Interactions of the bacterial pathogen *Mycobacterium avium* with amoebae

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ABSTRACT: Mycobacterium avium is an opportunistic bacterial pathogen that is known to survive in drinking water systems. It has been reported that M. avium is able to infect the amoeba Acanthamoeba castellanii, but little is known about the environmental relevance of this interaction. The objectives of this research were: (i) to determine if M. avium is able to infect a range of acanthamoeba strains including recent environmental isolates; (ii) to assess the stability of infections; and (iii) to determine the effect of co-culture on disinfection efficacy of M. avium using the drinking water disinfectant monochloramine. Infections were monitored and quantified using staining and fluorescence microscopy as well as by culturing methods. In this work, we found that M. avium was able to maintain stable infections in the eight acanthamoeba strains tested, including four recent environmental isolates. M. avium persisted in acanthamoeba and were still culturable after four weeks of co-culture, even under nutrient-poor conditions. During exposure of cultures to the disinfectant monochloramine, intracellular M. avium was found to be more resistant to monochloramine than free-living M. avium. This suggests that acanthamoeba-associated growth may serve as a mechanism to protect bacteria from disinfectants in drinking water systems. To test the rate of acanthamoeba infection in complex biofilm communities, acanthamoeba were allowed to graze on multispecies bacterial communities which had been spiked with M. avium at percentages of 1, 10, 25, 50, and 75% of total bacteria. After a 60 hour period, infection rates of acanthamoeba were proportional to the abundance of M. avium in the biofilm, suggesting a density-dependent infection rate.

INTRODUCTION

Growth of microorganisms is often observed in drinking water distribution systems (DWDS) despite the presence of disinfectant residual (Berry et al., 2006; LeChevallier et al., 1987). As a result, pathogenic bacteria have the potential to be present in tap water, which is a substantial public health concern. There are a variety of reasons for the discrepancies observed between the survival of bacteria in DWDS and in typical laboratory experiments performed to determine inactivation kinetics. The interaction of bacterial pathogens with protozoa may be one such reason and may constitute an important survival strategy for bacterial pathogens in the presence of disinfectants in DWDS (Berry et al., 2006). Intracellular growth of bacteria has been shown to select for virulence factors that affect pathogenesis in protozoon hosts (Molmeret et al., 2005) and diverse protozoa have been observed in DWDS (Sibille et al., 1998; Thomas et al., 2004). Therefore, an increased understanding of protozoon interactions with bacterial pathogens will be an important step towards developing better pathogen control strategies.

The awardee institution for the current NSF award (No. 0613193) is The University of Michigan. The collaborative project examines the ecological complexities that confer bacterial resistance to disinfection. The collaboration has enhanced the current research capacity by developing a deeper ability to examine the symbiotic and antagonistic relationships of bacteria and protozoa under conditions relevant to drinking water treatment and distribution.

Our foreign collaborators are Drs. Matthias Horn and Michael Wagner, both of the Department of Microbial Ecology, Faculty of Life Sciences at the University of Vienna, located in Vienna, Austria. The Department of Microbial Ecology is internationally-recognized for their work in environmental microbiology and microbial ecology, and they have core capabilities in the study of symbiotic interactions of bacteria with higher organisms such as acanthamoebae.

Ph.D. student David Berry worked in Austria for 6 months (March through August, 2008) as part of the collaboration. Mr. Berry was selected because he has significant experience in both water quality engineering and environmental microbiology, so he was well qualified to work in the Department of Microbial Ecology.

RESEARCH ACTIVITIES AND ACCOMPLISHMENTS OF THE INTERNATIONAL COOPERATION (LIMIT: 1-1/2 PAGES)

Dr. Raskin and Mr. Berry visited Drs. Horn and Wagner in Vienna in August 2007 to begin planning for the research visit and research plan. Dr. Horn visited the University of Michigan in November 2007 to further refine research plans and participate in Mr. Berry's preliminary exam committee. Dr. Horn will make another visit to University of Michigan in June 2009 to participate in Mr. Berry's dissertation committee. Mr. Berry visited the host laboratory between March and August 2008. During this time, he was given access to all laboratory facilities and participated in departmental activities such as a weekly seminar series, journal club, and a special short-course on fluorescence *in situ* hybridization (FISH), the "International FISH course", offered annually by the department. In addition, post-doctoral researcher Dr. Diane Holder, who worked on the current NSF award (No. 0613193) from December 2005-January 2009, visited the Vienna laboratory for a short visit during Mr. Berry's stay (May 2008).

During his 6 month research visit, Mr. Berry developed methodologies for studying intracellular survival of *M. avium* within acanthamoeba. He used these methodologies to study the infectivity and infection stability of *M. avium* in a range of host *Acanthamoeba* strains, including several recent environmental isolates (Figure 1). The inactivation kinetics of intracellular *M. avium* exposed to the drinking water disinfectant monochloramine were also examined and compared to extracellular *M. avium* and to *Acanthamoeba* inactivation (Figure 2). A manuscript based on the work conducted during the international visit will be submitted to journal *Environmental Microbiology* in the near future. The work conducted is a direct extension of the focus of the current NSF award to study the molecular mechanisms of bacterial resistance to monochloramine. The extension involves the study of the ecological mechanisms that can contribute to survival of bacteria in chloraminated water, specifically the survival and sheltering of bacteria within acanthamoebae.

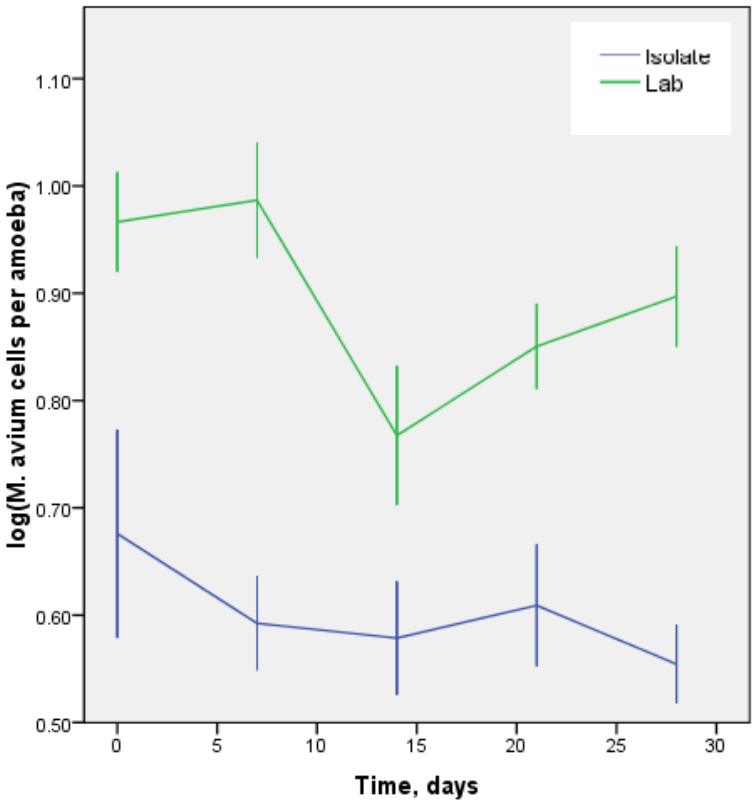

Figure 1: Infection stability of *M. avium* within *Acanthamoeba* lab strains (green) and recent environmental isolates (blue); the data are reported as the number of *M. avium* cells per infected amoeba cell.

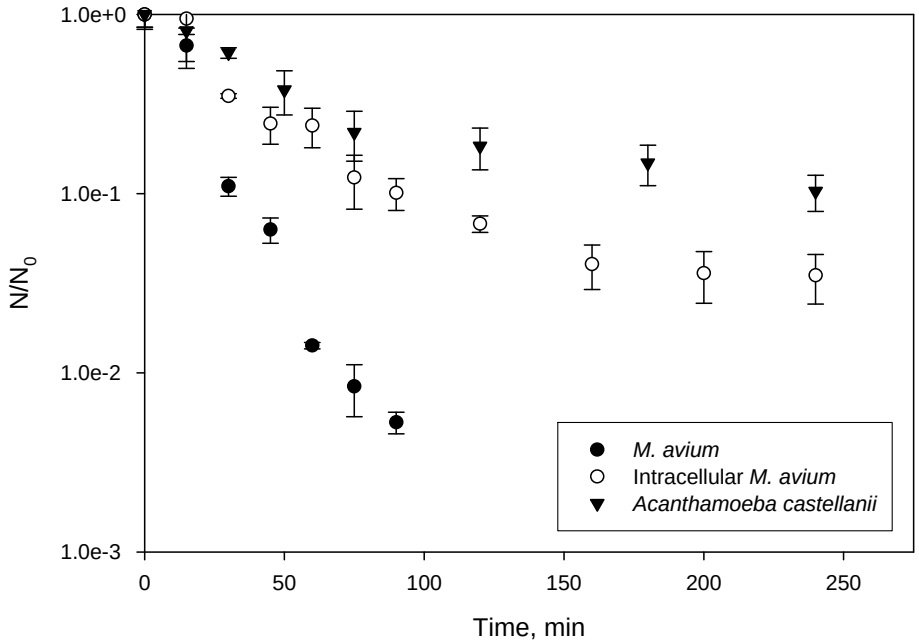

Figure 2: Inactivation kinetics of *M. avium* cultivated alone and intracellularly (within *A. castellanii*) and *A. castellanii* alone when exposed to 5 mg/L monochoramine. N/N_0 indicates the proportion of cells surviving inactivation with monochloramine for a given time of exposure.

BROADER IMPACTS OF THE INTERNATIONAL COOPERATION

The international collaboration facilitated a link between The University of Michigan and The University of Vienna that will be continued through further research on the role of acanthamoebae specifically and symbioses with higher organisms in general in drinking water treatment and distribution processes.

The international research experience broadened Mr. Berry's familiarity with the European research environment and European culture. Specifically, Mr. Berry began taking German lessons before traveling, took evening classes while abroad, and has continued lessons since his return to the US. He is now comfortable in conversational German, which will enhance any future research collaborations in Germany, Switzerland, or Austria. Mr. Berry actively participated in events in his host department, including a weekly seminar entitled "Future Directions in Microbial Ecology", the Department of Microbial Ecology Lecture Series, and seminars offered by the graduate school's seminar series in "Symbiotic Interactions". He also participated in the "International FISH Course 2009", an intensive one-week course hosted and taught by the Department of Microbial Ecology in Spring 2009. Through all these events, Mr. Berry benefited from interactions and exchanges with other researchers. He also attended two international conferences in Europe during his visit, the International Water Association World Water Congress in Vienna, which exposed him to regional and international environmental issues and policy trends around the world, as wells as the IWA Leading Edge Technology Conference in Zurich, at which he presented research (Berry et al., 2008).

Since his return to the US, Mr. Berry has contributed by teaching as a graduate assistant in a course taken by approx. 75 undergraduate students in Civil and Environmental Engineering, entitled "Environmental Process Engineering" (CEE 360 – Dr. Raskin is primary instructor). He also participates in a seminar and discussion series addressing engineering and societal challenges associated with achieving global sustainability, hosted by the Graham Environmental Sustainability Institute (http://www.provost.umich.edu/gesi/umes/).

DISCUSSION AND SUMMARY

The most signicant accomplishment of the international research experience was to enable the application of advanced techniques in microbiology and microbial ecology to environmental engineering research in drinking water treatment. The methodologies developed improve our understanding of the previously unconsidered role of amoeba-assosiated transport and fate of bacterial pathogens in drinking water treatment. Another valuable outcome of the collaboration was to expose graduate student David Berry to a long-term international research experience. This will undoubtedly have a significant impact on his future research career and has increased his interest in working aborad and pursuing international collaborations.

A useful extenstion of the IREE program would be to provide (partial) funds to host an international researcher from the host institute for an extended visit to the US institution. This would facilitate continued international collaboration and exchange between the US and international institutions.

ACKNOWLEDGEMENTS

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BRIEF BIOGRAPHIES OF RESEARCHERS

David Berry is pursuing a Ph.D. in Environmental Engineering at the University of Michigan. He holds a B.S. degree in Bioresource Engineering from Rutgers University and a M.S. degree in Environmental Engineering from the University of Michigan. He has received several scholarships and fellowships in recognition of his academic accomplishments, including most recently the Environmental Protection Agency STAR Fellowship and Graham Environmental Sustainability Institute Graduate Fellowship. He is an author or co-author of four journal publications and 12 conference presentations. Mr. Berry has significant experience in water quality engineering research and bioreactor operation as well as in microbiology and molecular biology techniques.

Matthias Horn is Professor for Microbial Symbioses at the Deparment of Microbial Ecology, University of Vienna, and has extensive experience studying bacterial endosymbionts of free-living amoebae. He has applied a full cycle rRNA approach including 16S rRNA gene sequencing and fluorescence in situ hybridization with 16S rRNA-targeted oligonucleotide probes assigned to symbionts of *Acanthamoeba* spp. and *Hartmannella* sp., finding five different evolutionary lineages within the *Proteobacteria*, the *Bacteroidetes*, and the *Chlamydiae*, respectively. Several of these bacterial symbiont groups are most closely related to bacterial pathogens of humans, and it has been suggested that some of them should be considered potential emerging pathogens. Complete genome sequence analysis of a chlamydia-related symbiont of *Acanthamoeba* sp. showed that this endosymbiont uses similar mechanisms for interaction with its eukaryotic host cell as well known bacterial pathogens of humans. Furthermore, phylogenetic analysis suggested that these mechanisms have been invented by the ancestor of the amoeba symbionts in interplay with ancient unicellular eukaryotes. Currently, functional genomics of chlamydial symbionts, including global transcription and proteome analysis is a major research focus in Dr. Horn's laboratory. Dr. Horn has 34 publications in peer-reviewed journals and eight book chapters and other publications. He has given 15 invited talks at university seminars and international conferences.

Lutgarde Raskin is a Professor of Environmental Engineering in the Department of Civil and Environmental Engineering at the University of Michigan. During the past 15 years, Professor Raskin has developed a leading edge and productive research program that is based on her expertise in molecular microbial ecology, environmental microbiology, and biological process engineering. A significant part of her work has focused on the development of molecular methods that allow for the quantification and visualization of microorganisms in complex microbial systems. Recent work has focused on techniques that can monitor growth and activity levels of microbial populations. Major applications of Professor Raskin's work have been in anaerobic waste treatment processes, filamentous foaming problems in activated sludge systems, and use of biologically active carbon to remove nitrate and perchlorate from drinking water. Her research has resulted in 80 refereed journal publications, one book, and six book chapters. She has presented over 50 invited lectures at university seminars and international conferences.

Michael Wagner is the head of the Department of Microbial Ecology, University of Vienna. He has experience discovering the identity and ecophysiology of microorganisms within selected environmental systems and to deciphering their interactions among each other and with eukaryotes, particularly through the development of novel molecular and microscope techniques. These studies are complemented by (eco-)genome, transcriptome, and proteome analyses of microbial key players. Dr. Wagner has 137 publications in peer-reviewed journals and 31 book chapters and other publications; the papers published during the last 10 years received 4,094 citations according to ISI Web of Science (rank 35 of all microbiologists). He also has given 88 invited talks at university seminars and international conferences.

Chuanwu Xi is an Assistant Professor in the Department of Environmental Health Sciences at the University of Michigan. He obtained his Ph.D. in molecular microbiology in 2000 from the University of Leuven, Belgium. He has extensive experience in microbial genetics, molecular biology, and flow cytometry. During his Ph.D. work, he constructed a bi-functional genetic tool containing *gusA* and *gfp* genes for studying gene expression and bacterial tracing simultaneously. More than 60 research groups around the world have requested this genetic tool. His Ph.D. and postdoctoral research have resulted in nine publications in highly ranked journals including two in the *Proceedings of the National Academy of Sciences*. He uses advanced imaging tools and nanotechnology to study biofilm development, the spread of antibiotic resistance, and control of biofilm-related infections. His research interests include the elucidation of molecular mechanisms of persistence and resistance of pathogens in natural, engineered and industrial environments