	0.39	<2 fold	0.44		[0.54 , 1.46]
rpoE	b2573	0.35	<2 fold	0.40		**[1.54 , 2.46]**
rpsI	b3230	0.50	<2 fold	0.50		**[0 , 0.66]**
rpsS	b3316	0.31	<2 fold	0.29		[0.54 , 1.46]
ruvC	b1863	0.44	0.25	<2 fold		**[0.04 , 0.96]**
speB	b2937	0.49	<2 fold	0.42		**[1.54 , 2.46]**
sugE	b4148	0.49	<2 fold	0.47		**[1.54 , 2.46]**
tauC	b0367	0.46	<2 fold	0.46		[0.54 , 1.46]
trxB	b0888	1.94	4.26	<2 fold	**[1.04 , 1.96]**	
yadR	b0156	0.06	<2 fold	0.28		[0.54 , 1.46]
yaeH	b0163	0.45	<2 fold	0.44		**[0.04 , 0.96]**
yagJ	b0276	0.47	<2 fold	0.49		**[0.04 , 0.96]**
yajK	b0423	0.40	0.37	<2 fold		**[0.04 , 0.96]**
yajO	b0419	0.34	<2 fold	0.45		[1.54 , 2.46]
yajR	b0427	0.48	0.48	0.51		[1.54 , 2.46]
ybbC	b0498	2.29	1.99	<2 fold	**[1.04 , 1.96]**	
ybcV	b0558	0.19	<2 fold	0.49		**[0.04 , 0.96]**
ybgF	b0742	1.99	<2 fold	2.11	**[1.04 , 1.96]**	
ybiR	b0818	2.62	6.54	<2 fold	[0.54 , 1.46]	
ybjP	b0865	0.50	0.41	0.48		[0.54 , 1.46]
ycbJ	b0919	2.54	6.44	<2 fold	[0.54 , 1.46]	

ycdX	b1034	0.23	<2 fold	0.47		[0.54 , 1.46]
ycfM	b1105	0.49	0.49	<2 fold		**[3.54 , 4.46]**
ycgR	b1194	0.39	<2 fold	0.47		**[1.54 , 2.46]**
ydaQ	b1346	0.41	<2 fold	0.48		[0.54 , 1.46]
ydeR	b1503	0.50	0.51	<2 fold		**[0.04 , 0.96]**
ydgE	b1599	0.48	<2 fold	0.50		[0.54 , 1.46]
yeaM	b1790	0.46	<2 fold	0.48		**[0.04 , 0.96]**
yeaX	b1803	1.95	<2 fold	1.98	[0.54 , 1.46]	
yecN	b1869	0.29	0.45	<2 fold		**[0 , 0.71]**
yedE	b1929	0.36	0.49	<2 fold		**[0.04 , 0.96]**
yegB	b2077	0.27	<2 fold	0.35		**[1.54 , 2.46]**
ygbA	b2732	0.14	<2 fold	0.45		**[0 , 0.66]**
ygdD	b2807	0.51	0.38	<2 fold		[0.54 , 1.46]
ygjF	b3068	0.04	<2 fold	0.31		**[3.54 , 4.46]**
yhbL	b3209	0.42	0.46	<2 fold		**[4.54 , 5.46]**
yhcC	b3211	0.42	0.42	<2 fold		[0.54 , 1.46]
yheM	b3344	0.36	<2 fold	0.35		**[1.54 , 2.46]**
yhfK	b3358	0.40	0.47	0.49		**[3.54 , 4.46]**
yiaA	b3562	0.44	<2 fold	0.44		**[1.54 , 2.46]**
yicE	b3654	0.08	<2 fold	0.40		**[1.54 , 2.46]**
yicM	b3662	0.37	0.32	<2 fold		-
yigG	b3818	1.98	3.28	<2 fold	**[1.04 , 1.96]**	
yihA	b3865	0.28	<2 fold	0.44		-

yihT	b3881	0.49	0.32	<2 fold		**[0 , 0.71]**
yihU	b3882	0.42	0.51	<2 fold		[0.54 , 1.46]
yjgL	b4253	3.28	9.72	<2 fold	**[1.04 , 1.96]**	
yjhB	b4279	3.09	8.47	2.01	**[1.04 , 1.96]**	
yjhS	b4309	1.97	2.28	<2 fold	[0.54 , 1.46]	
yjjK	b4391	0.19	<2 fold	0.40		[0.54 , 1.46]
ynhA	b1679	2.21	<2 fold	2.12	**[1.04 , 1.96]**	
yqaE	b2666	0.46	<2 fold	0.42		**[1.54 , 2.46]**
yqjE	b3099	0.47	0.29	<2 fold		**[0.04 , 0.96]**
yrbF	b3195	0.38	<2 fold	0.51		[0.54 , 1.46]
ytfG	b4211	0.49	<2 fold	0.48		**[1.54 , 2.46]**

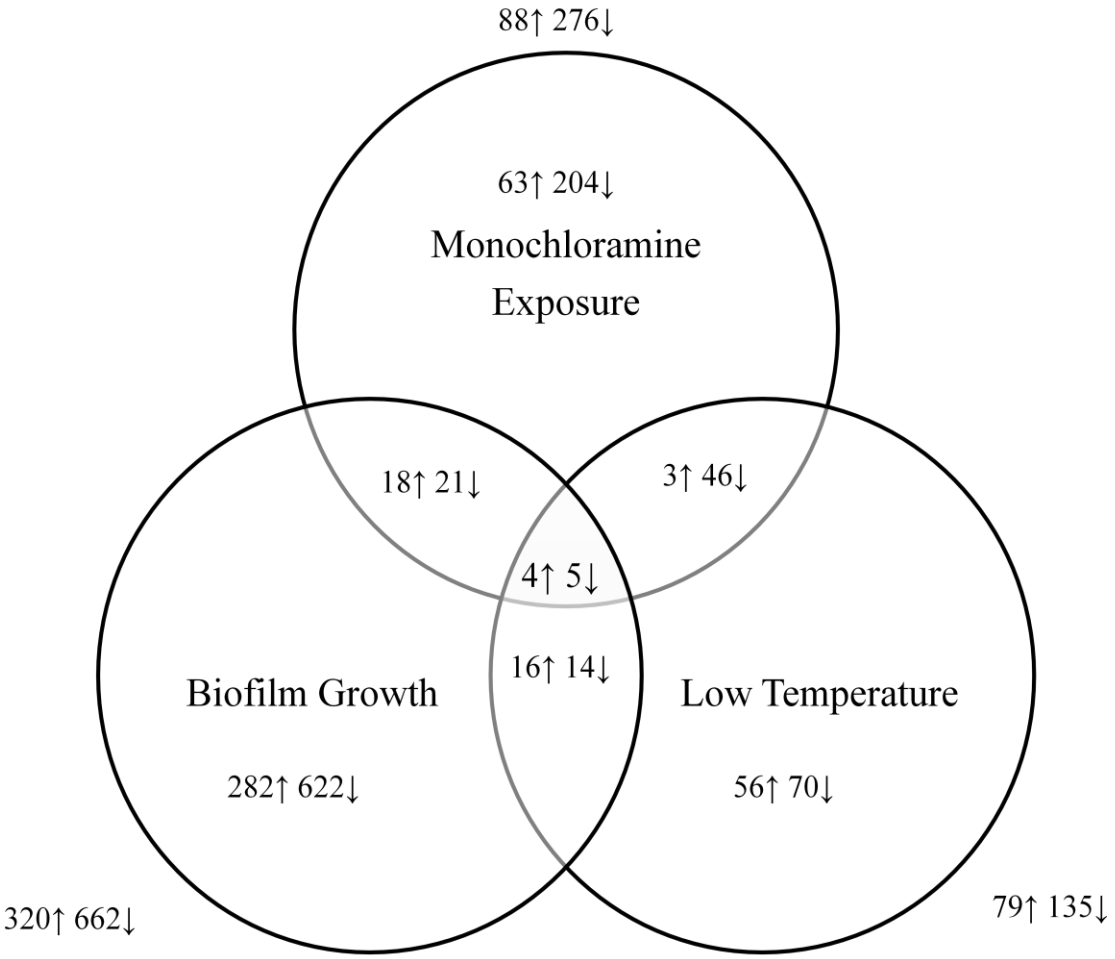

Figure 5.1 Venn diagram of number of differentially-expressed genes for each condition and common to more than one condition. Numbers outside of the circles indicate the number of differentially-expressed genes for each condition.

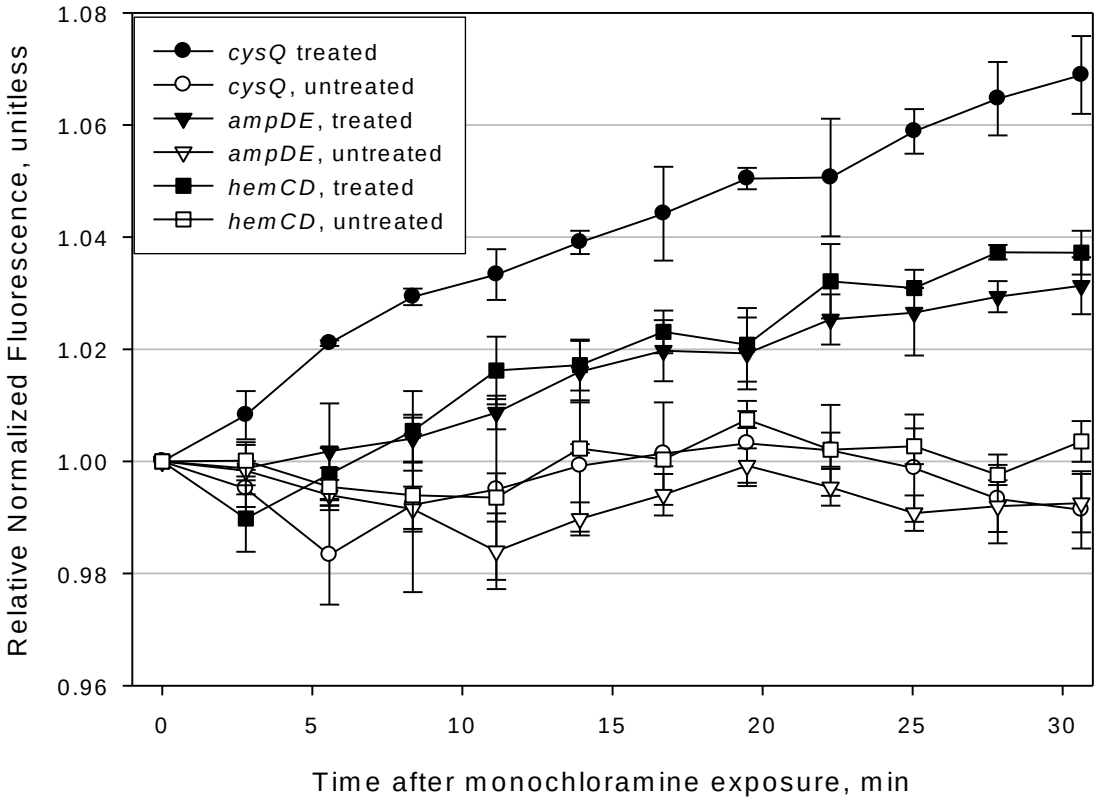

Figure 5.2 GFP-tagged promoter analysis of selected genes. Relative change in fluorescence intensity over time after exposure to monochloramine. Black points are cells treated with monochloramine and white cells are untreated. Error bars indicate standard deviation.

Table 5.S1 *E. coli* genes differentially-expressed during 15 min exposure to 1.0 mg/L (as Cl_2) monochloramine. The threshold for a gene to be considered differentially-expressed was a two-fold expression ratio with a statistical confidence of P<0.05.

Blattner Number	Gene	Fold-change (log2)
b0003	*thrB*	1.3
b0015	*dnaJ*	-1.5
b0023	*rpsT*	-4.2
b0027	*lspA*	-1.2
b0044	*fixX*	-1.3
b0053	*surA*	-1.6
b0060	*polB*	-1.8
b0068	*tbpA*	1.0
b0072	*leuC*	1.3
b0077	*ilvI*	-1.3
b0079	*fruL*	1.1
b0087	*mraY*	-1.3
b0111	*ampE*	1.3
b0112	*aroP*	1.5
b0121	*speE*	1.6
b0123	*yacK*	1.3
b0129	*yadI*	1.7
b0134	*panB*	1.0
b0147	*yadP*	1.0
b0149	*mrcB*	1.0
b0151	*fhuC*	1.2
b0152	*fhuD*	1.0
b0156	*yadR*	-3.9
b0163	*yaeH*	-1.2
b0164	*yaeI*	1.5
b0179	*lpxD*	-3.4
b0186	*ldcC*	1.0
b0213	*yafS*	1.4
b0227	*yafL*	1.2
b0238	*gpt*	-1.5
b0250	*ykfB*	1.5
b0262	*afuC*	1.2
b0276	*yagJ*	-1.1
b0278	*yagL*	1.1
b0338	*cynR*	1.0
b0340	*cynS*	1.2
b0349	*mhpC*	2.1
b0353	*mhpT*	-1.4
b0365	*tauA*	1.0
b0367	*tauC*	-1.1
b0375	*yaiV*	1.0
b0401	*brnQ*	-1.1
b0402	*proY*	-1.6
b0405	*queA*	-1.0
b0417	*thiL*	-3.4
b0419	*yajO*	-1.6
b0422	*xseB*	-1.8
b0423	*yajK*	-1.3
b0427	*yajR*	-1.1
b0428	*cyoE*	-1.3
b0432	*cyoA*	-1.0
b0436	*tig*	1.0
b0437	*clpP*	1.0
b0440	*hupB*	-1.2
b0464	*acrR*	-1.7
b0466	*ybaM*	-1.0
b0473	*htpG*	1.1
b0474	*adk*	-1.1
b0488	*ybbJ*	-3.0
b0498	*ybbC*	1.2
b0500	*ybbD*	-2.0
b0523	*purE*	-1.1
b0526	*cysS*	-1.6
b0558	*ybcV*	-2.4
b0570	*cusS*	1.2
b0571	*ylcA*	1.2
b0573	*ylcC*	1.0
b0581	*ybdK*	-1.1
b0584	*fepA*	-1.0
b0594	*entE*	1.4
b0622	*crcA*	-1.2
b0625	*ybeH*	-1.2
b0640	*holA*	-1.5
b0659	*ybeY*	-1.6
b0661	*yleA*	-1.1
b0693	*speF*	-1.6
b0694	*kdpE*	1.3
b0699	*ybfA*	-1.0
b0709	*ybgH*	-3.0
b0712	*ybgK*	-3.0
b0731	*hrsA*	-2.6
b0733	*cydA*	-1.6
b0735	*ybgE*	-1.7
b0742	*ybgF*	1.0
b0755	*gpmA*	-1.7
b0761	*modE*	1.3
b0762	*ybhT*	-1.2
b0818	*ybiR*	1.4
b0846	*ybjK*	-1.1
b0854	*potF*	-1.7
b0865	*ybjP*	-1.0
b0887	*cydD*	1.1
b0888	*trxB*	1.0
b0916	*ycaQ*	1.3
b0919	*ycbJ*	1.3
b0931	*pncB*	-1.2
b0939	*ycbR*	-1.2
b0946	*ycbW*	1.1
b0996	*torC*	-1.2
b1014	*putA*	-1.7
b1024	*ycdS*	1.1
b1034	*ycdX*	-2.1
b1035	*ycdY*	-1.6
b1037	*csgG*	-1.2
b1068	*mviM*	1.0
b1079	*flgH*	-1.0
b1084	*rne*	-1.0
b1092	*fabD*	-1.9
b1094	*acpP*	-2.5
b1105	*ycfM*	-1.0
b1107	*ycfO*	-1.1
b1123	*potD*	-1.7
b1131	*purB*	-2.4
b1140	*intE*	-1.3

b1148	*ymfM*	-1.3
b1190	*dadX*	-1.1
b1194	*ycgR*	-1.3
b1210	*hemA*	-1.9
b1219	*ychN*	-4.4
b1225	*narH*	-1.3
b1232	*purU*	-1.9
b1236	*galU*	-1.2
b1259	*yciG*	-1.9
b1270	*btuR*	1.0
b1278	*pgpB*	1.1
b1282	*yciH*	-1.3
b1304	*pspA*	-1.7
b1307	*pspD*	-1.4
b1341	*ydaM*	-5.4
b1346	*ydaQ*	-1.3
b1347	*ydaC*	1.0
b1421	*trg*	-1.1
b1464	*yddE*	-1.3
b1466	*narW*	-1.0
b1478	*adhP*	-3.1
b1503	*ydeR*	-1.0
b1542	*ydfI*	-1.3
b1548	*nohA*	-1.0
b1576	*ydfD*	1.0
b1585	*ynfC*	1.0
b1594	*mlc*	-1.5
b1599	*ydgE*	-1.1
b1664	*ydhQ*	-1.6
b1679	*ynhA*	1.1
b1701	*ydiD*	-1.2
b1713	*pheT*	-1.0
b1724	*ydiZ*	-1.8
b1736	*celC*	-1.4
b1770	*ydjF*	1.1
b1774	*ydjJ*	-1.4
b1784	*yeaH*	-1.4
b1790	*yeaM*	-1.1
b1792	*yeaO*	-1.3
b1803	*yeaX*	1.0
b1809	*yoaB*	-1.1
b1818	*manY*	1.5
b1830	*prc*	-1.0
b1853	*yebK*	-1.4
b1857	*yebL*	-1.2
b1863	*ruvC*	-1.2
b1869	*yecN*	-1.8
b1878	*flhE*	-1.3
b1879	*flhA*	-1.3
b1882	*cheY*	-2.0
b1897	*otsB*	-1.2
b1916	*sdiA*	1.0
b1929	*yedE*	-1.5
b1930	*yedF*	-1.6
b1944	*fliL*	-1.7
b1959	*yedA*	-1.4
b2003	*yeeT*	-1.1
b2021	*hisC*	-1.0
b2022	*hisB*	-1.0
b2026	*hisI*	1.0
b2061	*wzb*	-1.4
b2069	*yegD*	-1.2
b2072	*yegK*	-1.4
b2077	*yegB*	-1.9
b2095	*gatZ*	-1.7
b2102	*yegX*	1.1
b2127	*yehV*	1.4
b2141	*yohJ*	-2.2
b2145	*yeiS*	-1.3
b2150	*mglB*	-1.0
b2168	*fruK*	-1.0
b2190	*yejO*	1.3
b2196	*ccmF*	-1.0
b2198	*ccmD*	-1.6
b2199	*ccmC*	-3.0
b2252	*ais*	1.2
b2277	*nuoM*	-1.0
b2287	*nuoB*	1.1
b2288	*nuoA*	-1.1
b2323	*fabB*	-1.3
b2324	*yfcK*	1.0
b2358	*yfdO*	-1.2
b2388	*glk*	-1.2
b2414	*cysK*	-2.8
b2416	*ptsI*	-1.0
b2424	*cysU*	-1.6
b2452	*eutH*	-4.7
b2457	*cchA*	-1.4
b2466	*ypfG*	-1.1
b2474	*ypfI*	-1.3
b2497	*uraA*	-1.0
b2500	*purN*	-2.8
b2508	*guaB*	-2.0
b2513	*yfgM*	-1.4
b2515	*gcpE*	-1.2
b2532	*yfhQ*	-1.0
b2540	*hcaC*	-1.8
b2541	*hcaB*	-1.7
b2542	*hcaD*	-2.1
b2543	*yphA*	-1.5
b2556	*yfhK*	-1.5
b2573	*rpoE*	-1.5
b2608	*yfjA*	-1.0
b2643	*yfjX*	-2.1
b2645	*yfjZ*	-1.7
b2666	*yqaE*	-1.1
b2667	*ygaV*	-1.5
b2713	*hydN*	-1.1
b2720	*hycF*	-1.0
b2723	*hycC*	-6.7
b2732	*ygbA*	-2.9
b2777	*ygcF*	-1.3
b2783	*chpR*	-2.4
b2790	*yqcA*	-1.4
b2807	*ygdD*	-1.0
b2821	*ptr*	1.0
b2850	*ygeF*	1.3
b2855	*ygeK*	1.1
b2895	*fldB*	1.4
b2906	*visC*	-1.6
b2915	*yqfE*	-1.0
b2923	*yggA*	-1.4
b2926	*pgk*	-1.1

b2933	*cmtA*	-1.0
b2937	*speB*	-1.0
b2943	*galP*	-1.5
b2958	*yggN*	-1.4
b2961	*mutY*	-1.3
b2965	*speC*	1.1
b2966	*yqgA*	1.0
b2991	*hybF*	-1.7
b2992	*hybE*	-1.9
b3040	*ygiE*	-1.0
b3049	*glgS*	-1.1
b3059	*ygiH*	-3.2
b3068	*ygjF*	-4.8
b3095	*yqjA*	-1.3
b3099	*yqjE*	-1.1
b3145	*yraK*	-3.1
b3155	*yhbQ*	-1.7
b3156	*yhbS*	-1.2
b3161	*mtr*	-1.0
b3172	*argG*	-1.6
b3178	*hflB*	-1.1
b3183	*yhbZ*	-1.5
b3184	*yhbE*	-1.0
b3195	*yrbF*	-1.4
b3209	*yhbL*	-1.2
b3211	*yhcC*	-1.3
b3229	*sspA*	-1.0
b3230	*rpsI*	-1.0
b3234	*degQ*	-1.0
b3237	*argR*	-1.3
b3238	*yhcN*	-1.0
b3265	*acrE*	-1.3
b3287	*def*	-1.8
b3299	*rpmJ*	-1.0
b3305	*rplF*	-1.5
b3308	*rplE*	-1.8
b3313	*rplP*	-1.0
b3315	*rplV*	-2.0
b3316	*rpsS*	-1.7
b3344	*yheM*	-1.5
b3347	*fkpA*	-1.5
b3358	*yhfK*	-1.3
b3368	*cysG*	-1.2
b3407	*yhgF*	-1.2
b3408	*feoA*	-2.7
b3458	*livK*	1.1
b3466	*yhhL*	-1.0
b3468	*yhhN*	-1.1
b3475	*yhhU*	-1.1
b3487	*yhiI*	1.4
b3497	*yhiQ*	-1.9
b3506	*slp*	-1.0
b3538	*yhjU*	-1.6
b3562	*yiaA*	-1.2
b3566	*xylF*	-1.1
b3588	*aldB*	-2.0
b3609	*secB*	1.1
b3616	*tdh*	-1.1
b3619	*rfaD*	-3.4
b3623	*rfaK*	1.2
b3654	*yicE*	-3.6
b3662	*yicM*	-1.4
b3664	*yicO*	-3.4
b3666	*uhpT*	-1.4
b3668	*uhpB*	-1.3
b3669	*uhpA*	-3.3
b3679	*yidK*	1.0
b3689	*yidR*	-1.0
b3724	*phoU*	-1.1
b3727	*pstC*	-1.0
b3746	*yieN*	-1.6
b3775	*ppiC*	-1.2
b3779	*gppA*	-1.0
b3791	*wecE*	-1.5
b3792	*wzxE*	-1.8
b3794	*wecG*	1.6
b3795	*yifK*	1.4
b3804	*hemD*	1.0
b3818	*yigG*	1.0
b3820	*yigI*	-3.3
b3847	*pepQ*	-1.2
b3862	*yihG*	1.1
b3865	*yihA*	-1.8
b3881	*yihT*	-1.0
b3882	*yihU*	-1.3
b3937	*yiiX*	-1.6
b3939	*metB*	-1.1
b3956	*ppc*	-2.4
b3972	*murB*	-2.3
b3980	*tufB*	-1.1
b3983	*rplK*	-1.3
b3985	*rplJ*	1.0
b4012	*yjaB*	-2.0
b4044	*dinF*	-3.7
b4051	*qor*	-1.6
b4063	*soxR*	-3.0
b4090	*rpiB*	-1.2
b4093	*phnO*	-1.0
b4100	*phnH*	-1.0
b4101	*phnG*	-1.1
b4106	*phnC*	-1.5
b4108	*phnA*	-1.1
b4111	*proP*	-1.0
b4133	*cadC*	1.2
b4137	*cutA*	-2.0
b4148	*sugE*	-1.0
b4186	*yjfC*	-1.0
b4202	*rpsR*	-1.9
b4211	*ytfG*	-1.0
b4214	*cysQ*	1.0
b4221	*ytfN*	-1.0
b4243	*yjgF*	1.0
b4245	*pyrB*	-1.1
b4253	*yjgL*	1.7
b4260	*pepA*	1.1
b4264	*idnR*	1.0
b4267	*idnD*	1.1
b4277	*yjgZ*	-2.6
b4279	*yjhB*	1.6
b4309	*yjhS*	1.0
b4318	*fimF*	-1.0
b4321	*gntP*	-1.5
b4362	*dnaT*	-2.8

b4389	*sms*	-2.2
b4391	*yjjK*	-2.4

b4397	*creA*	-1.0
b4409	*blr*	-1.0

Table 5.S2 *E. coli* genes differentially-expressed during 14 d biofilm growth. The average log2-transformed fold-change is listed for each gene. The threshold for a gene to be considered differentially-expressed was a two-fold expression ratio with a statistical confidence of P<0.05.

Blattner Number	Gene	Fold-change (log2)
b0003	*thrB*	-1.8
b0010	*yaaH*	-1.1
b0013	*yaaI*	-1.4
b0031	*dapB*	-1.0
b0036	*caiD*	1.0
b0040	*caiT*	1.8
b0046	*yabF*	1.8
b0049	*apaH*	-1.0
b0050	*apaG*	-1.1
b0061	*araD*	-1.6
b0063	*araB*	-1.0
b0072	*leuC*	-1.9
b0073	*leuB*	-1.2
b0074	*leuA*	-1.1
b0076	*leuO*	-1.4
b0078	*ilvH*	-1.5
b0079	*fruL*	1.4
b0086	*murF*	-1.3
b0088	*murD*	-1.1
b0093	*ftsQ*	-1.4
b0095	*ftsZ*	-1.2
b0103	*yacE*	1.3
b0107	*hofB*	-1.3
b0108	*ppdD*	1.5
b0109	*nadC*	-1.1
b0111	*ampE*	4.7
b0112	*aroP*	-1.1
b0113	*pdhR*	1.8
b0114	*aceE*	2.2
b0117	*yacH*	-1.0
b0120	*speD*	1.5
b0123	*yacK*	-2.0
b0130	*yadE*	1.7
b0131	*panD*	-1.1
b0134	*panB*	1.5
b0144	*yadB*	2.9
b0146	*sfsA*	2.1
b0147	*yadP*	-1.1
b0148	*hrpB*	1.9
b0152	*fhuD*	2.1
b0161	*htrA*	-1.0
b0170	*tsf*	1.7
b0174	*yaeS*	1.6
b0182	*lpxB*	-1.4
b0185	*accA*	-1.0
b0188	*tilS*	-1.2
b0194	*proS*	1.4
b0195	*yaeB*	-1.4
b0197	*metQ*	1.0
b0198	*metI*	-1.7
b0209	*yafD*	1.0
b0211	*dniR*	2.0
b0212	*gloB*	1.2
b0221	*yafH*	-1.1
b0223	*yafJ*	1.2
b0225	*yafQ*	1.2
b0226	*dinJ*	1.4
b0227	*yafL*	-1.6
b0228	*yafM*	-1.5
b0231	*dinB*	1.0
b0234	*yafP*	2.1
b0236	*prfH*	-1.2
b0237	*pepD*	1.1
b0239	*yafA*	1.0
b0253	*ykfA*	1.1
b0255	*yi91a*	1.7
b0283	*yagQ*	-1.2
b0285	*yagS*	-1.3
b0289	*yagV*	1.1
b0300	*ykgA*	-1.3
b0304	*ykgC*	-1.2
b0307	*ykgF*	1.4
b0308	*ykgG*	-1.4
b0311	*betA*	2.1
b0312	*betB*	1.9
b0314	*betT*	-1.0
b0317	*yahC*	-1.6
b0320	*yahF*	1.3
b0327	*yahM*	1.8
b0335	*prpE*	-1.0
b0338	*cynR*	-1.7
b0340	*cynS*	-1.0
b0341	*cynX*	-2.1
b0343	*lacY*	-1.0
b0345	*lacI*	-1.0
b0346	*mhpR*	1.1
b0349	*mhpC*	-2.4
b0351	*mhpF*	1.1
b0352	*mhpE*	-1.1
b0363	*yaiP*	-1.1
b0365	*tauA*	-2.0
b0366	*tauB*	-1.4
b0376	*yaiH*	-1.1
b0379	*yaiY*	1.2
b0380	*yaiZ*	-1.5
b0391	*yaiE*	-1.2
b0396	*araJ*	-2.0
b0402	*proY*	-1.4
b0411	*tsx*	1.1
b0413	*ybaD*	1.2
b0418	*pgpA*	1.9
b0423	*yajK*	-1.4
b0427	*yajR*	-1.1
b0441	*ybaU*	-1.2
b0442	*ybaV*	1.4
b0444	*ybaX*	-1.5
b0445	*ybaE*	-1.5
b0449	*mdlB*	1.3
b0451	*amtB*	-2.9
b0452	*tesB*	-1.4
b0453	*ybaY*	-1.0

b0467	*priC*	-1.2
b0468	*ybaN*	-1.1
b0470	*dnaX*	-1.1
b0472	*recR*	-2.2
b0473	*htpG*	1.4
b0476	*ybaC*	1.4
b0485	*ybaS*	-1.8
b0486	*ybaT*	-2.0
b0490	*ybbL*	-1.1
b0494	*tesA*	-1.3
b0495	*ybbA*	1.3
b0497	*rhsD*	1.4
b0498	*ybbC*	1.0
b0504	*ybbS*	-1.2
b0511	*allP*	-1.1
b0512	*ybbX*	-1.6
b0513	*ybbY*	-1.4
b0516	*ylbB*	-1.9
b0517	*ylbC*	3.0
b0522	*purK*	-1.5
b0531	*sfmC*	1.0
b0543	*emrE*	-1.3
b0544	*ybcK*	2.2
b0545	*ybcL*	1.5
b0546	*ybcM*	1.3
b0550	*rus*	-1.2
b0551	*ybcQ*	-1.3
b0559	*ybcW*	1.4
b0561	*ybcX*	-1.0
b0562	*ybcY*	1.3
b0567	*ybcH*	-1.9
b0569	*nfrB*	1.5
b0570	*cusS*	1.4
b0573	*ylcC*	-2.0
b0576	*pheP*	1.1
b0584	*fepA*	1.3
b0588	*fepC*	-2.0
b0590	*fepD*	-1.2
b0593	*entC*	-1.0
b0594	*entE*	-1.5
b0596	*entA*	-1.2

b0602	*ybdN*	-1.3
b0604	*dsbG*	-1.1
b0607	*ybdQ*	2.0
b0610	*rnk*	-1.1
b0611	*rna*	1.3
b0612	*ybdS*	-1.7
b0613	*citG*	-1.3
b0616	*citE*	-1.6
b0619	*citA*	1.6
b0623	*cspE*	1.7
b0624	*crcB*	-1.1
b0626	*ybeM*	-1.5
b0627	*ybeC*	-1.5
b0629	*ybeF*	1.9
b0632	*dacA*	1.3
b0633	*rlpA*	1.6
b0634	*mrdB*	-1.5
b0641	*rlpB*	1.0
b0643	*ybeL*	2.2
b0644	*ybeQ*	1.3
b0645	*ybeR*	3.6
b0649	*ybeV*	-1.3
b0651	*ybeK*	1.7
b0653	*gltK*	-1.6
b0655	*ybeJ*	1.6
b0660	*ybeZ*	-1.9
b0683	*fur*	-1.6
b0684	*fldA*	-1.0
b0685	*ybfE*	1.9
b0686	*ybfF*	-2.2
b0687	*seqA*	-1.8
b0692	*potE*	-1.1
b0694	*kdpE*	1.4
b0696	*kdpC*	-2.0
b0697	*kdpB*	-1.6
b0700	*rhsC*	1.5
b0705	*ybfL*	-1.6
b0706	*ybfD*	1.0
b0707	*ybgA*	-1.5
b0721	*sdhC*	1.1
b0722	*sdhD*	-1.0

b0736	*ybgC*	-1.4
b0739	*tolA*	-1.1
b0750	*nadA*	1.6
b0751	*pnuC*	-2.2
b0755	*gpmA*	1.1
b0756	*galM*	-1.1
b0761	*modE*	-1.5
b0765	*modC*	-1.0
b0767	*ybhE*	-1.1
b0770	*ybhI*	-1.1
b0771	*ybhJ*	1.0
b0772	*ybhC*	-1.2
b0775	*bioB*	-2.7
b0785	*moaE*	-1.6
b0786	*ybhL*	-1.3
b0792	*ybhR*	-1.6
b0798	*ybiA*	-1.5
b0802	*ybiJ*	-1.6
b0806	*ybiM*	1.9
b0811	*glnH*	-1.5
b0812	*dps*	-1.1
b0814	*ompX*	-1.2
b0818	*ybiR*	2.7
b0820	*ybiT*	-1.4
b0823	*ybiW*	-1.1
b0826	*moeB*	-1.2
b0838	*yliJ*	-1.5
b0840	*deoR*	-1.1
b0845	*ybjJ*	-2.5
b0851	*mdaA*	-1.7
b0857	*potI*	-1.2
b0861	*artM*	-1.7
b0862	*artQ*	-1.2
b0863	*artI*	-1.1
b0864	*artP*	-1.5
b0865	*ybjP*	-1.3
b0866	*ybjQ*	-1.1
b0870	*ltaE*	1.0
b0875	*aqpZ*	-1.4
b0876	*ybjD*	-1.3
b0884	*infA*	1.0

b0886	*cydC*	-1.0
b0888	*trxB*	2.1
b0890	*ftsK*	-1.1
b0892	*ycaJ*	-1.2
b0897	*ycaC*	1.0
b0901	*ycaK*	1.2
b0906	*ycaP*	2.1
b0913	*ycaI*	1.3
b0914	*msbA*	-1.2
b0919	*ycbJ*	2.7
b0922	*mukF*	2.4
b0933	*ycbE*	-1.0
b0934	*ycbM*	-1.2
b0936	*ycbO*	1.2
b0938	*ycbQ*	-1.3
b0940	*ycbS*	-1.2
b0945	*pyrD*	1.8
b0947	*ycbX*	1.1
b0952	*ymbA*	-1.2
b0956	*ycbG*	-2.4
b0957	*ompA*	1.3
b0959	*yccR*	-1.0
b0964	*yccT*	-2.1
b0966	*yccV*	1.8
b0969	*yccK*	1.1
b0970	*yccA*	-1.0
b0972	*hyaA*	-1.6
b0973	*hyaB*	-1.5
b0982	*yccY*	-1.0
b0986	*ymcC*	1.3
b0990	*cspG*	-1.2
b0991	*sfa*	3.0
b0993	*torS*	-1.1
b1000	*cbpA*	-1.3
b1013	*ycdC*	-1.5
b1019	*ycdB*	-1.1
b1033	*ycdW*	-1.1
b1041	*csgB*	-1.2
b1042	*csgA*	-1.0
b1043	*csgC*	-1.3
b1047	*ymdD*	1.1
b1053	*yceE*	-2.1
b1055	*yceA*	-1.3
b1058	*yceO*	-1.5
b1060	*yceP*	1.3
b1075	*flgD*	-2.0
b1079	*flgH*	-1.1
b1082	*flgK*	1.5
b1083	*flgL*	-1.3
b1088	*yceD*	-1.2
b1089	*rpmF*	-1.1
b1098	*tmk*	-1.4
b1102	*fhuE*	-1.6
b1103	*ycfF*	-1.0
b1105	*ycfM*	-1.0
b1111	*ycfQ*	-1.2
b1113	*ycfS*	-1.9
b1114	*mfd*	-1.3
b1115	*ycfT*	-1.2
b1119	*ycfX*	-1.5
b1121	*ycfZ*	-2.4
b1122	*ymfA*	-1.3
b1130	*phoP*	3.0
b1137	*ymfD*	2.7
b1139	*lit*	1.5
b1150	*ymfR*	-1.4
b1160	*ycgW*	-1.2
b1171	*ymgD*	-1.7
b1174	*minE*	-1.1
b1179	*ycgL*	-1.2
b1183	*umuD*	1.1
b1184	*umuC*	-1.7
b1191	*ycgO*	-1.3
b1195	*ymgE*	-1.7
b1197	*treA*	-1.0
b1203	*ychF*	-1.1
b1206	*ychM*	-1.4
b1212	*hemK*	-1.6
b1215	*kdsA*	-1.4
b1220	*ychP*	-1.6
b1221	*narL*	-1.6
b1222	*narX*	-1.2
b1225	*narH*	-1.3
b1226	*narJ*	-1.6
b1229	*tpr*	1.0
b1233	*ychJ*	1.2
b1238	*tdk*	3.4
b1239	*ychG*	-1.0
b1245	*oppC*	1.3
b1247	*oppF*	1.2
b1252	*tonB*	-1.4
b1253	*yciA*	-1.2
b1264	*trpE*	1.1
b1270	*btuR*	-2.9
b1271	*yciK*	-1.3
b1278	*pgpB*	1.5
b1285	*yciR*	3.6
b1289	*ycjD*	1.1
b1290	*sapF*	-2.1
b1292	*sapC*	-1.1
b1296	*ycjJ*	-1.5
b1301	*ordL*	-1.5
b1304	*pspA*	-1.2
b1305	*pspB*	-1.2
b1310	*ycjN*	-1.4
b1312	*ycjP*	-1.4
b1316	*ycjT*	-1.0
b1320	*ycjW*	-1.5
b1323	*tyrR*	-1.5
b1326	*ycjI*	-1.1
b1328	*ycjZ*	2.9
b1329	*mppA*	-1.0
b1340	*ydaL*	-1.3
b1342	*ydaN*	-1.1
b1352	*kil*	1.7
b1359	*ydaU*	-1.3
b1361	*ydaW*	-1.1
b1366	*ydaY*	-1.3
b1382	*ynbE*	-1.0
b1406	*ydbC*	-1.1
b1409	*ynbB*	-1.5
b1426	*ydcH*	1.5
b1429	*tehA*	-1.5

b1441	*ydcT*	-1.4
b1448	*yncA*	-1.3
b1460	*ydcC*	-1.2
b1461	*ydcE*	-1.7
b1463	*nhoA*	-2.2
b1464	*yddE*	1.1
b1465	*narV*	-1.7
b1468	*narZ*	-1.1
b1479	*sfcA*	-1.4
b1494	*pqqL*	-1.8
b1495	*yddB*	-1.1
b1503	*ydeR*	-1.0
b1507	*hipA*	-1.5
b1511	*ydeV*	-1.6
b1521	*uxaB*	-1.1
b1523	*yneG*	-1.2
b1526	*yneJ*	1.2
b1528	*ydeA*	-1.7
b1529	*ydeB*	-1.1
b1532	*marB*	1.9
b1537	*ydeJ*	-1.4
b1538	*dcp*	-1.4
b1539	*ydfG*	-2.0
b1540	*ydfH*	-1.2
b1541	*ydfZ*	1.4
b1543	*ydfJ*	-1.4
b1573	*ydfC*	-1.2
b1576	*ydfD*	-1.3
b1583	*ynfB*	-1.1
b1585	*ynfC*	-1.7
b1586	*ynfD*	1.6
b1588	*ynfF*	2.2
b1590	*ynfH*	1.2
b1591	*ynfI*	-1.2
b1593	*ynfK*	-1.6
b1597	*asr*	-1.4
b1604	*ydgH*	-1.6
b1606	*ydgB*	-1.0
b1610	*tus*	-1.4
b1625	*ydgT*	-1.0
b1626	*ydgK*	-1.6
b1630	*ydgO*	-1.7
b1635	*gst*	-1.2
b1636	*pdxY*	-1.0
b1638	*pdxH*	1.4
b1639	*ydhA*	-1.5
b1641	*slyB*	1.4
b1654	*ydhD*	-1.0
b1655	*ydhO*	-1.5
b1660	*ydhC*	1.4
b1661	*cfa*	1.3
b1663	*ydhE*	-1.8
b1672	*ydhW*	-1.0
b1675	*ydhZ*	-1.4
b1676	*pykF*	-1.1
b1683	*ynhE*	-1.1
b1689	*ydiL*	-1.5
b1698	*ydiR*	-1.0
b1700	*ydiT*	1.4
b1703	*ydiA*	-1.3
b1705	*ydiE*	-1.4
b1708	*nlpC*	-1.0
b1711	*btuC*	-1.0
b1714	*pheS*	2.0
b1715	*pheM*	1.0
b1717	*rpmI*	-1.1
b1724	*ydiZ*	1.4
b1725	*yniA*	-2.2
b1727	*yniC*	-1.1
b1743	*spy*	-1.2
b1750	*ydjX*	-1.0
b1753	*ynjA*	-1.2
b1755	*ynjC*	1.8
b1765	*ydjA*	-1.0
b1769	*ydjE*	1.2
b1776	*ydjL*	-1.6
b1787	*yeaK*	3.0
b1789	*yeaL*	-1.0
b1794	*yeaP*	-1.2
b1800	*yeaU*	-1.3
b1801	*yeaV*	1.7
b1802	*yeaW*	1.3
b1803	*yeaX*	-1.1
b1812	*pabB*	-1.1
b1818	*manY*	2.3
b1821	*yebN*	1.0
b1823	*cspC*	1.9
b1837	*yebW*	1.1
b1842	*holE*	-1.6
b1846	*yebE*	-1.0
b1847	*yebF*	-1.2
b1849	*purT*	1.5
b1852	*zwf*	-1.1
b1858	*yebM*	1.2
b1861	*ruvA*	-1.5
b1863	*ruvC*	-2.0
b1865	*ntpA*	1.2
b1866	*aspS*	-1.6
b1868	*yecE*	-1.1
b1869	*yecN*	-1.1
b1891	*flhC*	-1.2
b1902	*yecI*	2.4
b1919	*yedO*	-1.1
b1920	*fliY*	-1.1
b1922	*fliA*	-1.1
b1929	*yedE*	-1.0
b1930	*yedF*	1.0
b1932	*yedL*	-1.3
b1935	*yedM*	1.2
b1938	*fliF*	1.0
b1940	*fliH*	-1.0
b1942	*fliJ*	-1.4
b1946	*fliN*	-2.2
b1962	*yedJ*	-1.4
b1974	*yodB*	-1.0
b1988	*nac*	-1.0
b1991	*cobT*	1.4
b1992	*cobS*	-1.8
b1999	*yeeP*	-1.7
b2005	*yeeV*	-1.3
b2007	*yeeX*	1.0
b2008	*yeeA*	1.2
b2012	*yeeD*	-1.3

b2013	*yeeE*	-1.2
b2026	*hisI*	-1.1
b2032	*wbbK*	1.8
b2033	*wbbJ*	1.6
b2034	*wbbI*	1.6
b2052	*wcaG*	-1.0
b2061	*wzb*	1.7
b2062	*wza*	-1.1
b2068	*alkA*	-1.2
b2073	*yegL*	-2.1
b2076	*yegO*	-1.1
b2078	*baeS*	-1.3
b2086	*yegS*	-1.4
b2094	*gatA*	-2.1
b2100	*yegV*	-2.0
b2102	*yegX*	-1.7
b2107	*yohN*	-1.0
b2122	*yehQ*	-1.2
b2126	*yehU*	-1.1
b2127	*yehV*	-1.3
b2128	*yehW*	-1.1
b2130	*yehY*	-1.1
b2134	*pbpG*	-1.1
b2137	*yohF*	-1.5
b2142	*yohK*	-1.6
b2143	*cdd*	1.8
b2151	*galS*	-1.3
b2152	*yeiB*	-1.4
b2156	*lysP*	1.1
b2157	*yeiE*	-1.1
b2158	*yeiH*	2.1
b2173	*yeiR*	-1.3
b2180	*yejF*	-1.1
b2181	*yejG*	-1.0
b2188	*yejM*	-1.2
b2193	*narP*	1.2
b2195	*dsbE*	-1.0
b2198	*ccmD*	-2.4
b2201	*ccmA*	1.1
b2203	*napB*	1.0
b2205	*napG*	1.0
b2207	*napD*	1.6
b2209	*eco*	-1.0
b2212	*alkB*	-1.2
b2213	*ada*	1.3
b2216	*yojN*	1.3
b2221	*atoD*	1.0
b2225	*yfaP*	-1.0
b2226	*yfaQ*	-1.2
b2230	*yfaA*	-2.0
b2232	*ubiG*	-1.6
b2238	*yfaH*	2.0
b2239	*glpQ*	-1.0
b2240	*glpT*	-1.1
b2245	*yfaU*	-1.6
b2252	*ais*	2.1
b2256	*yfbH*	-1.0
b2259	*pmrD*	1.5
b2269	*elaD*	-1.8
b2279	*nuoK*	-1.4
b2285	*nuoE*	-1.3
b2287	*nuoB*	2.3
b2304	*yfcH*	-2.2
b2305	*yfcI*	-1.3
b2306	*hisP*	-1.9
b2312	*purF*	-2.0
b2313	*cvpA*	1.4
b2317	*dedA*	-1.2
b2320	*pdxB*	1.0
b2321	*div*	-1.1
b2327	*yfcA*	-1.3
b2328	*mepA*	-1.0
b2330	*yfcB*	1.7
b2346	*vacJ*	1.3
b2369	*evgA*	1.4
b2377	*yfdY*	-1.4
b2381	*ypdB*	-1.3
b2407	*xapA*	1.3
b2409	*yfeR*	-2.5
b2411	*lig*	-1.1
b2412	*zipA*	1.2
b2413	*cysZ*	-1.1
b2422	*cysA*	-1.2
b2424	*cysU*	-1.7
b2425	*cysP*	-2.5
b2435	*amiA*	2.4
b2437	*yfeG*	-1.1
b2456	*cchB*	-1.6
b2458	*eutI*	-1.6
b2476	*purC*	-1.6
b2483	*hyfC*	-1.3
b2487	*hyfG*	-2.1
b2488	*hyfH*	-1.2
b2489	*hyfI*	-1.8
b2496	*yfgE*	-2.5
b2509	*xseA*	-1.0
b2522	*sseB*	-1.7
b2526	*hscA*	-1.1
b2533	*suhB*	1.1
b2543	*yphA*	1.2
b2545	*yphC*	-1.3
b2547	*yphE*	-1.4
b2559	*tadA*	-1.2
b2572	*rseA*	1.1
b2574	*nadB*	-1.0
b2579	*yfiD*	-1.0
b2593	*yfiH*	-1.3
b2594	*sfhB*	1.3
b2595	*yfiO*	-1.3
b2597	*yfiA*	-1.4
b2598	*pheL*	1.6
b2614	*grpE*	-1.1
b2615	*yfjB*	-1.1
b2625	*yfjI*	1.4
b2629	*yfjM*	-1.4
b2634	*yfjR*	2.1
b2644	*yfjY*	-1.4
b2662	*gabT*	2.2
b2663	*gabP*	-1.2
b2665	*ygaU*	-1.9
b2668	*ygaP*	-1.4
b2671	*ygaC*	-1.0
b2676	*nrdF*	-1.2

b2677	*proV*	-1.3
b2688	*gshA*	1.0
b2696	*csrA*	1.3
b2699	*recA*	-1.1
b2701	*mltB*	-1.5
b2703	*srlE*	-1.2
b2705	*srlD*	-1.1
b2707	*srlR*	1.3
b2709	*ygaA*	-1.3
b2721	*hycE*	-1.6
b2722	*hycD*	-1.5
b2725	*hycA*	-1.4
b2728	*hypC*	-1.1
b2735	*ygbI*	-1.0
b2737	*ygbK*	-1.4
b2742	*nlpD*	1.1
b2746	*ygbB*	1.2
b2747	*ygbP*	-1.1
b2751	*cysN*	1.2
b2769	*ygcQ*	-1.6
b2771	*ygcS*	1.1
b2774	*ygcW*	-2.5
b2784	*relA*	1.5
b2794	*yqcD*	1.0
b2796	*sdaC*	-1.2
b2798	*exo*	-1.0
b2800	*fucA*	-1.5
b2807	*ygdD*	-1.4
b2813	*mltA*	-1.8
b2823	*ppdC*	-1.4
b2828	*lgt*	-1.0
b2840	*ygeA*	-1.0
b2846	*yqeH*	-1.1
b2848	*yqeJ*	1.0
b2851	*ygeG*	-1.5
b2867	*ygeT*	-1.3
b2870	*ygeW*	1.0
b2890	*lysS*	1.5
b2893	*dsbC*	-1.1
b2895	*fldB*	3.0
b2896	*ygfX*	-1.4
b2900	*yqfB*	-1.3
b2903	*gcvP*	-1.0
b2906	*visC*	1.4
b2912	*ygfA*	-1.1
b2920	*ygfH*	-1.5
b2925	*fba*	-1.2
b2927	*epd*	-1.3
b2929	*yggD*	-1.3
b2939	*yqgB*	-1.6
b2942	*metK*	2.0
b2945	*endA*	-1.0
b2947	*gshB*	-1.1
b2957	*ansB*	-1.0
b2963	*mltC*	-1.4
b2966	*yqgA*	-1.1
b2968	*yghD*	-1.7
b2969	*yghE*	-1.2
b2986	*yghT*	-1.1
b2993	*hybD*	-1.2
b2994	*hybC*	-1.1
b2996	*hybA*	-1.7
b3010	*yqhC*	-1.6
b3017	*sufI*	-1.2
b3019	*parC*	1.1
b3030	*parE*	1.3
b3035	*tolC*	1.2
b3042	*yqiC*	-2.0
b3054	*ygiF*	-1.0
b3055	*ygiM*	1.2
b3062	*ttdB*	-1.2
b3065	*rpsU*	1.0
b3074	*ygjH*	-1.3
b3080	*ygjK*	-1.1
b3081	*ygjL*	-1.5
b3083	*ygjN*	1.0
b3084	*ygjO*	-1.1
b3085	*ygjP*	-1.4
b3086	*ygjQ*	1.0
b3088	*ygjT*	-2.1
b3089	*ygjU*	-1.1
b3099	*yqjE*	-1.8
b3101	*yqjF*	-1.3
b3118	*tdcA*	-1.0
b3121	*yhaC*	1.4
b3129	*sohA*	-1.3
b3133	*agaV*	-1.8
b3137	*agaY*	-1.3
b3138	*agaB*	-1.5
b3139	*agaC*	-1.6
b3140	*agaD*	-1.4
b3142	*yraH*	-1.1
b3161	*mtr*	-1.2
b3165	*rpsO*	-1.2
b3166	*truB*	-1.2
b3167	*rbfA*	-1.3
b3173	*yhbX*	-1.6
b3187	*ispB*	-1.0
b3188	*nlp*	1.1
b3194	*yrbE*	-1.1
b3201	*yhbG*	-1.4
b3206	*ptsO*	2.2
b3209	*yhbL*	-1.1
b3210	*arcB*	1.1
b3211	*yhcC*	-1.3
b3217	*yhcE*	3.0
b3219	*yhcF*	-1.7
b3220	*yhcG*	-1.0
b3222	*yhcI*	-1.3
b3224	*nanT*	-1.0
b3231	*rplM*	-1.1
b3234	*degQ*	-1.6
b3235	*degS*	-1.2
b3241	*yhcQ*	1.0
b3242	*yhcR*	-1.1
b3243	*yhcS*	1.0
b3247	*cafA*	-1.5
b3257	*yhdT*	-1.9
b3261	*fis*	-3.3
b3263	*yhdU*	-1.0
b3265	*acrE*	-1.2
b3266	*acrF*	-1.4
b3281	*aroE*	-1.0

b3293	*yhdN*	-1.1
b3300	*prlA*	1.0
b3306	*rpsH*	-1.5
b3308	*rplE*	2.1
b3311	*rpsQ*	-1.3
b3317	*rplB*	1.2
b3320	*rplC*	-1.5
b3321	*rpsJ*	-1.0
b3335	*hofD*	-1.6
b3339	*tufA*	-1.7
b3341	*rpsG*	1.8
b3342	*rpsL*	-1.0
b3343	*yheL*	-1.0
b3346	*yheO*	1.5
b3348	*slyX*	-1.0
b3350	*kefB*	1.4
b3358	*yhfK*	-1.1
b3362	*yhfG*	-1.0
b3363	*ppiA*	-1.3
b3369	*yhfL*	-1.1
b3384	*trpS*	-1.4
b3395	*yrfD*	-1.2
b3396	*mrcA*	-1.2
b3401	*yrfI*	-1.2
b3403	*pckA*	-1.9
b3410	*yhgG*	-1.5
b3412	*bioH*	-1.0
b3417	*malP*	1.0
b3423	*glpR*	-1.0
b3425	*glpE*	-1.5
b3426	*glpD*	-1.9
b3433	*asd*	-1.0
b3437	*gntK*	-1.1
b3458	*livK*	1.3
b3459	*yhhK*	-1.9
b3463	*ftsE*	-1.6
b3465	*yhhF*	-1.3
b3477	*nikB*	-1.7
b3479	*nikD*	-1.0
b3482	*rhsB*	1.0
b3487	*yhiI*	-1.0
b3492	*yhiN*	-1.7
b3495	*uspA*	-1.3
b3496	*yhiP*	-1.2
b3499	*yhiR*	-1.0
b3501	*arsR*	-1.7
b3502	*arsB*	-1.3
b3503	*arsC*	1.4
b3507	*yhiF*	1.4
b3508	*yhiD*	-1.3
b3509	*hdeB*	-1.3
b3511	*hdeD*	-1.4
b3513	*yhiU*	-1.6
b3517	*gadA*	-1.3
b3522	*yhjD*	-1.5
b3524	*yhjG*	-1.2
b3527	*yhjJ*	-1.3
b3531	*yhjM*	-1.0
b3533	*yhjO*	1.0
b3539	*yhjV*	2.2
b3541	*dppD*	-1.1
b3542	*dppC*	-1.4
b3543	*dppB*	1.5
b3552	*yiaD*	-1.3
b3553	*yiaE*	-1.4
b3555	*yiaG*	-1.1
b3563	*yiaB*	-1.4
b3565	*xylA*	-1.1
b3571	*malS*	-2.0
b3576	*yiaL*	-1.2
b3580	*lyxK*	1.1
b3584	*yiaT*	-1.3
b3587	*yiaW*	-1.2
b3588	*aldB*	1.0
b3590	*selB*	-1.5
b3594	*yibA*	1.3
b3601	*mtlR*	-1.1
b3612	*yibO*	1.3
b3617	*kbl*	-1.4
b3621	*rfaC*	-1.3
b3626	*rfaJ*	-1.4
b3628	*rfaB*	2.0
b3641	*ttk*	-1.7
b3645	*dinD*	-1.0
b3647	*yicF*	-1.7
b3649	*rpoZ*	1.5
b3651	*spoU*	-1.6
b3652	*recG*	1.2
b3654	*yicE*	1.1
b3660	*yicL*	-1.5
b3662	*yicM*	-1.7
b3670	*ilvN*	-1.4
b3672	*ivbL*	-1.3
b3675	*yidG*	1.7
b3684	*yidP*	-2.0
b3687	*ibpA*	-1.7
b3704	*rnpA*	-1.0
b3709	*tnaB*	-1.3
b3710	*yidY*	-1.6
b3712	*yieE*	2.9
b3714	*yieG*	-1.4
b3723	*bglG*	-1.1
b3735	*atpH*	-1.1
b3737	*atpE*	-1.1
b3745	*yieM*	-1.6
b3747	*kup*	-1.7
b3755	*yieP*	-1.5
b3764	*yifE*	1.6
b3765	*yifB*	1.6
b3769	*ilvM*	1.0
b3771	*ilvD*	-1.0
b3774	*ilvC*	-1.4
b3778	*rep*	-1.0
b3780	*rhlB*	1.7
b3781	*trxA*	-2.4
b3787	*wecC*	1.0
b3788	*rffG*	-1.3
b3790	*wecD*	-1.2
b3793	*wecF*	-1.0
b3794	*wecG*	-1.6
b3801	*aslA*	1.0
b3804	*hemD*	1.9
b3805	*hemC*	-1.6

b3811	*xerC*	2.4
b3816	*corA*	-1.2
b3818	*yigG*	1.7
b3819	*rarD*	-1.4
b3824	*yigK*	-1.0
b3828	*metR*	1.6
b3829	*metE*	-1.4
b3831	*udp*	1.5
b3832	*yigN*	1.9
b3843	*ubiD*	1.5
b3849	*trkH*	-1.2
b3850	*hemG*	-1.2
b3857	*mobA*	-1.3
b3858	*yihD*	1.4
b3860	*dsbA*	1.5
b3861	*yihF*	1.8
b3863	*polA*	2.5
b3866	*yihI*	2.3
b3867	*hemN*	1.0
b3881	*yihT*	-1.7
b3882	*yihU*	-1.0
b3888	*yiiD*	-1.2
b3899	*frvB*	1.1
b3900	*frvA*	-2.2
b3904	*rhaB*	-1.8
b3906	*rhaR*	1.5
b3907	*rhaT*	-1.4
b3911	*cpxA*	1.0
b3919	*tpiA*	-1.4
b3927	*glpF*	-1.1
b3928	*yiiU*	-1.3
b3932	*hslV*	-1.4
b3941	*metF*	1.7
b3947	*ptsA*	-1.2
b3950	*frwB*	-1.7
b3956	*ppc*	1.8
b3957	*argE*	-1.0
b3960	*argH*	-1.6
b3962	*udhA*	1.2
b3983	*rplK*	1.1
b3991	*thiG*	-1.3
b3993	*thiE*	-1.0
b3994	*thiC*	1.6
b4001	*yjaH*	1.4
b4014	*aceB*	1.4
b4017	*arp*	1.4
b4031	*xylE*	1.0
b4044	*dinF*	1.1
b4045	*yjbJ*	-1.0
b4058	*uvrA*	1.4
b4062	*soxS*	-1.7
b4063	*soxR*	1.3
b4068	*yjcH*	-1.2
b4069	*acs*	-1.2
b4074	*nrfE*	-1.3
b4075	*nrfF*	-1.6
b4096	*phnL*	1.4
b4099	*phnI*	-1.4
b4111	*proP*	-1.1
b4116	*adiY*	1.7
b4117	*adiA*	1.2
b4139	*aspA*	1.3
b4152	*frdC*	-1.3
b4157	*yjeN*	-1.2
b4158	*yjeO*	-1.0
b4161	*yjeQ*	2.2
b4171	*miaA*	-1.0
b4172	*hfq*	1.1
b4174	*hflK*	1.4
b4176	*yjeT*	-1.0
b4178	*yjeB*	-1.4
b4188	*yjfN*	1.0
b4195	*ptxA*	-1.2
b4199	*yjfY*	1.7
b4207	*fklB*	1.2
b4208	*cycA*	1.8
b4214	*cysQ*	1.9
b4218	*ytfL*	1.7
b4224	*chpS*	1.0
b4225	*chpB*	1.9
b4231	*yjfF*	2.3
b4246	*pyrL*	2.3
b4248	*yjgH*	1.5
b4249	*yjgI*	1.5
b4253	*yjgL*	3.3
b4254	*argI*	1.5
b4255	*yjgD*	-1.1
b4263	*yjgR*	1.5
b4265	*idnT*	1.2
b4268	*idnK*	1.1
b4269	*yjgB*	1.2
b4276	*yjgY*	4.3
b4277	*yjgZ*	1.5
b4279	*yjhB*	3.1
b4289	*fecC*	2.1
b4291	*fecA*	-1.7
b4293	*fecI*	-1.7
b4298	*yjhH*	1.2
b4301	*sgcE*	-1.3
b4302	*sgcA*	2.3
b4306	*yjhP*	2.1
b4309	*yjhS*	1.2
b4314	*fimA*	1.5
b4317	*fimD*	-1.4
b4325	*yjiC*	1.5
b4326	*yjiD*	-1.4
b4327	*yjiE*	-1.4
b4329	*yjiG*	-1.9
b4330	*yjiH*	-1.4
b4332	*yjiJ*	1.0
b4334	*yjiL*	-1.2
b4341	*yjiS*	-1.5
b4346	*mcrB*	-1.6
b4347	*yjiW*	-1.1
b4358	*yjjN*	-1.2
b4359	*mdoB*	-1.1
b4365	*yjjQ*	1.3
b4374	*yjjG*	-1.4
b4380	*yjjI*	-1.0
b4383	*deoB*	-1.0
b4384	*deoD*	-1.2
b4385	*yjjJ*	-1.7
b4388	*serB*	1.2

b4392	*slt*	-1.4
b4394	*yjjX*	1.2
b4401	*arcA*	1.2
b4460	*araH*	-1.7

b4467	*glcF*	-1.3
b4474	*frlC*	-1.1
b4476	*gntU*	-1.7
b4481	*rffT*	1.0

b4482	*yigE*	1.0
b4486	*yjiV*	1.2

Table 5.S3 *E. coli* genes differentially-expressed during growth in continuous culture at 20 °C. The average log2-transformed fold-change is listed for each gene. The threshold for a gene to be considered differentially-expressed was a two-fold expression ratio with a statistical confidence of P<0.05.

Blattner Number	Gene	Fold-change (log2)
b0027	*lspA*	-1.0
b0111	*ampE*	1.6
b0122	*yacC*	-1.2
b0130	*yadE*	1.1
b0150	*fhuA*	1.0
b0156	*yadR*	-1.8
b0163	*yaeH*	-1.2
b0226	*dinJ*	1.9
b0248	*yafX*	-1.0
b0249	*ykfF*	-1.8
b0251	*yafY*	1.6
b0253	*ykfA*	1.7
b0276	*yagJ*	-1.0
b0290	*yagW*	1.3
b0320	*yahF*	1.1
b0327	*yahM*	-1.3
b0344	*lacZ*	-1.2
b0347	*mhpA*	-1.0
b0352	*mhpE*	1.2
b0367	*tauC*	-1.1
b0393	*yaiD*	-1.1
b0401	*brnQ*	-1.2
b0411	*tsx*	1.0
b0419	*yajO*	-1.1
b0427	*yajR*	-1.0
b0428	*cyoE*	-1.1
b0438	*clpX*	1.0
b0511	*allP*	1.0
b0519	*ylbE*	1.3
b0553	*nmpC*	-1.7
b0558	*ybcV*	-1.0
b0562	*ybcY*	1.0
b0565	*ompT*	-1.0
b0589	*fepG*	-1.2
b0606	*ahpF*	1.9
b0641	*rlpB*	-1.2
b0649	*ybeV*	1.1
b0675	*nagD*	-1.0
b0685	*ybfE*	-1.1
b0689	*ybfP*	-1.0
b0695	*kdpD*	-1.1
b0742	*ybgF*	1.1
b0770	*ybhI*	-1.1
b0774	*bioA*	-1.0
b0819	*ybiS*	1.4
b0854	*potF*	-1.5
b0865	*ybjP*	-1.1
b0909	*ycaL*	1.3
b0916	*ycaQ*	-1.0
b0929	*ompF*	-1.0
b0964	*yccT*	1.8
b0980	*appA*	-1.2
b0992	*yccM*	-1.8
b1005	*ycdF*	1.1
b1013	*ycdC*	1.0
b1014	*putA*	-1.3
b1034	*ycdX*	-1.1
b1042	*csgA*	-1.2
b1115	*ycfT*	-1.0
b1131	*purB*	-1.2
b1194	*ycgR*	-1.1
b1198	*ycgC*	1.0
b1222	*narX*	1.0
b1237	*hns*	1.4
b1243	*oppA*	1.5
b1257	*yciE*	-1.0
b1258	*yciF*	-1.0
b1281	*pyrF*	1.1
b1302	*goaG*	-1.0
b1307	*pspD*	-1.2
b1313	*ycjQ*	-1.1
b1346	*ydaQ*	-1.0
b1428	*ydcK*	1.0
b1442	*ydcU*	1.2
b1452	*yncE*	-1.0
b1497	*ydeM*	1.0
b1572	*ydfB*	1.1
b1599	*ydgE*	-1.0
b1644	*ydhJ*	-2.1
b1659	*ydhB*	-1.0
b1668	*ydhS*	1.0
b1679	*ynhA*	1.1
b1685	*ydiH*	1.2
b1709	*btuD*	1.3
b1712	*himA*	1.2
b1790	*yeaM*	-1.1
b1802	*yeaW*	-1.0
b1803	*yeaX*	1.0
b1825	*yebO*	1.3
b1851	*edd*	1.3
b1870	*yecO*	1.2
b1908	*yecA*	-1.1
b1957	*yodC*	-1.3
b1958	*yedI*	1.0
b2029	*gnd*	-1.0
b2077	*yegB*	-1.5
b2096	*gatY*	1.0
b2137	*yohF*	-1.0
b2142	*yohK*	1.4
b2163	*yeiL*	1.1
b2165	*yeiN*	1.4
b2166	*yeiC*	1.4
b2170	*yeiO*	1.0
b2209	*eco*	-1.0
b2252	*ais*	1.4
b2257	*arnT*	1.3
b2260	*menE*	-1.0
b2265	*menF*	-2.6
b2287	*nuoB*	-1.4
b2289	*lrhA*	-1.0
b2312	*purF*	1.1

b2346	*vacJ*	1.1
b2347	*yfdC*	1.1
b2457	*cchA*	-1.3
b2493	*perM*	-1.1
b2502	*ppx*	-1.0
b2537	*hcaR*	-1.1
b2540	*hcaC*	-1.6
b2555	*yfhG*	-1.2
b2571	*rseB*	-1.2
b2573	*rpoE*	-1.3
b2597	*yfiA*	1.0
b2631	*yfjO*	-1.3
b2662	*gabT*	1.1
b2664	*ygaE*	-1.2
b2666	*yqaE*	-1.3
b2669	*stpA*	-1.4
b2706	*gutM*	-1.0
b2720	*hycF*	-1.1
b2723	*hycC*	-2.0
b2732	*ygbA*	-1.1
b2745	*ygbO*	-1.3
b2783	*chpR*	-1.0
b2828	*lgt*	1.6
b2927	*epd*	1.1
b2937	*speB*	-1.3
b2946	*yggJ*	-1.0
b2968	*yghD*	1.1
b2975	*yghK*	-1.1
b3002	*yqhA*	-1.8
b3003	*yghA*	-1.3
b3031	*yqiA*	-1.2
b3049	*glgS*	-1.2
b3068	*ygjF*	-1.7
b3075	*ebgR*	-1.0
b3152	*yraR*	-1.3
b3161	*mtr*	-1.1
b3185	*rpmA*	-1.0
b3186	*rplU*	-1.0
b3195	*yrbF*	-1.0
b3198	*yrbI*	-1.0
b3222	*yhcI*	1.2
b3230	*rpsI*	-1.0
b3250	*mreC*	-1.1
b3265	*acrE*	-1.1
b3300	*prlA*	-1.3
b3305	*rplF*	-1.1
b3316	*rpsS*	-1.8
b3320	*rplC*	-1.0
b3344	*yheM*	-1.5
b3351	*yheR*	-1.8
b3358	*yhfK*	-1.0
b3368	*cysG*	-1.0
b3394	*yrfC*	1.0
b3395	*yrfD*	-1.0
b3416	*malQ*	-1.7
b3449	*ugpQ*	1.1
b3532	*yhjN*	-1.0
b3562	*yiaA*	-1.2
b3586	*yiaV*	1.1
b3590	*selB*	-1.4
b3618	*htrL*	1.1
b3646	*yicG*	-1.4
b3654	*yicE*	-1.3
b3681	*glvG*	-1.2
b3715	*yieH*	-1.5
b3729	*glmS*	-1.0
b3733	*atpG*	-1.3
b3752	*rbsK*	-1.4
b3780	*rhlB*	1.2
b3793	*wecF*	-1.0
b3804	*hemD*	1.0
b3822	*recQ*	-1.5
b3827	*yigM*	-1.3
b3860	*dsbA*	1.5
b3865	*yihA*	-1.2
b3940	*metL*	1.0
b3956	*ppc*	-1.0
b3972	*murB*	-1.1
b3983	*rplK*	-1.2
b4049	*yjbN*	1.1
b4071	*nrfB*	1.2
b4105	*phnD*	-1.5
b4148	*sugE*	-1.1
b4150	*ampC*	-1.3
b4166	*yjeS*	-1.3
b4190	*yjfP*	1.3
b4199	*yjfY*	1.1
b4211	*ytfG*	-1.1
b4236	*cybC*	1.0
b4252	*yjgK*	1.1
b4265	*idnT*	1.4
b4275	*yjgX*	1.5
b4276	*yjgY*	1.1
b4279	*yjhB*	1.0
b4281	*yjhD*	-1.1
b4295	*yjhU*	1.0
b4332	*yjiJ*	-1.0
b4339	*yjiQ*	-1.1
b4341	*yjiS*	-1.1
b4364	*yjjP*	-1.0
b4391	*yjjK*	-1.3
b4398	*creB*	1.0
b4411	*ecnB*	1.2

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Chapter 6
Infectivity and intracellular survival of *Mycobacterium avium* in environmental *Acanthamoeba* strains and dynamics of inactivation with monochloramine

6.1 Abstract

Infections of several *Acanthamoeba* strains with *Mycobacterium* avium were examined to determine the infectivity, stability, and viability of intracellular *M. avium* in *Acanthamoeba* hosts for a variety of conditions. M. avium was able to infect all tested *Acanthamoeba* strains, and one strain, *Acanthamoeba castellanii* Neff, was used to study how frequently acanthamoebae are infected with *M. avium* during grazing of multispecies bacterial consortia containing this bacterial pathogen. Kinetics of inactivation during exposure to the drinking water disinfectant monochloramine were determined for *M. avium* and *A. Castellannii* Neff. Intracellular *M. avium* exhibited greater resistance to the disinfectant than free living *M. avium*, and the inactivation kinetics of *M. avium* inside *A. castellanii* Neff were similar to inactivation kinetics of *A. castellanii* Neff, suggesting that acanthamoebae inactivation is a useful conservative surrogate for intracellular *M. avium* inactivation.

6.2 Introduction

The interaction of protozoa with bacteria, including pathogenic bacteria, is a potentially very important ecological relationship that has received little attention in drinking water treatment research. Acanthamoebae are a group of protozoa widely distributed in the environment - they are present in water, soil, and air (Marciano-Cabral and Cabral, 2003) - and generally function ecologically as predators of bacteria (Rodríguez-Zaragoza, 1994). However, numerous types of bacteria are resistant to predation by acanthamoebae, many of which are also human pathogens. The ability of bacteria to

utilize amoebae as hosts has lead to the development of the theory that amoebae acts as a "training ground"(Molmeret et al., 2005) or "biological gymnasium" (Dixon, 2006) for the development of human bacterial pathogens. One such amoeba-resisting bacterium is *Mycobacterium avium*.

Mycobacterium avium complex (MAC) includes a number serovars of closely related species that are important agents of opportunistic infections in immunocompromised individuals (Inderlied et al., 1993; Primm et al., 2004). Environmental mycobacteria have been associated with high incidence rates of nosocomial infections (Vaerewijck et al., 2005) and *M. avium* is responsible for several types of infections including pulmonary, bacteremia in AIDS patients, cervical lmphadenitis in children, and tenosynovitis (Falkinham, 2002). Potentially pathogenic mycobacteria have been observed in drinking water and drinking water distribution systems (Falkinham et al., 2001; Torvinen et al., 2004), and *Mycobacterium avium* subspecies *paratuberculosis* (Map) has been observed to persist for long durations in drinking water and in drinking water sources (Pickup et al., 2005; Whittington et al., 2005).

It is known that *M. avium* can persist within both *Acanthamoeba polyphaga* trophozoites and cysts (Steinert et al., 1998). Several strains of *M. avium* have also been observed to replicate within *Acanthamoeba castellanii* for at least 9 days when cultured at temperatures above 24 °C (Cirillo et al., 1997). *Acanthamoeba* cultures undergo many physiological changes after several passages in the laboratory (Mazur and Hadaś, 1994; Hughes et al., 2003; Koehsler et al., 2008), but it has not been determined if there is reduced infectivity of *M. avium* in recent *Acanthamoeba* isolates, which is important for assessing the relevance of associations between *Acanthamoeba* and *M. avium* in the environment.

Acanthamoebae are resistant to inactivation with a range of disinfectants, including chlorine (De Jonckheere and van de Voorde, 1976; Cursons et al., 1980; Dawson and Brown, 1987; Thomas et al., 2004), chlorine dioxide (Cursons et al., 1980; Dawson and Brown, 1987; Thomas et al., 2004), monochloramine (Thomas et al., 2004), and ozone

(Cursons et al., 1980; Thomas et al., 2004). Bacteria within acanthamoebae are shielded from exposure to disinfectants (King et al., 1988). *M. avium* growing within *A. castellanii* is more resistant to the antimicrobials rifabutin, azithromycin, and clarithromycin (Miltner and Bermudez, 2000). Map surviving within *A. polyphaga* is more resistant to 2 mg/L of free chlorine than pure cultures of Map (Whan et al., 2006) and survival within *Acanthamoeba* hosts has been observed to increase bacterial resistance to free chlorine (King et al., 1988; Howard and Inglis, 2005) and monochloramine (Howard and Inglis, 2005) for a variety of bacteria. Disinfection studies typically examine inactivation of either bacteria or acanthamoebae, so an understanding of the relative rates and characteristics of inactivation are not well understood.

The present research was undertaken to extend knowledge of interactions of *M. avium* with acanthamoebae, particularly to elucidate the role of different *Acanthamoeba* strains (both laboratory strains and recent environmental isolates) in *M. avium* infectivity and infection stability. *M. avium* infections in eight *Acanthamoeba* strains (four laboratory strains and four recent environmental isolates) were monitored for 28 days under high nutrient and low nutrient conditions. Since infections occur in the environment during grazing of acanthamoebae on bacteria, the infectivity of *M. avium* when present at different proportions within a multispecies microbial consortium was also examined. Finally, inactivation kinetics upon exposure to the drinking water disinfectant monochloramine were compared for *M. avium* in pure culture, *M. avium* in co-culture within *A. castellanii* Neff, and *A. castellanii* Neff in pure culture.

6.3 Results and discussion

6.3.1 *M. avium* infections in *Acanthamoeba* strains

M. avium was able to infect all tested *Acanthamoeba* strains (Fig. 6.5), with the proportion of infected amoeba (P_I) values varying between 0.33 and 0.77 (Fig. 6.2a) and the number of *M. avium* cells per infected amoeba (N_C) values varying between 1.5 and 18.4 for all strains and conditions (Fig. 6.2b). This is within the range of 1-20 previously found for the number of *M. avium* serotype 4 cells per infected *A. polyphaga* cell (Steinert et al., 1998) and similar to *M. avium* serotype 1 cells per infected *A. castellanii* cell (Cirillo et al., 1997). Infections persisted in all eight *Acanthamoeba* strains for the

duration of the four week experiment. Survival of *M. avium* within acanthamoebae has previously been observed for *M. avium* serotype 4 for 14 days within *A. polyphaga* (Steinert et al., 1998), and *M. avium* subspecies *avium* for 15 days within *A. polyphaga* (Drancourt et al., 2007). During the four week experiment in the current study, the concentration of viable *M. avium* in the co-culture medium (N_V) remained relatively constant (2.4-9.4*104 cfu/mL), suggesting that *M. avium* remains viable during long-term infections, but that it exhibits little or no net positive growth (Fig. 6.2c). This result agrees with an observation of *M. avium* serotype 4 within *A. polyphaga* exhibiting no net growth (Steinert et al., 1998), although Cirillo et al. report replication of several *M. avium* strains at temperatures above 24 °C (Cirillo et al., 1997).

A three-way ANOVA was used to determine the statistical significance of the explanatory variables: 'amoeba strain', 'type of culture medium' (a nutrient rich medium (PYG) vs. a non-nutrient buffer (PAS)), and 'time post-infection' (0, 7, 14, 21, and 28 days). PYG is a typical nutrient rich laboratory medium used for *Acanthamoeba* cultivation, whereas PAS is a non-nutrient salts buffer which should induce starvation conditions typical of oligotrophic aquatic systems. Interestingly, the variable 'type of culture medium' did not have a statistically significant effect on P_I, N_C, or N_V ($P>0.05$ for all cases). This provides evidence that long-term stable associations between *M. avium* and acanthamoebae are possible in low nutrient aquatic environments such as oligotrophic freshwater and drinking water. The variable 'type of culture medium' was removed from the ANOVA model and further analysis was simplified to a two-way ANOVA. P_I, N_C, and N_V all varied with time post-infection ($P<0.05$), but no steady increase or decrease in values was observable over the four week period. 'Amoeba strain' was also an important factor explaining variance for all three dependent variables ($P<0.05$), indicating that different amoeba strains have appreciably different tolerances to *M. avium* infection. The eight strains tested were divided into two groups of four based on whether they had been passaged many times in the laboratory or had been isolated recently from the environment. This analysis was performed to determine whether recent environmental isolates are more resistant to infection, or differ in other infection characteristics from commonly used model strains. To test whether recent environmental isolates have an increased tolerance against infection, a two-way ANOVA was conducted

using only 'time post-infection' and 'type of amoeba strain' (laboratory strain vs. recent isolate) as explanatory variables. The 'type of amoeba strain' was found to be statistically significant in this analysis ($P<0.05$). PI was clearly affected by the type of amoeba strain, with recent environmental isolates exhibiting a consistently lower level of infection in co-culture (Fig. 6.2a). N_C was greater for laboratory strains than recent isolates at all time points, although a decrease in N_C in the laboratory strains was observed from day 7 to day 14, followed by an increase during the second 14 days of the experiment (Fig. 6.2b). Similarly, a previous report found that a reduction in the number of Map in the first several days of infection of *A. polyphaga* was followed by a recovery in numbers, as measured using qPCR (Mura et al., 2006). The effect of strain type on N_V was not as straightforward. Initially, laboratory strains had a higher N_V than recent isolates, but on days 7 and 14, the opposite was observed. On days 21 and 28, there was no significant difference between the two strain types (Fig. 6.2c). Interestingly, the drop in N_V in laboratory strain co-cultures, which took place between days 1 and 7, preceded the reduction in N_C for laboratory strains between days 7 and 14, suggesting that some of the *M. avium* cells observed in P_I and N_C measurements were inactivated by the amoebae during the first 7 days of the experiment. Subsequent digestion by acanthamoebae would then explain the drop in P_I and N_C between days 7 and 14 (Figs. 6.2a and 6.2b).

6.3.2 Infection of *Acanthamoeba* strains during grazing on mixed-species consortia

Intracellular survival is one mechanism of bacterial resistance to protozoon predation (Matz and Kjelleberg, 2005), so it is expected the *M. avium* infections would be acquired during acanthamoebal grazing. In order to evaluate the infectivity of *M. avium* in mixed-species consortia subject to acanthamoebal grazing, *A. castellanii* Neff was allowed to graze for 60 hours on two different consortia: a mixture of fluorescently-labeled *M. avium* spiked in different proportions into (i) a pure culture of *Escherichia coli* and (ii) a complex microbial community from a laboratory-scale biologically-active carbon (BAC) filter used for drinking water treatment (bacterial community composition described in (Li et al., 2009)). *M. avium* was spiked into these consortia at different concentrations to a final amount of between 1-83% of the total consortia (determined by mass). The P_I value after 60 hours of grazing was affected by the proportion of *M. avium* in the consortia: it increased linearly with an increasing concentration of *M. avium* ($R^2=0.97$),

but remained below approximately 25% (Fig. 6.3). The P_I value was much greater ($P<0.001$) for grazing experiments with pure cultures of *M. avium*, averaging 0.79 (95% CI [0.75, 0.83]), which suggests that in the presence of edible bacteria *A. castellanii* is able to avoid infection by *M. avium*. *E. coli* is known to be an excellent food source for acanthamoebae (Weekers et al., 1993; Josué de and Silvia, 2008), so these results indicate that *A. castellanii* is preferentially feeding on edible bacteria. Selective grazing of protozoa is a well-studied phenomenon (Hahn and Höfle, 2001) and selective grazing of *A. castellanii* has been observed to alter the bacterial community composition of a soil microcosm (Ronn et al., 2002). This density-dependent behavior of infection during grazing of multispecies consortia is consistent with the concept that not every interaction between bacterial pathogen and host yields an infection, but that the probability of infection increases with the density of the bacterial pathogen because there are more opportunities for infection. Declerck and co-workers previously observed that the infection intensity of *A. castellanii* with *Legionella pneumophila* was reduced when other bacteria were present, although they did not evaluate different ratios of *L. pneumophila* to other bacteria (Declerck et al., 2005). Relationships between the proportion of infected amoebae as a function of the proportion of *M. avium* in the consortium were remarkably similar for the simple, dual species bacterial consortium with *E. coli*, and for the BAC community. This may suggest that the abundance of *M. avium* in a consortium is more important than the overall community composition for predicting grazing-acquired acanthamoebae infection levels.

6.3.3 Inactivation kinetics of *M. avium* and *A. castellanii* Neff

Inactivation kinetics were determined for *M. avium* alone, intracellular *M. avium* (within *A. castellanii* Neff), and *A. castellanii* Neff when exposed to 5 mg/L (as Cl_2) of the commonly used drinking water disinfectant monochloramine (Figure 6.4). This concentration of monochloramine was chosen because it is close to the US EPA Maximum Residual Disinfectant Level of 4 mg/L of chloramines (as Cl_2) (http://www.epa.gov/ogwdw/disinfection/chloramine/pdfs/chloramine2.pdf) and has been shown previously to effectively inactivate *M. avium* under similar conditions (Luh et al. 2008). Inactivation of *M. avium* alone proceeded as a pseudo first-order Chick-Watson reaction (Watson 1908) (Equation 6.1):

$$N/N_0 = e^{-kCt} \quad (6.1)$$

where N/N_0 is the fraction of organisms surviving, k is the rate of inactivation, C is the concentration of disinfectant, and t is time of exposure to the disinfectant. The pseudo-first order rate constant (k = 0.0126 $L*mg^{-1}*min^{-1}$, R^2 = 0.97) was close to the value previously reported (k = 0.0123 $L*mg^{-1}*min^{-1}$) for conditions similar to the current study (Luh et al. 2008). Intracellular *M. avium* and *A. castellanii* Neff inactivation both followed biphasic kinetics characterized by a pseudo first-order reaction during the first 90 min (intracellular *M. avium*: k = 0.0054 $L*mg^{-1}*min^{-1}$, R^2 = 0.94; *A. castellanii* Neff: k = 0.0038 $L*mg^{-1}*min^{-1}$, R^2 = 0.99) followed by a slower pseudo first-order inactivation (intracellular *M. avium*: k = 0.0011 $L*mg^{-1}*min^{-1}$, R^2 = 0.77; *A. castellanii* Neff: k = 0.0010 $mg^{-1}min^{-1}$, R^2 = 0.98), a phenomenon known as "tailing", which is commonly observed in disinfection of bacterial spores (Cerf 1977) and protozoon (oo)cysts (Craik et al. 2001). Tailing of acanthamoebae cultures has been observed for inactivation with free chlorine, chlorine dioxide, ozone (Loret et al. 2008), polyaminopropyl biguanide (Burger et al. 1994), and UV (Maya et al. 2003; Hijnen et al., 2006). Interestingly, the tailing observed in the current study begins around the same time for intracellular *M. avium* and *A. castellanii* Neff, suggesting that mechanisms which protect *A. castellanii* Neff from inactivation, such as cyst formation, are also responsible for protection of *M. avium*.

6.4 Conclusion

This work has demonstrated that *M. avium* can infect a range of *Acanthamoeba* strains, including recent environmental isolates, and maintains its viability within acanthamoebae, remaining culturable for a period of at least 28 days. *M. avium* infections were stable even under low nutrient conditions, suggesting that this is a phenomenon relevant to survival within drinking water. Interestingly, infections can also occur during grazing of acanthamoebae on multispecies bacterial consortia, though to a lower level than when *M. avium* is the only bacteria present. Intracellular *M. avium* was found to be much more resistant to monochloramine than *M. avium* alone, and the inactivation kinetics of intracellular *M. avium* exposed to monochloramine closely matched the inactivation kinetics of *Acanthamoeba castellanii* Neff. Taken together,

these results suggest that acanthamoebae-facilitated fate and transport processes of *M. avium* may be an important phenomenon in health-related water microbiology. Further work is needed to determine the relevance of our observations in actual water treatment and distribution systems. More generally, more research is needed to understand the importance of amoeba-bacterial pathogen interactions in the context of drinking water treatment. The results of the monochloramine inactivation kinetics in the present study suggest that acanthamoebal inactivation may be a useful surrogate for intracellular *M. avium* inactivation. While additional work is necessary to determine whether this correlation between amoebae host and intracellular bacterial inactivation holds for other intracellular bacterial pathogens, it is suggested to be a simple and useful conservative indicator of bacterial pathogen inactivation.

6.5 Materials and methods

6.5.1 Strains and growth conditions

Mycobacterium avium subsp. *hominissuis* 104, an isolate from an AIDS patient (Bermudez et al., 1997), was provided by Gerard Cangelosi (Seattle Biomedical Research Institute, Seattle, Washington), where the strain was verified using a large sequence polymorphism (LSP)-based genotyping test (Horan et al., 2006) (personal communication). *M. avium* was cultured on Middlebrook 7H9 broth (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) supplemented with Middlebrook OADC medium (oleic acid, albumin, dextrose, catalase (Sigma-Aldrich Chemie GmbH, Steinheim, Germany)) at 37 °C for 5 days. Prior to infection of acanthamoebae, the bacterial cells were washed in 1X phosphate-buffered saline (PBS) and passed through a 26 gauge needle ten times to disperse aggregates. *M. avium* cultures were inspected microscopically to monitor dispersal of aggregates and quantified using a cell counting chamber (C-Chip DHC-F01, INCYTO Co., Cheonan, Korea). *Acanthamoeba* strains were cultured axenically in tryptic soy yeast broth (TSY) and were found to be free of endosymbionts using DAPI staining (Heinz et al., 2007). *Acanthamoeba* isolates were isolated less than two months before the experiments and were passaged no more than three times in this period. 10^5 *Acanthamoeba* cells were inoculated into individual wells of 24-well plates. Acanthamoebae were given one hour to attach and form mono-layers

and then *M. avium* was added at a multiplicity of infection of 10:1. Multi-well plates were centrifuged at 164 x g for 15 min at 20 °C to facilitate uptake of bacteria. Following centrifugation, acanthamoebae were washed with Page's amoeba saline buffer (PAS) to remove extracellular bacteria, followed by a two hour amikacin (100 µg/ml) antibiotic treatment, which was previously determined to not have a cytotoxic effect on acanthamoebae or intracellular *M. avium* (Cirillo et al., 1997). Co-cultures were washed with PAS and then either TSY or PAS (for the starvation assay) was added. Co-cultures were incubated at 20 °C in the dark and were washed and treated with amikacin every week to minimize the possibility of bacterial growth outside of acanthamoebae. Cultures were monitored daily throughout the experiment using phase-contrast microscopy and no growth of *M. avium* outside of acanthamoebae was observed.

6.5.2 *Acanthamoeba* strains, sequencing, and phylogenetic analysis

Eight *Acanthamoeba* strains were used in this study, four of which were recently isolated from a number of environments (biofilm from a drinking water distribution system, forest soil, and marsh sediment) and four "laboratory strains" which had been passaged many times on nutrient-rich media (Table 6.1). The strains were classified to genotype according to the 95% sequence similarity threshold for 18S rRNA genes (Stothard et al., 1998). Six of the eight strains were members of sequence Type T4, which is the most commonly found sequence type and is known to harbor bacterial symbionts (Schmitz-Esser et al., 2008). Of these six strains, three had not previously been classified: *Acanthamoeba* sp. DWDS, *Acanthamoeba* sp. MSA, and *Acanthamoeba* sp. MSC. *Acanthamoeba* sp. F2B had not previously been classified and was classified as Type T13, a sequence type which also harbors bacterial symbionts (Horn et al., 1999). *Acanthamoeba* hatchetii was classified previously as Type T11 (Walochnik et al., 2000) (Fig. 6.1). All of the strains were determined to be free of symbionts prior to the M. avium infection assays. All eight *Acanthamoeba* strains were infected with M. avium at a multiplicity of infection of 10:1 and then cultured on nutrient rich (PYG) and nutrient poor (PAS) media for four weeks. P_I, N_C, and N_V were determined at the start of the experiment and monitored weekly for four weeks.

DNA was extracted from *Acanthamoeba* strains as described previously (Heinz et al., 2007) and PCR was conducted targeting a fragment of the 18S rRNA gene using either the *Acanthamoeba* -specific primer pair JDP1 (5'-GGCCCAGATCGTTTACCGTGAA) and JDP2 (5'-TCTCACAAGCTGCTAGGGAGTCA) (Schroeder et al., 2001) or a general *Eukarya* primer pair 18S-U16F (5'-AACCTGGTTGATCCTGCCAGT) and 18S-U1511R (5'-GATCCTTCTGCAGGTTCACCTAC) as described previously (Gast, 2006). Amplified target DNA was cloned using the Topo TA kit (Invitrogen, Carlesbad, CA) and then sequenced with an ABI 3130xl DNA sequencer using the BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA). The nearest matching sequence in public databases was determined using a nucleotide BLAST search of the GenBank database (accessed February 2009). Phylogenetic analysis was performed using ARB software (Ludwig et al., 2004) and the TREE PUZZLE algorithm (HKY nucleotide substitution model) (Strimmer and von Haeseler, 1996). A filter was imposed to only compare positions conserved in the majority of all amoebal 18S rRNA gene sequences.

6.5.3 Harvesting, staining, and plating

Acanthamoeba cultures were harvested by repeatedly pipetting the supernatant up and down to disperse *Acanthamoeba* mono-layers. The harvested cells were centrifuged at 4,427 x g for 10 min at 4 °C and washed with PAS. A portion of the culture was used for acid-fast staining using a modified Ziehl-Neelson staining protocol (Giménez, 1964). Briefly, cells were heat-fixed onto glass slides and stained with a hot carbol-fuchsin solution (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for 15 min and then washed with a 1% HCl ethanol solution for 2 min and rinsed in distilled water. The number of infected amoebae and the number of intracellular mycobacteria were counted using a 100X objective microscope (Axioplan 2, Carl Zeiss Microimaging GmbH, Jena, Germany), and at least 50 amoebae were counted for each time point. Phenolic acridine orange fluorescence staining (Smithwick et al., 1995) was also used in combination with confocal laser scanning microscopy (CLSM) for localization of intracellular M. avium. Briefly, cells were incubated for 15 min with a staining solution of 50 mg/ml phenol and 1 mg/ml acridine orange and then washed with an acid alcohol de-staining and counterstaining solution (70% ethanol, 19% hydrochloric acid, and 2 mg/ml methylene

blue) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Dried samples were mounted using VECTASHIELD (Vector Laboratories, Inc., Burlingame, CA) and imaged at 100X objective using a CLSM with two helium-neon-lasers (543 nm and 633 nm) and an argon laser (458-514 nm) (LSM 510 Meta, Carl Zeiss Microimaging GmbH, Jena, Germany). For mycobacteria viability assays, the harvested acanthamoebae were re-suspended in PYG buffer and lysed using a 3-min vortexing step with sterile glass beads. The cells were then centrifuged at 164 x g for 5 min to separate the acanthamoebae and mycobacteria. The supernatant was passed through a 26 gauge needle ten times to disperse mycobacteria aggregates and was plated in duplicate on Middlebrook 7H9 agar supplemented with Middlebrook OADC (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for 14 days before enumeration of colony-forming units.

6.5.4 Inactivation kinetics assays

Monochloramine was prepared as described previously (Berry et al., 2009) and was quantified using the DPD titrimetric method (Eaton et al., 1995) and did not change significantly between the start and end of each experiment. To test the inactivation kinetics of *M. avium* alone, the bacteria were cultured as described above, then pelleted, washed, and resuspended in PBS (pH 8.0) at a concentration of approximately 107 cfu/mL and exposed to 5 mg/L monochloramine (as Cl2) for several time durations at a temperature of 20 °C. Monochloramine was quenched by the addition of 0.12% sodium thiosulfate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and dilutions of the samples collected were plated as described above in duplicate to monitor changes in viability. To monitor inactivation kinetics of intracellular *M. avium*, acanthamoebae were first infected as described above, including media exchange and amikacin treatment to minimize viable extracellular bacteria. Infected acanthamoebae were then pelleted, washed, and re-suspended in PBS (pH 8.0) at a concentration of approximately 106 cells/mL and exposed to 5.0 mg/L monochloramine (as Cl2) for several time durations. Monochloramine was quenched by the addition of 0.12% sodium thiosulfate. In order to lyse amoebae cells, 500 mg sterile glass beads were added to each sample and tubes were vortexed for 3 min, followed by several passages through a 26 gauge needle (B. Braun Melsungen AG, Melsungen, Germany). Supernatant was then plated in duplicate as described above.

Acanthamoeba viability tests were conducted as previously described (Haider et al.) using the fluorescent nucleic acid stain propidium iodide (PI), which is excluded by viable cells and only penetrates cells whose membrane integrity is disrupted, and therefore considered dead. *A. castellanii* Neff cells were seeded in 8-well Lab-Tek™ Chambered Coverglass (Nunc, Roskilde, Denmark) containing 300 µl medium per well. After attachment of acanthamoebae, cells were rinsed once with 1x PBS and exposed to 5 mg/L monochloramine (as Cl2) for several time durations. Monochloramine was quenched by the addition of 0.12% sodium thiosulfate and 150 µl of a 1.5 µM PI solution (Molecular Probes Inc., Eugene, OR) in 1x PBS (1:1000 dilution of stock solution) was added per well. The cells were exposed to PI in the dark at room temperature (~21 °C) for 50 min and subsequently inspected by inverse fluorescence microscopy using a confocal laser scanning microscope (LSM 510 Meta, Carl Zeiss Microimaging GmbH, Jena, Germany). Fluorescence was monitored by excitation at 485 nm and emission at 580 nm.

6.5.5 Multispecies grazing assays

M. avium was stained with a stable intracellular fluorescent dye according to the manufacturer's instructions (Vybrant CFDA Cell Tracer Kit, Molecular Probes Inc., Eugene, OR). Fluorescence was stable for at least one week (data not shown). Fluorescently-labeled *M. avium* were mixed with either *E. coli* or a complex microbial community harvested from a laboratory-scale BAC filter treating drinking water contaminated with nitrate and perchlorate (this system is described in detail in (Li et al., 2009)). Proportions of *M. avium* in spiked consortia ranged from 0.01 to 0.83. The proportions were calculated on the basis of wet biomass weight (calculated as the weight of the wet cell pellet) because the morphological heterogeneity and presence of aggregates in the BAC community did not allow for cell counting. Mixtures were spread evenly on non-nutrient agar plates and *A. castellanii* Neff were added to the plates and allowed to graze for 60 hours in the dark at room temperature (~21 °C). After the grazing period, the numbers of total and infected acanthamoebae were quantified using epifluorescence microscopy (10X objective). At least 300 acanthamoebae were counted for each plate, and tests were conducted in triplicate.

6.5.6 Data analysis

All data analyses were performed with SPSS Statistics 17.0 (SPSS Inc., Chicago IL).

6.5.7 Accession numbers

Newly determined partial 18S rRNA gene sequences were deposited in GenBank under the Accession Nos. FJ807647–FJ807651.

6.6 Tables and figures

Table 6.1 *Acanthamoeba* strains used in this study. The environments from which recently isolated strains were obtained are briefly described. For strains not previously in GenBank, the database strain with the greatest 18S rRNA gene sequence similarity to the submitted strain is listed.

***Acanthamoeba* sp. Isolate and ATCC no.**	**Source**	**18S rRNA gene GenBank accession no.**	**Greatest 18S rRNA gene sequence similarity (sequence similarity and Genbank accession no.)**	**Reference**
A. castellanii Neff (ATCC 50373)	Lab strain	U07416	N.A.	(Gast et al. 1996)
A. sp. UCW1	Lab strain	AM941721	N.A.	Horn, M, unpublished data
A. hatchetti 4RE	Lab strain	AF251937	N.A.	(Walochnik et al. 2000)
A. polyphaga DOME	Lab strain	FJ807648	*Acanthamoeba* sp. ACA10 (99%, AF132136)	Horn, M, unpublished data
A. sp. DWDS	Biofilm from drinking water distribution system, Ann Arbor, MI (residual disinfectant is chloramine)	FJ807647	*Acanthamoeba polyphaga* Nagington (98%, AF019062)	This study
A. sp. MSA	Marsh sediment, Austria	FJ807650	*Acanthamoeba* sp. ATCC 30868 (AC021) (99%, AY549558)	This study
A. sp. MSC	Marsh sediment, Austria	FJ807651	*Acanthamoeba* sp. ATCC 30868 (AC021) (99%, AY549558)	This study
A. sp. F2B	Forest soil, Austria	FJ807649	*Acanthamoeba* sp. UWET39 (97%, AF132136)	This study

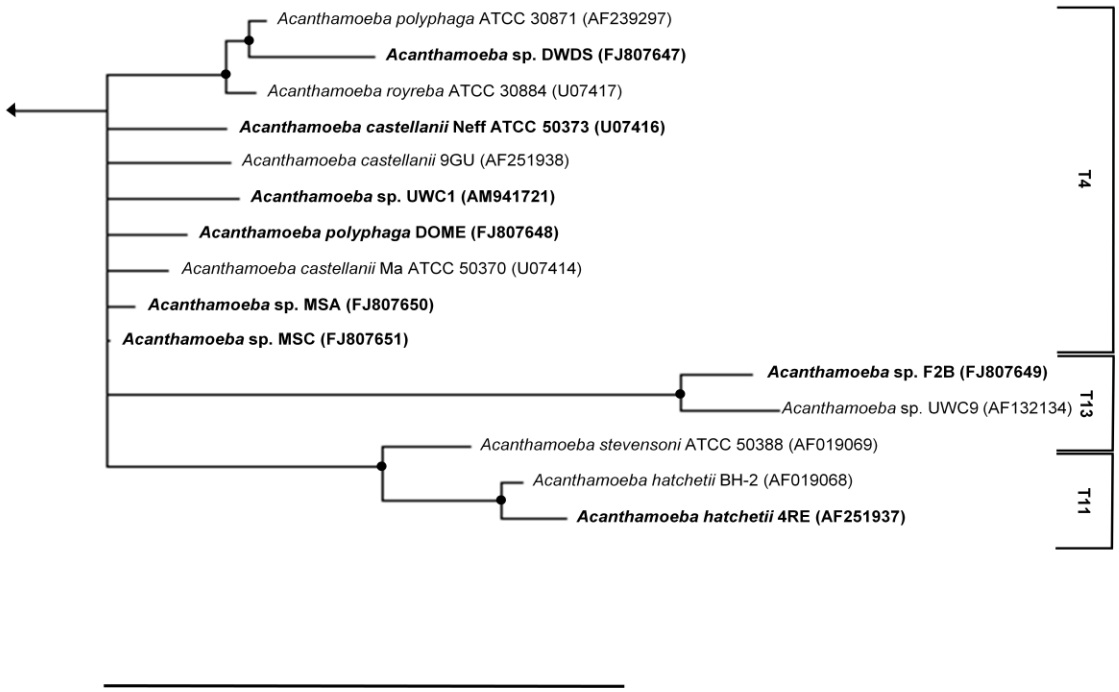


Figure 6.1 An 18S rRNA gene TREE-PUZZLE phylogenetic tree of *Acanthamoeba* strains used in this study (in bold font) and related strains. Black dots indicate at least 75% bootstrap support (1,000 runs) and the scale bar at the bottom indicates 5% sequence divergence. Sequence Type classifications are indicated by the brackets on the right.

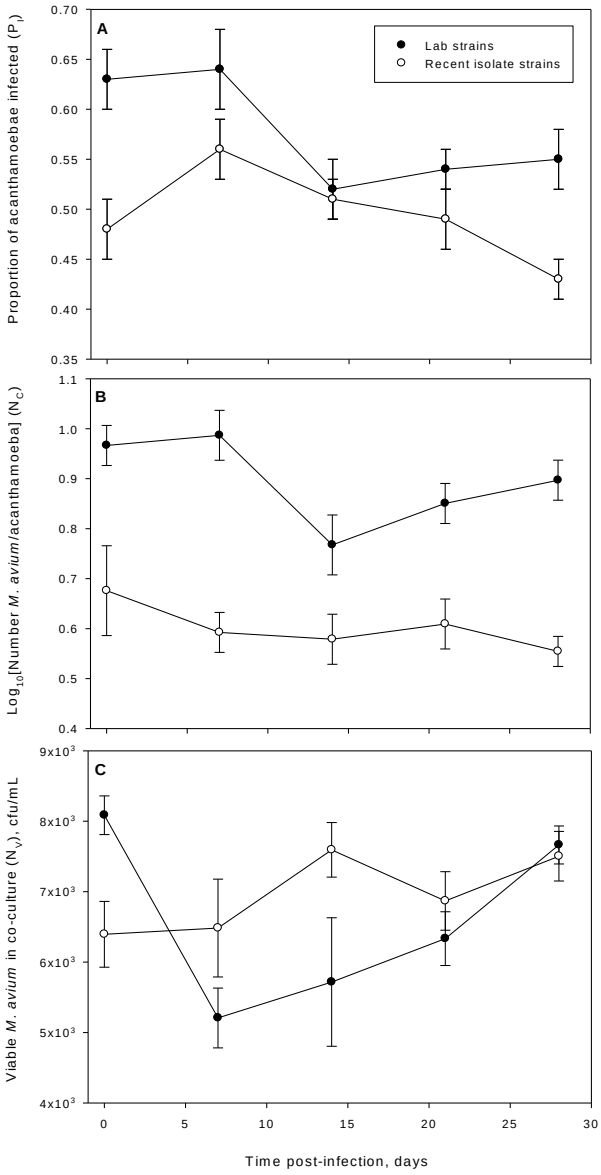


Figure 6.2. Infection dynamics of Mycobacterium avium within lab strains (black dots) and recent environmental isolates (white dots) of Acanthamoeba. (A) Proportion of Acanthamoeba strains infected after initial infection, (B) average number of M. avium cells per infected Acanthamoeba cell over time, and (C) viability of M. avium in co-culture with Acanthamoeba strains over time. Data points are mean average values for four strains of Acanthamoeba (either recent environmental isolates or laboratory strains), and each strain was tested in triplicate. Viability was assessed as colony forming units (cfu) per mL of culture medium in co-culture and samples at each time point were plated in duplicate. ANOVA analysis indicated that the variables 'time' and 'strain type' were significant explanatory factors for all response variables presented ($P<0.05$). Error bars indicate 95% confidence intervals.

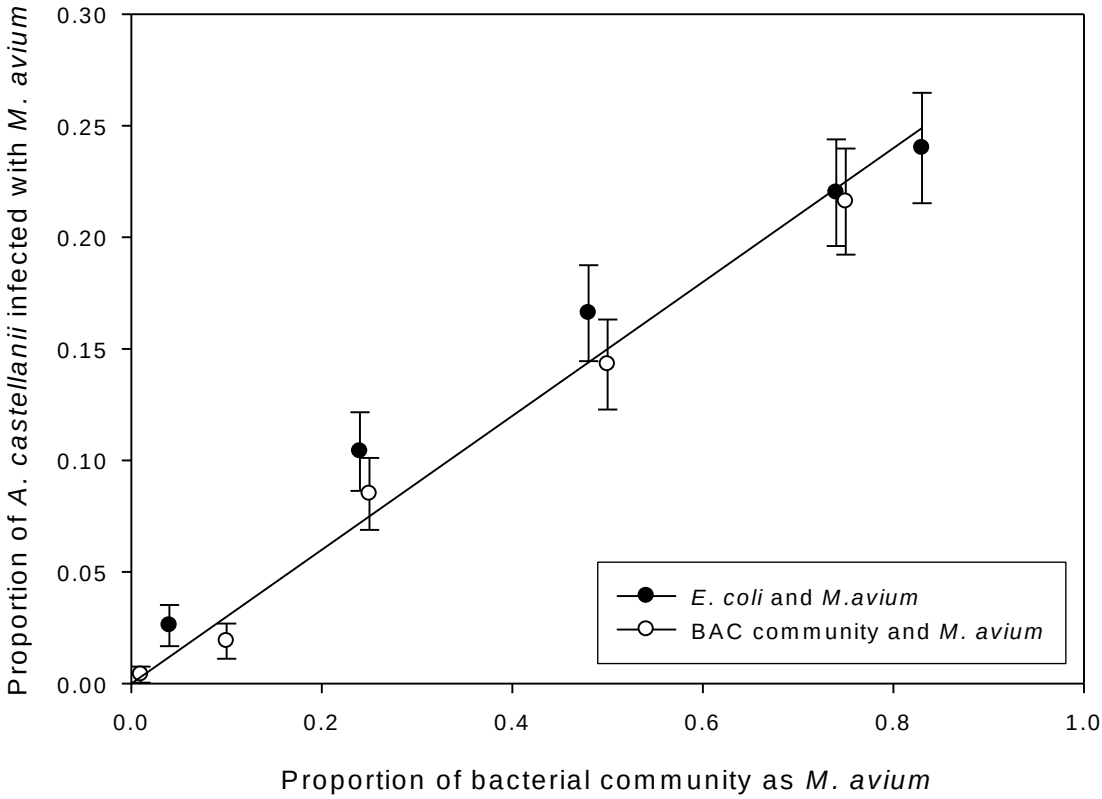


Figure 6.3 Proportion of *A. castellanii* Neff harboring ingested *Mycobacterium avium* after 60 hours of grazing on two bacterial consortia. An *E. coli* culture and a biologically active carbon (BAC) filter biofilm community were spiked with fluorescently-labeled *M. avium* at proportions between 0.01 and 0.82 on a per mass basis. Error bars indicate 95% confidence intervals.

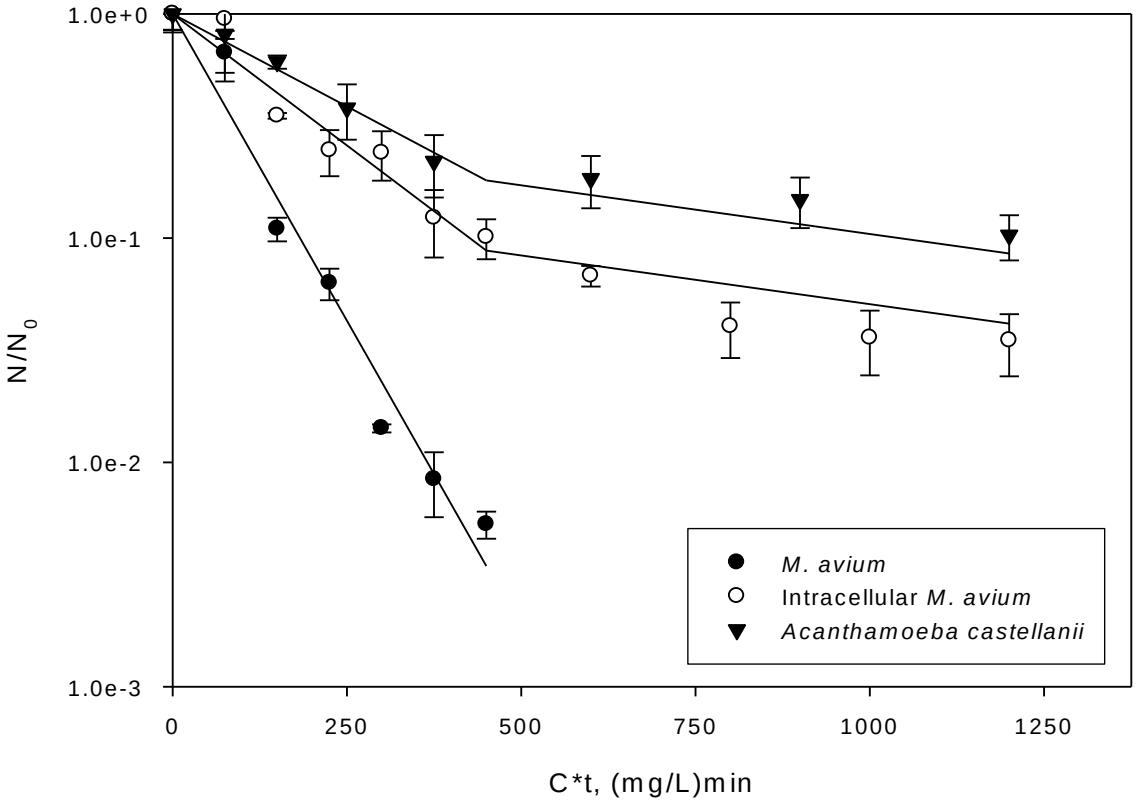
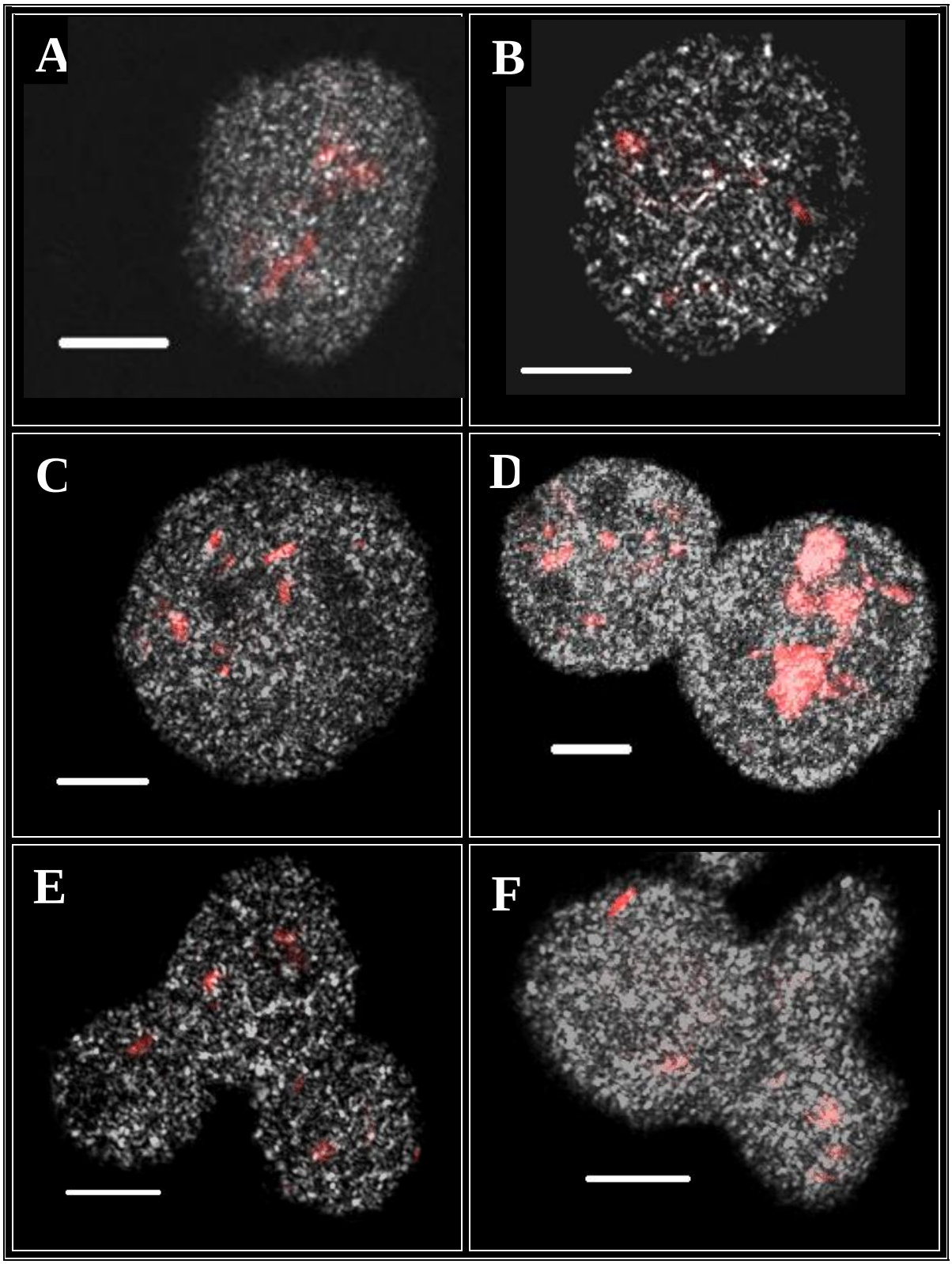


Figure 6.4 Inactivation kinetics of *Mycobacterium avium* in pure culture and when in co-culture with *A. castellanii* Neff (quantified using viability plating and expressed as CFU/CFU_O), and *A. castellanii* Neff (quantified using viability staining and expressed as N/N_O). Error bars indicate 95% confidence intervals.


A
B
C
D
E
F

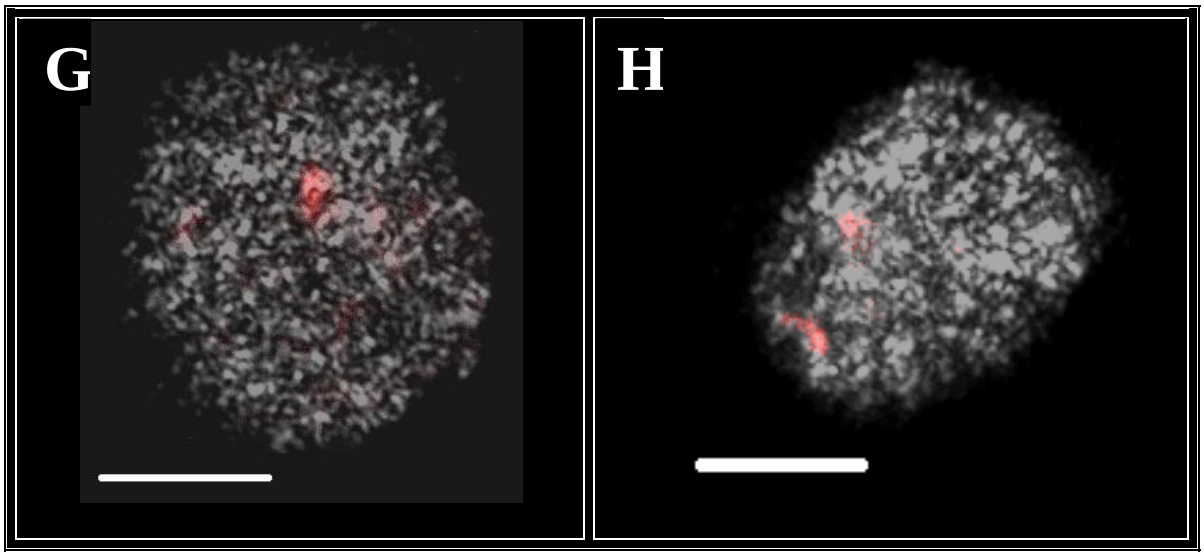


Figure 6.5 Confocal scanning laser microscopy images of intracellular *M. avium* using a phenol/acridine orange staining. *M. avium* (in red) infecting (A) *Acanthamoeba castellanii* Neff (B), *Acanthamoeba* sp. UCW1, (C) *Acanthamoeba polyphaga* DOME, (D) *Acanthamoeba hatchetii* 4RE, (E) *Acanthamoeba* sp. DWDS, (F) *Acanthamoeba* sp. MSA, (G) *Acanthamoeba* sp. MSC, (E) *Acanthamoeba* sp. F2B. Scale bars are 5 µm.

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Chapter 7
Conclusions and implications

This research has been undertaken to contribute to our understanding of the response and molecular and ecological mechanisms of resistance of bacteria to drinking water disinfection. Several major conclusions and promising future research directions emerge from this work.

The first theme highlighted in this research is that bacterial sensing and response to inactivation with monochloramine are rapid, dynamic processes. Exposure of *M. avium* to a sub-lethal dose of monochloramine (0.5 mg/L as Cl_2) resulted in rapid changes to cell wall permeability, intracellular thiol concentration, and gene expression. In addition, the oxidative stress (OxyR) response was induced very strongly and rapidly in response to monochloramine exposure. Furthermore, many virulence-associated genes were upregulated soon after exposure to monochloramine. It is unclear at this point whether this response increases *M. avium* virulence to humans. The mammalian immune system can produce several strong oxidants including protein chloramines and monochloramine to kill invading bacteria, a response called the respiratory burst (Ogino et al., 2009). Therefore virulence-associated genes in *M. avium* may either be upregulated as a direct detoxification response to damage from monochloramine, or, alternatively, *M. avium* may use monochloramine as an indicator that it is in an intracellular environment and experiencing a respiratory burst and therefore should engage its virulence gene expression program. Future work should be conducted to investigate the role(s) and importance of virulence-associated genes in disinfection response and how this induction of virulence-associated genes may increase bacterial virulence. A better understanding of whether exposure to monochloramine and more broadly drinking water disinfection can induce bacterial virulence is important for gauging the impact of drinking water treatment on human health, especially among immunocompromised populations.

The second major theme addressed in this work evaluated whether relevant environmental conditions can induce bacterial resistance to monochloramine. *E. coli*

grown in either biofilm mode or at a sub-optimal temperature of 20 °C exhibited increased resistance to monochloramine. Most previous research has studied bacterial inactivation efficiency at optimal growth conditions and the results presented in this dissertation suggest that testing inactivation at optimal conditions may lead to underestimations of bacterial resistance to disinfection. Therefore it is advisable to test the inactivation of bacteria cultured at environmentally relevant conditions, e.g., conditions bacteria experience in drinking water distribution systems. The transcriptional profile of resistant cells was determined in order to characterize the response of *E. coli* to the "drinking water stressome". A comparative transcriptional fingerprint of cells grown in biofilm mode, at 20 °C, or after monochloramine exposure was characterized by widespread metabolic inhibition, regulation of redox-active genes, and regulation of osmotic and cell envelope stress responses. Overall, there appears to be extensive overlap between stress due to monochloramine exposure and other stresses such as general oxidative stress and osmotic stress. These results indicate a very complex response to monochloramine that includes not only a direct response to oxidation of sensitive cellular targets and an attempt to maintain redox homeostasis, but also a loss of cell membrane integrity and a widespread "shutdown" of normal cellular functions. In addition to genes with known functions, the function of some genes identified in this study in resistance to monochloramine is not-well understood and future research is needed in order to elucidate the roles of these genes.

The third major theme researched in this dissertation involves the interaction between bacteria and microbial eukaryotes in increasing resistance to monochloramine inactivation. The sheltering of *M. avium* within *Acanthamoeba* is potentially a significant survival strategy during drinking water treatment and distribution. This work demonstrated that *M. avium* can infect a range of *Acanthamoeba* strains and maintains its viability within acanthamoebae for an extended period even under nutrient-poor conditions. Intracellular *M. avium* was much more resistant to monochloramine than *M. avium* alone. Inactivation kinetics of intracellular *M. avium* exposed to monochloramine closely matched the inactivation kinetics of an *Acanthamoeba* strain, suggesting that acanthamoebal inactivation may be a useful surrogate for intracellular *M. avium* inactivation. Taken together, these results suggest that acanthamoebae-facilitated fate

and transport processes of *M. avium* may be an important phenomenon in health-related water microbiology. However, further work is needed to determine the relevance of these observations in actual drinking water treatment and distribution systems. More generally, research is needed to understand the importance of amoeba-bacterial pathogen interactions in the context of drinking water treatment. There is still a lack of quantitative information about the presence of free-living amoebae in drinking water treatment and distribution (Bichai et al., 2008). Also, amoebae have two morphological stages: an active trophozite form and a stress-resistant cyst form, and questions remain about the relative importance of each of these forms in bacterial pathogen fate and transport throughout the drinking water treatment and distribution process. Additional work is necessary to determine whether inactivation studies of acanthamoebae alone can be used generally as a surrogate for determining inactivation rates for a range of intracellular bacterial pathogens, which would be a simple and useful conservative indicator of bacterial pathogen inactivation.

This research underscores the importance of biological processes in drinking water treatment and distribution, characterizing the biological complexity of bacterial response to monochloramine, the complexities emerging from response to conditions typically found in drinking water distribution, and the interactions of bacterial pathogens with acanthamoebae. The observed increased resistance to monochloramine of bacteria grown under environmentally-relevant conditions and when associated with acanthamoebae certainly has implications for drinking water treatment and distribution system process design and operation. It may not be possible to use knowledge of bacterial resistance mechanisms to directly improve the efficacy of chlorine-based disinfection. Rather, attention should be focused on rigorous and/or alternative treatment and nutrient reduction in order to minimize the bacterial growth potential or to reduce potential risks associated with conventional treatment technologies in treated water. Disinfection should be viewed as a final polishing step and not the panacea of drinking water safety.

Many conceptual and methodological challenges remain on the road towards achieving safer drinking water for all, including immunocompromised populations, and this research serves as a helpful step on this journey.

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Appendix
Preliminary microbial community characterization of Ann Arbor drinking water distribution system biofilms

A.1 Introduction

The Ann Arbor Drinking Water Distribution System (DWDS) serves the City of Ann Arbor with a network of approximately 440 miles of pipeline and using monochloramine (approximately 2.7 mg/L) as a residual disinfectant. Characterization of the biofilm attached to DWDS pipes was conducted in order to discover the identity of dominant bacterial and eukaryotic organisms.

This research is intended to be a preliminary step in characterizing the microbial community of the Ann Arbor DWDS. The knowledge gained through this work helps improve our understanding of biological processes in drinking water distribution systems and suggests future lines of research.

A.2 Materials and methods

A.2.1 Sampling campaigns

Biofilm samples were obtained in August 2007 from two locations in the City of Ann Arbor's DWDS (Packard Street and Geddes Heights). The internal surfaces of pipes removed from the DWDS during fire hydrant replacement were aseptically scraped and removed biofilm and inorganic scale was stored in sterile containers on ice (Figures A.1a and A.1b). The samples were immediately transported to the laboratory and stored at -80 °C.

A.2.2 DNA extraction, PCR and sequence analysis

DNA was extracted using a low pH, hot phenol-chloroform method (Alm et al., 2000). A fragment of the 16S rRNA gene was amplified using universal bacterial primers (5'-AGAGTTTGATCMTGGCTCAG-3' and 5'-GGTTACCTTGTTACGACTT-3') (Lane,

1991) and a fragment of the 18S rRNA gene was amplified using conserved eukaryotic primers (5'-AACCTGGTTGATCCTGCCAG-3' and 5'-GATCCTTCTGCAGGTTCACCTAT-3') (Gast et al., 1994). Clone libraries were constructed from PCR products and shipped to Washington University Genome Sequencing Center for sequencing. Vector sequences were removed and sequences were trimmed using a threshold quality score of 20. Partial 16S rRNA gene sequences (~600 bases) were aligned using Greengenes (DeSantis et al., 2006) and classified using the Ribosomal Database Project classifier, which is based on a Naïve Bayesian rRNA classifier (Wang et al., 2007). Phylogenetic trees were created using the "drawtree" program in Mobyle (http://mobyle.pasteur.fr/cgi-bin/portal.py?form=treealign). Forward and reverse primer sequences of 18S rRNA gene sequence were concatenated (~1200 bases), aligned and classified according to the nearest neighbor in Silva (Pruesse et al., 2007) (July 2009). Clones from both sampling sites were combined for analysis for a total of approximately 300 clones for each library.

A.3 Results and discussion

A.3.1 Bacterial community

The *Gammaproteobacteria* were the dominant members of the bacterial clone library, comprising 83% of clones (Figure A.2). The *Gammaprotebacteria* clones were dominated by the genus of *Pseudomonas*, which constituted 98% of gammaproteobacterial clones. *Pseudomonas* spp. were more abundant in this study than any previously reported study of DWDSs (reviewed in Berry et al., 2006). A distance matrix of aligned sequences revealed that *Pseudomonas* clones clustered into two tightly-defined clades (<0.1% difference) with an average of 2.8% difference with each other (Figure A.3). *Betaproteobacteria spp.* comprised approximately 15% of clones, and *Alphaproteobacteria*, *Deltaproteobacteria*, and *Firmicutes* were detected at marginal levels (Figure 2). The major genera of the *Betaproteobacteria* were *Burkholderia* (33%) and *Variovorax* (33%), as well as unclassified members of the *Oxalobacteraceae* (20%).

A.3.2 Eukaryotic community

The major constituents of the eukaryotic clone library were the kingdoms *Plantae* (38%) and *Rhizaria* (38%) (Figure A.4). The *Plantae* in the library were all classified in the

order *Poales*, which consists of flowering monocot plants such as grasses and sedges (Linder and Rudall, 2005). This is assumed to be DNA from seeds of plants that have entered the DWDS and organisms within the *Poales* are presumably not playing an active role in the DWDS environment. The *Rhizaria* in the library were all classified in the genus *Cercomonas*, which are unicellular amoeboflagellates (Adl et al., 2005). All Fungi in the library (17%, Figure 3) were classified in the phylum *Ascomycota*, which is a large and heterogenous group of unicellular and multicellular organisms (Schoch et al., 2009). It is not clear whether the fungi are metabolically active in the DWDS or whether fungal spores have entered the DWDS similarly to the *Poales* clones and are either dormant or dead. The *Stramenopiles* in the library were all classified in the genus *Poterioochromonas*, which are unicellular photosynthetic protists that also can feed on bacteria.

A.4 Conclusions and future directions

A preliminary characterization of the bacterial and eukaryotic communities in the Ann Arbor DWDS was performed. The dominant bacterial genus observed was *Pseudomonas*. The dominant eukaryotic genus was the unicellular amoeboflagellate *Cercomonas*. Many *Plantae* clones were also observed, but are suspected to be recalcitrant DNA from dormant or dead plant seeds. Future work should use more targeted primers to elucidate the presence of specific groups (e.g., *Acanthamoeba*) and use complementary methods such as fluorescence in situ hybridization (FISH) and stab le isotope probing to verify clone library work and identify metabolically active groups.

A.5 Figures

Figure A.1 Drinking water distribution system sampling. (a) Fire hydrant with connection to main line and (b) close-up of main line pipe and biofilm interspersed with pipe corrosion.

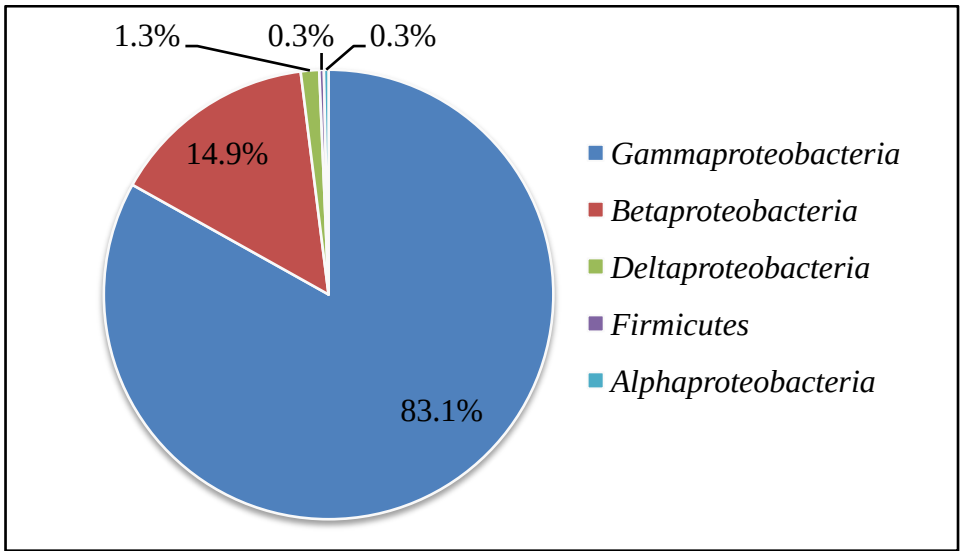

Figure A.2 Composition of DWDS bacterial clone library

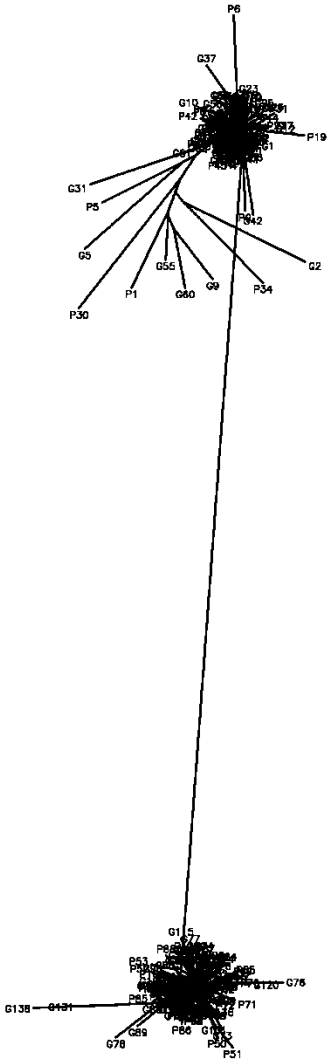

Figure A.3 Unrooted phyogenetic tree of *Pseudomonas* spp. clones. Clones cluster into two tightly-defined groups found at both sampling sites (Clone ID beginning with "G" is Geddes Heights and "P" is Packard St. sampling sites).

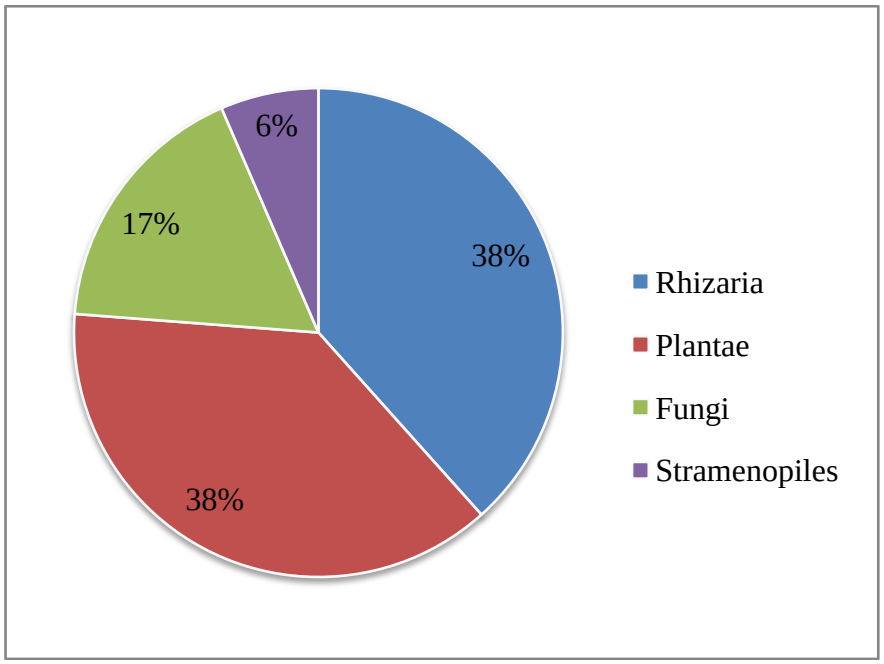

Figure A.4 Composition of DWDS eukaryotic clone library

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