

THE DETECTION, CHARACTERIZATION, AND CULTIVATION OF  
NONCULTURABLE *HELICOBACTER PYLORI*

by

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A dissertation submitted in partial fulfillment  
of the requirements for the degree

of

Doctor of Philosophy

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## TABLE OF CONTENTS

1. SCOPE OF THE DISSERTATION.....	1
References.....	5
2. INTRODUCTION.....	6
Background.....	6
<i>H. pylori</i> Epidemiology.....	8
<i>H. pylori</i> Routes of Transmission.....	10
Oral-Fecal Route of transmission.....	11
Environmental Transmission.....	12
Factors Responsible for Colonization and Survival of Harsh Gastric Conditions .....	15
Concluding Remarks.....	19
References.....	21
3. DETECTION OF MYCOBACTERIA, LEGIONELLA, AND HELICOBACTER IN DRINKING WATER AND ASSOCIATED BIOFILMS ON THE CROW RESERVATION, MONTANA, USA.....	34
Contribution of Authors and Co-Authors.....	32
Manuscript Information Page.....	33
Title Page.....	34
Abstract.....	35
Introduction.....	36
Materials and Methods.....	38
Study Area.....	38
Sample Collection and Processing.....	39
Quantification of Fecal Indicator Bacteria and Heterotrophic Bacteria.....	40
Control Bacterial Strains and Growth Conditions.....	41
Culture of Drinking Water and Biofilm Samples.....	42
DNA Extraction from Biofilm and Water Samples.....	43
PCR amplification, Sequencing, and Phylogenetic Analysis.....	44
Statistical Analysis.....	45
Results.....	45
Physical Characteristics of Sampled Drinking Water.....	45
Detection of Heterotrophic Bacteria and Fecal Indicator Bacteria.....	46
Presence of <i>Mycobacteria</i> , <i>Legionella</i> , and <i>Helicobacter</i> .....	47
Interactions Between Potentially Pathogenic Genera.....	50

## TABLE OF CONTENTS – CONTINUED

Discussion.....	50
Fecal Coliforms and HPC Bacteria in Drinking Water.....	50
Sampling Strategy Influences Detection of <i>Mycobacteria</i> , <i>Legionella</i> , and <i>Helicobacter</i> .....	54
Health Consequences of <i>Mycobacteria</i> , <i>Legionella</i> , and <i>Helicobacter</i> in Drinking Water.....	57
Acknowledgements.....	59
References.....	60
4. MULTIPLE PROCESSES GOVERN SWITCH TO NONCULTURABLE STATE IN <i>HELICOBACTER PYLORI</i> .....	78
Contribution of Authors and Co-Authors.....	76
Manuscript Information Page.....	77
Title Page.....	78
Abstract.....	79
Introduction.....	80
Materials and Methods.....	83
Bacterial Strain and Culture Conditions.....	83
Stress Exposure and Enumeration of Total and Viable Cells.....	84
RNA Isolation, Microarray Hybridization and Analysis.....	85
Assessment of Transcriptional Changes of Nonculturable Cells After Incubation in a Regrowth Medium .....	89
Reliability and Validity of Array Data .....	90
Urease Activity Assay.....	91
Results.....	92
Effect of Aging, Oxygen Stress, and Nutrient Deprivation on the Conversion to Coccoid and Viability of <i>H. pylori</i> .....	92
Global Gene Expression Analysis.....	93
Oxygen Stress Alone Causes a Faster Transcriptional Response than Nutrient Deprivation.....	95
Virulence Gene Transcription was Affected by Stress Treatments.....	96
Stressed cells Exhibit Different Levels of Virulence Gene Transcription when Presented with Favorable Growth Conditions.....	97
Discussion.....	99
Acknowledgements.....	107
References.....	107

## TABLE OF CONTENTS – CONTINUED

5. OPTIMIZING THE GROWTH OF STRESSED <i>HELICOBACTER PYLORI</i> .....	129
Contribution of Authors and Co-Authors.....	127
Manuscript Information Page.....	128
Title Page.....	129
Abstract.....	130
Introduction.....	130
Materials and Methods.....	132
Routine Culturing.....	132
Preliminary Testing.....	133
Biphasic Slant Culture and Stress Treatment.....	134
Optimized Regrowth Medium (R broth).....	135
Total and Culturable Cell Counts.....	137
Selective and Differential Growth Medium.....	137
Statistical Analysis.....	138
Results.....	139
Preliminary Results.....	139
Culture of Stressed <i>H. pylori</i> .....	141
Selective and Differential Growth Medium.....	143
Discussion.....	144
Acknowledgements.....	149
References.....	150
6. THESIS SYNTHESIS AND FUTURE DIRECTIONS.....	157
Future Directions.....	160
References.....	162
7. APPENDICES	
APPENDIX A: Community-Based Participatory Research in Indian Country: Improving Health through Water Quality Research and Awareness.....	164
APPENDIX B: Permission to reproduce .....	186
APPENDIX C: Supplementary table of complete list of significantly regulated genes.....	190

## LIST OF TABLES

Table	Page
3.1. Primer Sequences, References and PCR Conditions.....	69
3.2. Range and Arithmetic Mean of HPC Bacteria, Total Coliforms, and <i>E. coli</i> .....	70
3.3. Statistical Analysis of the Interactions between HPC Bacteria in Drinking Water and Response Variable.....	71
3.4. Statistical Analysis of the Interactions between HPC Bacteria in Biofilms and Response Variables.....	72
3.5. Statistical Analysis of the Interactions between Drinking Water Source (Treated Municipal or Groundwater Well) and Response Variables.....	72
4.1. Oxygen Stress and Nutrient Deprivation Stress Up Regulated Genes of <i>H. pylori</i> .....	117
4.2. Comparison of the Log <sub>2</sub> Fold Change Associated with Microarray and qRT-PCR.....	123
5.1. Comparison of the Differences in Total Cells and Viable Cells Between the Oxygen Stress and Nutrient Deprivation Treatments Before and After Resuscitation.....	154

## LIST OF FIGURES

Figure	Page
3.1. Phylogenetic Relationship of PCR Isolates Isolated from Drinking Water and Associated Biofilms and Specific for the <i>Mycobacterium</i> 16s rRNA gene.....	73
3.2. Phylogenetic Relationship of PCR Isolates Isolated from Drinking Water and Associated Biofilms and Specific for the <i>Legionella</i> 16s rRNA gene.....	74
3.3. Phylogenetic Relationship of PCR Isolates Isolated from Drinking Water and Associated Biofilms and Specific for the <i>Helicobacter</i> 16s rRNA gene.....	75
4.1. Relationships Between the Morphologic Characteristic of the Cell and Viability of <i>H. pylori</i> Under Aging and Stress Conditions.....	115
4.2. Fluorescence Micrographs Showing <i>H. pylori</i> in Various Stages of Transformation to Coccoid Cell Morphology.....	116
4.3. Expression Analysis of Statistically Significant, Differentially Expressed Genes in Oxygen Stressed and Nutrient Deprived <i>H. pylori</i> .....	124
4.4. Transcription of <i>ureA</i> Gene and Urease Activity Before and After Treatment with Nutrients and Human Erythrocytes.....	125
4.5. Transcription of the Gene <i>vacA</i> in Oxygen Stressed and Nutrient Deprived <i>H. pylori</i> Before and After Treatment with Nutrients and Human Erythrocytes.....	126
5.1. Depicts the Total and Viable <i>H. pylori</i> Cells Before and After Resuscitation.....	155
5.2. Depicts the Selective and Differential R Slant.....	156

## ABSTRACT

Transmission of a bacterial pathogen from host to host is a complex process that may involve survival of the pathogen outside the host for considerable lengths of time. The bacterium *Helicobacter pylori* causes severe gastritis and gastric ulcers, and infection can increase the risk of stomach cancer. The main mode of transmission is believed to be the oral-oral route, however other routes of transmission such as drinking water have been implicated but have not been proven due to difficulty in culturing this organism. In this dissertation, the environmental transmission of *H. pylori* was investigated using several approaches.

A primary objective of this study was to determine if *H. pylori* could be detected in an environmental reservoir readily consumed by humans, such as drinking water. *H. pylori* was detected by PCR but not culture in drinking water and biofilms that were obtained from groundwater and municipal systems. *H. pylori* contamination was sporadic and not associated with measured environmental factors, such as pH or temperature. Growth curve analysis of laboratory grown *H. pylori* showed that the cells exhibited a switch from a spiral to coccoid morphology as they aged or were exposed to stressful culture conditions. However, results showed that cell morphology was not indicative of culturability, with spiral forms dominant in early nonculturable samples. Microarray analysis of the transition to a nonculturable state showed that cells under oxygen stress quickly modified their transcriptional activity while the cells exposed to nutrient deprivation had nearly undetectable changes in transcriptional activities. Resuscitation of the stressed cells showed that type of stress and length of exposure affected regrowth of *H. pylori*. The oxygen stressed cells increased virulence factor transcription while nutrient deprived cells decreased transcription of the same factors. This observation led to the conclusion that oxygen stressed and nutrient deprived cells are metabolically active but react differently to *in vitro* culture conditions with starved cells likely undergoing nutrient shock. Collectively these data suggest that *H. pylori* can persist and are metabolically active under stressful conditions posed by the environmental mode of transmission.

## CHAPTER 1

## SCOPE OF THE DISSERTATION

The main goal of the research presented in this dissertation was to increase our understanding of *H. pylori* transmission via the environmental route. Initially, the study of *H. pylori* in drinking water was requested by concerned individuals on the Crow Reservation, MT. The implementation of the research described in Chapter 3 used community based participatory research (CBPR) to achieve the goal of drinking water assessment. The process of building a CBPR project is described in a manuscript published in *Family and Community Health*, 2010, (2) and is included in Appendix A. The intent of the research at the Crow Reservation was to detect *H. pylori* in drinking water as this was a concern to the community. A secondary goal was to sample for the presence of the opportunistic pathogens, *Legionella pneumophila* and *Mycobacterium avium*, in drinking water and associated biofilms. Both *Legionella pneumophila* and *Mycobacteria avium* are known to be harbored in drinking water systems and pose a threat to young, elderly, and immunocompromised people (5).

The content of Chapter 3 has been submitted to *Applied and Environmental Microbiology* (7) and the work showed that all three species could be detected in drinking water and associated biofilms. For all three organisms, DNA based methods were superior to culture methods. This is likely because environmental bacteria will not always grow on the rich nutrient media used for their culture (6). Both *H. pylori* and *L. pneumophila* are known to enter a viable but not culturable (VBNC) state when exposed

to unfavorable conditions (1, 3, 4). The mysterious nature of this phenomenon seen in *H. pylori* motivated the research described in Chapter 4, which will be submitted to the *Journal of Bacteriology* (9).

The research presented in Chapter 4 describes the characterization of two morphologically distinct populations of coccoid and spiral shaped cells that occur in stressed and nonculturable *H. pylori*. The specific hypotheses that motivated this work were 1) coccoid *H. pylori* were responsible for the loss in culturability seen in stressed cells, and 2) the conversion of the cell to a coccoid morphology was regulated at the transcriptional level. Two stress conditions that cause a nonculturable state, oxygen stress and nutrient deprivation (also incubated under atmospheric levels of oxygen), were examined for changes in cell morphology and gene transcription. The two treatments caused a differential response regarding the morphological conversion of the cells; oxygen stress promoted the formation of coccoid cells while nutrient deprivation did not (< 45% coccoid after 2 weeks).

Whole genome microarray analysis showed that the transcriptomes of these two populations were very different. A large change in gene transcription was observed in cultures at the onset of the conversion to a coccoid morphology which indicated an active mechanism for adapting to environmental conditions. Many of the significantly up regulated genes in both treatments were related to cell envelope functions including outer membrane proteins and enzymes involved in the biosynthesis and modification of peptidoglycan. Cells that did not convert to a coccoid morphology showed little change in transcription until approximately seven days of stress exposure. The data presented in

Chapter 4 also showed that coccoid cells induced by stress had decreased transcription of many important virulence factors including *cag* pathogenicity island genes, vacuolating cytotoxin, and urease genes. The cells under nutrient deprivation did not convert as readily to a coccoid morphology and had a much slower decrease in transcription of the same factors. Overall, these experiments show that conversion of the cell morphology is an adaptive process and uses an active mechanism involving gene transcription.

Chapter 5 follows up on this work by optimizing a culturing technique for environmentally stressed *H. pylori* which has been submitted to the Journal of Microbiological Methods (8). Broth media did not support reliable growth of healthy *H. pylori* without the addition of trace minerals and human blood products. Fresh lysed human erythrocytes and serum were an absolute requirement for the recovery of stressed *H. pylori* in a broth growth medium. Complete recovery of the culturability of all stressed cells was not achieved and recovery depended on the type of stress and the length of time the cells were exposed to that stress. However, the data presented in Chapter 5 suggests that at least a portion of these “nonculturable” cells can be recovered under certain growth conditions.

Chapter 6 discusses the conclusions of this dissertation and suggests further research directions.

In summary, this dissertation has shown that *H. pylori* and other potential pathogens can be detected in drinking water and associated biofilms in rural Montana, U.S.A. Additionally, environmentally stressed *H. pylori* seem to have coping mechanisms specific for the type of stress encountered. It is possible that the unique set

of genes up regulated during stress provide *H. pylori* with a mechanism for surviving transmission and colonization of a new host, compensating for the decrease in virulence factors by increasing adherence capabilities. The recovery of previously nonculturable *H. pylori* cells onto conventional agar media using a resuscitation technique supports the idea that some of the cells retain viability and the potential to colonize a host via the environmental route of transmission.

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References

1. Adams, B. L., T. C. Bates, and J. D. Oliver. 2003. Survival of *Helicobacter pylori* in a natural freshwater environment. *Appl. Environ Microbiol.* 69:7462-7466.
2. Cummins, C., J. Doyle, L. Kindness, M. J. Lefthand, U. Walk, A. L. Bends, S. C. Broadaway, A. K. Camper, R. Fitch, T. E. Ford, S. Hamner, A. R. Morrison, C. L. Richards, S. L. Young, and M. J. Eggers. 2010. Community-based participatory research in indian country improving health through water quality research and awareness. *Fam. Comm. Health.* 33:166-174.
3. Diederer, B. M. W., C. M. A. de Jong, I. Aarts, M. F. Peeters, and A. van der Zee. 2007. Molecular evidence for the ubiquitous presence of *Legionella* species in Dutch tap water installations. *J. Water Health* 5:375-383.
4. Klont, R. R., A. J. M. Rijs, A. Warris, P. D. J. Sturm, W. J. G. Melchers, and P. E. Verweij. 2006. *Legionella pneumophila* in commercial bottled mineral water. *FEMS Immunol. Med. Microbiol.* 47:42-44.
5. Kusnetsov, J., E. Torvinen, O. Perola, T. Nousiainen, and M. L. Katila. 2003. Colonization of hospital water systems by legionellae, mycobacteria and other heterotrophic bacteria potentially hazardous to risk group patients. *APMIS* 111:546-556.
6. Oliver, J. D. 2005. The viable but nonculturable state in bacteria. *J. Microbiol.* 43:93-100.
7. Richards, C. L., S. C. Broadaway, M. J. Eggers, E. Colgate, J. Doyle, B. H. Pyle, A. K. Camper, and T. E. Ford. 2010. Detection of *Mycobacteria*, *Legionella*, and *Helicobacter* in drinking water and associated biofilms on the Crow Reservation, Montana, USA. (Manuscript in Submission) *Appl. Environ. Microbiol.*
8. Richards, C. L., B. J. Buchholz, T. E. Ford, S. C. Broadaway, B. H. Pyle, and A. K. Camper. 2010. Optimizing the growth of stressed *Helicobacter pylori*. (Manuscript in Submission) *J. Microbiol. Methods.*
9. Richards, C. L., K. Williamson, T. E. Ford, and A. K. Camper. 2010. Multiple processes govern the switch to nonculturable state in *H. pylori*. (Manuscript in preparation) *J. Bacteriol.*

## CHAPTER 2

## INTRODUCTION

Background

*Helicobacter pylori* is the primary cause of gastritis, peptic and duodenal ulcers in humans around the world. It is also known to increase the risk of the development of gastric cancer in infected people (8). *H. pylori* was first isolated and identified in 1983 and has since been designated a class-1 carcinogen for stomach cancer by the World Health Organization (8, 66). The incidence of *H. pylori* infection worldwide is assumed to be approximately 50%, with developing countries having a higher infection rate than developed countries (70, 78). The mode of transmission is not well understood, but sources from the environment as well as fecal-oral and oral-oral routes have been implicated (108). The primary reservoir appears to be the human stomach, especially the antrum.

The genus *Helicobacter* includes several species that are pathogenic to mammals such as *H. felis*, isolated from the gastric mucosa of cats and dogs, *H. mustelae*, known to colonize ferrets, and *H. nemestrinae*, isolated from the pig tailed macaque (31). In general, *Helicobacter* species tend to be host specific and have fastidious *in vitro* growth requirements (12, 95). Similar to most *Helicobacter* species, the human pathogen *H. pylori* has a strict requirement for a microaerophilic atmosphere (54). Aerobiosis causes stress to *H. pylori* and eventually leads to a loss in culturability (60). However, small amounts of oxygen are required for growth *in vitro*. Donelli and co-workers (35)

characterized the effect of oxygen on growth and cell morphology of *H. pylori* and showed that >1% oxygen was required for adequate growth and an atmospheric concentration of 85% N<sub>2</sub>, 5% O<sub>2</sub>, and 10% CO<sub>2</sub> was optimal. Additionally, the cells converted their cell morphology from a spiral to a coccoid form very quickly under suboptimal oxygen concentrations but under extremely low oxygen concentrations, the cells appeared to form filamentous, long and undivided rods. Another study compared varied combinations of CO<sub>2</sub> and H<sub>2</sub> concentrations while maintaining approximately 5% O<sub>2</sub> and found that there was no significant difference in growth as long as O<sub>2</sub> levels were maintained (12). The major difference was in the length of time taken to generate the appropriate atmosphere at the onset of culturing. The Campygen<sup>TM</sup> sachet system (Becton Dickonson) requires approximately 30 minutes to generate an appropriate atmosphere, while the use of a modular atmosphere controlled workstation allows cultures to be manipulated and incubated without excessive exposure to atmospheric oxygen.

Culture of *H. pylori* is the most accurate way of assessing viability, and isolation of the organism can allow testing for pathogenicity, antibiotic susceptibility, and for analyzing differences between strains (83, 95, 98). *H. pylori* will grow *in vitro* on rich culture media supplemented with whole or lysed blood or serum (4). Solid agars, such as tryptic soy agar, brain heart infusion agar, Columbia base agar, and Brucella agar, are commonly used for investigations with this organism and support efficient growth but are not very convenient for harvesting large numbers of cells (6, 9, 12, 33, 59). The growth requirements of *H. pylori* in a broth medium have been investigated by many groups; in

defined and complex broth media, *H. pylori* has a strict requirement for certain trace minerals as well as blood serum or cholesterol for reliable growth (2, 3, 73, 97). The fastidious nature of *H. pylori* explains the difficulty in reproducibly culturing the organism from clinical, laboratory, and environmental sources and accounts for the lack of understanding of the main route of transmission.

### *H. pylori* Epidemiology

*H. pylori* infects the stomach of humans and is thought to have colonized humans early on in the evolution of *Homo sapiens*. Because genetic differences between strains isolated from different geographic locations have been observed, it is thought that *H. pylori* coevolved with humans (29, 55, 93). Suerbaum and co-workers (93) analyzed three gene fragments from *H. pylori* strains isolated from Germany, Canada, and South Africa (*flaA*, *flab*, and *vacA*). They concluded that *H. pylori* is one of the most diverse bacterial species so far reported with the highest known rate of intraspecific recombination (93). Interestingly, they found that clonal descent of species was observed only in strains isolated from paired family members. The transmission of *H. pylori* strains between family members has been examined and it has been shown that only in some cases are identical strains involved while many familial groups carry genetically different strains (46, 94).

In general, infection occurs globally, but differences in prevalence are observed between developed and developing countries (70). *H. pylori* is acquired during childhood and infection can continue throughout the life of the host. Infection commonly occurs in

children but varies depending on many factors (58, 79). The prevalence of active infection in children under 10 years old is approximately 0-5% for those that reside in developed countries and 13-60% for those that reside in developing countries (70). While infection commonly occurs in childhood, the overall prevalence of infection increases with the age of the population, indicating a risk for acquisition throughout adulthood (32, 100). Ethnicity is a factor in infection with the age-adjusted prevalence of infection in non-Hispanic black and Mexican Americans substantially higher compared to non-Hispanic whites (44, 58). Diet and socioeconomic status can play a role in infection as well (58, 107).

*H. pylori* infection is accepted as the cause of the most common form of chronic gastritis. The organism is responsible for alterations of the gastric mucosa that can result in peptic and duodenal ulceration (34, 66, 107). *H. pylori* colonizes both the antrum and corpus compartments of the stomach and the duodenum but is most commonly found in the antrum (34). Initial infection causes acute gastritis and hypochloridia, characterized by vomitus with neutral pH. Acute gastritis can progress to active chronic gastritis, which is dependent on the failure to eradicate *H. pylori* via the host immune response. Chronic infection may eventually lead to atrophy, which is the loss of glandular tissue caused by progressive mucosal injury (34). There is some evidence that host autoimmune responses increase atrophy and the synergy of host response and bacterial effects increase the likelihood of ulcer formation (18, 34, 103). Chronic inflammation and direct effects of bacterial colonization cause the gastric and duodenal mucosa to be more vulnerable to acid, which eventually leads to the formation of ulcers in some

individuals. Over time, extended atrophic gastritis leads to intestinal metaplasia and the formation of gastric cancer. In 2002, 20% of the global burden of cancers were attributable to infectious diseases with *H. pylori* being the leading cause (5.5% of all cancers) (71). It is important to note that many individuals are asymptomatic, never showing symptoms throughout their lives, and spontaneous clearance of infection has been observed as well (56, 64, 100).

### *H. pylori* Routes of Transmission

The elucidation of the mode of transmission has been hindered for several reasons. First, *H. pylori* is extremely variable in its culturability (1, 12, 83). It converts to a viable but not culturable (VBNC) state when in most environments outside the human stomach. This loss in culturability is often coincident with the morphological conversion of the cells from spiral to coccoid (60, 83). Microbiologists have traditionally relied on culturing an organism to prove the organism is viable and capable of infection, but due to the loss in culturability, the mode of transmission for this organism is difficult to pinpoint. Second, due to the potential for serious health consequences such as cancer, there have been very few human trials that adequately measure the infectious dose or investigate potential transmission sources. One study utilizing human subjects found that a dose of  $10^4$  CFU was adequate to cause infection (48). Other studies have utilized primate or mice models to study *H. pylori* transmission and infection (85, 88). Currently, several animal models are used in *H. pylori* research including the mouse, Mongolian gerbil, guinea pig, gnotobiotic piglets, and non-human primate models (61). Coccoid *H.*

*pylori* in the VBNC state are capable of infecting mice and producing ulcer and inflammatory cell infiltration although with decreased efficacy compared to healthy cells (85). Natural infection of humans may occur differently from animal models; more study is required to fully understand *H. pylori* transmission.

#### Oral-Fecal Route of Transmission

There is evidence for and against the oral-fecal route of transmission.

Investigations of the transmission of *H. pylori* have shown that survival through the intestines is not likely. Bile has a lethal effect on *H. pylori* and bile exposure would likely decrease the survival of the organism when passing through the intestines (49). Investigations have rarely identified *H. pylori* from feces and culture of the organism from fecal matter has been rare (43, 49, 76, 81, 98). However, when molecular methods were used, Queralt et al. (81) detected *H. pylori* DNA in 33% of 36 human fecal samples and 66% of wastewater samples obtained from north-east Spain. Enroth and co-workers (43) used immunomagnetic separation (IMS) and PCR to detect *H. pylori* DNA from water and stool samples. They observed that as the bacteria aged, more bacteria per ml were required for detection by IMS and PCR and hypothesized that coccoid forms seen in stationary phase had different antigenicity and DNA content than early, spiral forms. One factor that may promote the passage of *H. pylori* in fecal matter is malnourishment or diarrhea of the host. Successful culture from infected individuals was increased when patients were given a cathartic to induce diarrhea (76). More study is required to fully understand *H. pylori* survival through the intestines and in feces.

*H. pylori* has been consistently identified primarily using molecular methods from vomitus, saliva, and dental plaque (39, 40, 76, 90, 91). The organism was detected by PCR and culture in several studies that included people with healthy and diseased mouth states, as well as those with confirmed gastritis (40, 45, 62). Whether the occurrence of this organism in the human oral cavity is responsible for primary person to person infection or recurrence of infection after antibiotic treatment is currently unknown. Other research on *H. pylori* in the oral cavity has shown that some bacteria that normally reside in the human oral cavity (i.e. *Fusobacterium* sp.) can promote the coaggregation of *H. pylori* in *in vitro* culture (5, 52). It is unknown if the ability to form coaggregates with certain oral bacteria will lead to greater biofilm formation by *H. pylori* in the oral cavity. It has been hypothesized that forming or integrating into biofilms allows *H. pylori* to survive in adverse environments long enough to find a new host (9, 10, 47). This characteristic makes it likely that *H. pylori* would take advantage of the oral cavity as a reservoir for infection due to the abundance of natural biofilms established there. *H. pylori* has been found to be associated with dental plaque but the extent of its colonization, metabolic activity and virulence in that environment has never been characterized.

#### Environmental Transmission

There have been numerous studies documenting various pathogens and their persistence in water distribution systems. Distribution system biofilms have been implicated as environmental reservoirs that could serve as possible routes of human infection. For example, the bacterial pathogen *Vibrio cholerae*, which is the principal

cause of cholera epidemics, has been shown to exist in aquatic biofilms and their residence in that matrix may protect them to some extent from protozoan predation (67). Another pathogen that is a notable biofilm former is *Campylobacter jejuni* a close relative of *H. pylori*; this pathogen colonizes the gastrointestinal tract and is the most common cause of diarrheal illness in the United States (53). Similar to *H. pylori*, both *V. cholera* and *C. jejuni* are known to enter into a VBNC state under stressful conditions, which may permit the survival and persistence of these organisms in a metabolically dormant state (20, 102).

*H. pylori* DNA has been found in many different environments such as well, spring, river, and pond water, as well as, soil, house flies, and cattle feces (22, 77, 84, 101, 105). Untreated well water has also been implicated in clinical infections in the United States (15). *H. pylori* DNA has been found in plumbing systems biofilms in England, as well as in other types of water receptacles such as water pots in Keneba, West Africa (22, 105). Areas with poor water quality may be more likely to have higher rates of water-borne transmission, especially in children (22, 105). However, culture of *H. pylori* from drinking water and associated biofilms is a rare occurrence and links between water and infection have relied on epidemiological evidence (16, 68).

There have been several studies investigating the survival of *H. pylori* in the environment. To examine *H. pylori* survival in a natural freshwater environment, Adams and co-workers (1) used membrane diffusion chambers inoculated with *H. pylori* suspended in sterile creek water. The chambers were then suspended and anchored in the same creek. They monitored viability and culturability by staining the cells with the

LIVE/DEAD *Baclight*<sup>TM</sup> viability kit and by plating on Brucella agar, respectively. Although the number of culturable cells decreased dramatically, *Baclight*<sup>TM</sup> staining showed that a large number of cells retained viability and that on average the total number of cells stayed the same as exposure time to natural freshwater increased. They also noted that the cell morphology was not indicative of culturability, with both spiral and coccoid forms being present during stress exposure.

Azevedo and co-workers (9, 11-14, 47) have investigated the survival and biofilm forming characteristics of *H. pylori* in model drinking water systems. Some of the conclusions are that the characteristic change in cell morphology from a spiral to coccoid form occurs under the stress conditions seen in a model drinking water system, and is likely a form of adaptation to the environment (9, 11, 13). They also concluded that water stressed *H. pylori* are subject to nutrient shock when traditional culture techniques are used and that the quick establishment of a microaerophilic atmosphere is essential for recovery of these cells (12). Similar to Adams et al. (1), Azevedo and co-workers (9) have observed both spiral and coccoid forms representing “nonculturable” cells under conditions simulating a drinking water system.

*H. pylori* is a classic biofilm forming organism and has been found to persist in monospecies as well as multispecies biofilms (14, 28). The ability of *H. pylori* to adhere to gastric epithelial cells is crucial to colonization and subsequent infection (110-112). This ability to adhere may allow *H. pylori* to persist outside the body, providing an environmental reservoir in plumbing and potable water systems. There have been several studies of *H. pylori* biofilm formation on different materials, with reports of glass, 304

stainless steel, and polypropylene being suitable biofilm substrata (11, 47). Azevedo et al. (13) observed that small aggregates of cells formed over time with three dimensional structures only forming occasionally. Additionally high shear stress inhibited the formation of biofilms. These observations led to the conclusion that *H. pylori* could likely colonize a drinking water system, particularly one that has low shear stress, such as a groundwater well (13). When a biofilm was established with autochthonous aquatic bacteria suspended in sterile tap water, *H. pylori* was able to incorporate, persist, and agglomerate for up to 31 days without a loss in total cell numbers (47). The presence of other bacteria seemed to negate the influence of shear stress seen in the monospecies biofilm experiment (47).

While some headway has been made on the subject of environmental transmission of *H. pylori*, many aspects are still not well understood. Of particular interest and the least understood are the cues that allow *H. pylori* to switch from the nondividing, unculturable forms seen during stressful, environmental conditions to an actively dividing cell capable of causing disease.

#### Factors Responsible for Colonization and Survival of Harsh Gastric Conditions

Successful colonization of the host requires transmission, adherence to a specific niche, avoidance of host defense mechanisms, and acquisition of nutrients (96). While the mode of transmission remains under investigation, the mechanism of colonization has been extensively studied; several key factors allow *H. pylori* to survive and proliferate in

the human gastric environment as described below. Urease enzyme production, flagella and heat shock proteins are key colonization and virulence factors for *H. pylori*.

Although the urease enzyme is not required for viability *in vitro*, it is an important colonization factor that is necessary for survival and attachment to the gastric mucosa *in vivo* (37, 38, 72). The breakdown of urea into ammonia and bicarbonate by urease allows *H. pylori* to maintain a microenvironment with a higher pH than the surrounding environment (36). The urease enzyme is a high molecular weight multisubunit metalloenzyme that has been exploited to provide presumptive identification during culture from environmental samples (30). A color changing pH indicator can show that urea in the growth medium is broken down to yield ammonia and bicarbonate. Urease mutants constructed by allelic exchange mutagenesis are viable *in vitro* but avirulent in animal studies (87). Stressed *H. pylori* show decreased transcription and expression of urease both *in vitro* and *in vivo* compared to healthy cells (74, 85).

*H. pylori* cells normally possess a polar bundle of two to six sheathed flagella that are critical for colonization and survival; without motility *H. pylori* cannot penetrate the mucous layer found in the stomach (41, 65, 92). Flagella are composed of flagellin, encoded by *flaA* and *flaB* (92, 106). Eaton and co-workers (41, 42) found that non-motile *H. pylori* variants were less likely to colonize gnotobiotic piglets (25% colonization versus 90% for motile *H. pylori*) and had decreased survival overall. Another study has shown that *flaA* mRNA transcription level correlates with *H. pylori* colonization efficiency in gnotobiotic piglets (65). Interestingly, coccoid cells originating from late stationary phase cultures and those obtained from artificially induced cultures (i.e.

antibiotic stress) rarely possess flagella (19, 106). This observation could account for the decrease in colonization efficacy observed when mice were infected with coccoid *H. pylori* cells (24, 85).

Yamaguchi and co-workers (109) have demonstrated that heat shock proteins promote colonization of gastric epithelial cells by increasing adhesion. It has been hypothesized that upon exposure to stress, expression of heat shock proteins (Hsps) may be induced so that *H. pylori* is primed for adhering to the gastric epithelia (110-112). Indeed, Han and co-workers (51) observed up regulation of Hsps in response to growth temperature variation. Additionally, Thompson and co-workers (99) observed an increase in Hsp transcription during the switch from logarithmic to stationary phases. However, Merell and co-workers (69) observed that Hsps were significantly down regulated during acid stress. The conflicting results regarding *H. pylori* transcription of Hsps has shed doubt on the role of these proteins in stress modulation. Additionally, the levels of transcription seen under different conditions point to the utilization of varying mechanisms for coping with specific stresses. Therefore, the importance of these proteins for survival of *H. pylori* during stressful conditions and colonization of the host requires more study.

*H. pylori* has a wide variety of cell envelope proteins that are thought to promote adherence and colonization (96). The composition of the cell envelope of *H. pylori* is similar to other Gram- negative bacteria, with a cytoplasmic membrane, a peptidoglycan periplasm, and an outer membrane. The outer membrane is composed of peptidoglycan and lipopolysaccharide (LPS) (7, 61). In *H. pylori*, LPS is composed of lipid A, core

oligosaccharide, and O side chains; the O side chain is responsible for phase variation and is thought to be adaptive to host conditions. The O side chain can be fucosylated, imitating the Lewis blood group antigens (Le<sup>x</sup> and Le<sup>y</sup>) and is thought to be a part of the evasion of the host immune response via molecular mimicry.

Protein, lipid and antigen profiles change as the cells convert to the coccoid cell morphology, however the mechanism and purpose of this change are not well understood (17, 23). Expression of outer membrane proteins is known to promote adhesion to host cells and the specific changes seen in coccoid cells may promote survival and adhesion during transmission from host to host (17, 21, 26, 86, 89, 106).

Other factors are responsible for virulence in *H. pylori* but are not carried by all strains and are not necessary for colonization. Certain toxins produced by *H. pylori* such as vacuolating cytotoxin, VacA, and the CAG (cytotoxin associated gene) pathogenicity island lead to cytoskeletal rearrangements in the host cell, and the presence of these genes is associated with severe health outcomes (50, 75, 80, 82). A whole genome microarray study evaluating gene transcription during the switch in growth phase from logarithmic to stationary phase was presented by Thompson et al. (99) and demonstrated a growth phase dependent switch in virulence. In that study, up regulation of several virulence genes occurred at the onset of the stationary phase of growth which also coincided with the morphological conversion of the cell to the coccoid form.

Coccoid forms of *H. pylori* adhere to human gastric epithelial cells *in vitro*, stimulate cytoskeletal rearrangements, induce tyrosine phosphorylation of host proteins, and lead to successful infection in mice (24, 25, 27, 57, 63, 104). However, these effects

take longer and cause a decreased host immune response compared to healthy, spiral cells. The overall decrease in colonization factors such as flagella and Hsps may provide an explanation why stressed, coccoid forms of *H. pylori* have slower colonization and infection of mice compared to a healthy cell (24, 85). To fully understand how *H. pylori* colonize a host, *in vivo* studies will be important to elucidate the role of colonization and virulence factors in transmission.

### Concluding Remarks

Although *H. pylori* has been extensively studied, the mode of transmission remains unknown. The organism is highly virulent which makes *in vivo* study potentially dangerous to volunteers. The successful transmission of *H. pylori* requires i) exit of the host ii) survival outside the host for a period of time and iii) colonization of a new individual. Evidence suggests that direct passage of *H. pylori* from person to person is possible with cells being cultured commonly from saliva and vomitus. Indeed, *H. pylori* seems ideally suited to adhere to dental biofilms due to the plethora of outer membrane and adhesion proteins it expresses. However, this does not rule out the possibility that the organism will have to survive outside the host for a period of time. The identification of *H. pylori* specific DNA sequences and the occasional culture of this organism from both oral and environmental samples indicate that the organism can successfully leave its specific niche and perhaps survive long enough for colonization. Successful colonization of the host can lead to a variety of health outcomes ranging from asymptomatic infection to gastric cancer.

Current techniques for diagnosing infections rely mainly on markers for the urease enzyme, anti-*H. pylori* antibodies, and *H. pylori* antigens as well as biopsy and direct culture. Therapeutic approaches usually involve triple antibiotic therapy, which has drawbacks such as promoting the development of multi-drug resistant strains and adverse effects on the host. Studies using whole genome approaches are important for understanding the biochemistry and physiology of the organism. Key changes related to environmental conditions can be pinpointed using this approach and may lead to the discovery of genes that allow survival under adverse conditions. This information is useful because it may allow for the identification of novel therapeutic targets such as unique outer membrane proteins specific to coccoid cells.

The objective of this research was to increase our knowledge of the natural life history of *H. pylori*. This bacterium is interesting due to its harsh and narrow ecological niche yet apparently diverse mechanisms for coping with environmental stress and host immune responses. Understanding the nature of the survival of *H. pylori* outside the human host is important for optimizing culture techniques and assessing viability.

References

1. Adams, B. L., T. C. Bates, and J. D. Oliver. 2003. Survival of *Helicobacter pylori* in a natural freshwater environment. *Appl. Environ. Microbiol.* 69:7462-7466.
2. Albertson, N., I. Wenngren, and J. E. Sjostrom. 1998. Growth and survival of *Helicobacter pylori* in defined medium and susceptibility to Brij 78. *J. Clin. Microbiol.* 36:1232-1235.
3. Andersen, A. P., D. A. Elliott, M. Lawson, P. Barland, V. B. Hatcher, and E. G. Puszkin. 1997. Growth and morphological transformations of *Helicobacter pylori* in broth media. *J. Clin. Microbiol.* 35:2918-22.
4. Andersen, L. P., and T. Wadstrom. 2001. Basic bacteriology and culture, p. 27-38. *In* H. L. T. Mobley, G. L. Mendz, and S. L. Hazell (ed.), *Helicobacter pylori* physiology and genetics. American Society for Microbiology, Washington D. C.
5. Andersen, R. N., N. Ganeshkumar, and P. E. Kolenbrander. 1998. *Helicobacter pylori* adheres selectively to *Fusobacterium* spp. *Oral Microbiol. Immunol.* 13:51-54.
6. Anonymous. 2005. Product information sheet for ATCC<sup>®</sup> 700392. American Type Culture Collection, Manassas, VA.
7. Appelmelk, B. J., S. L. Martin, M. A. Monteiro, C. A. Clayton, A. A. McColm, P. Y. Zheng, T. Verboom, J. J. Maaskant, D. H. van den Eijnden, C. H. Hokke, M. B. Perry, C. Vandembroucke-Grauls, and J. G. Kusters. 1999. Phase variation in *Helicobacter pylori* lipopolysaccharide due to changes in the lengths of poly(C) tracts in alpha 3-fucosyltransferase genes. *Infect. Immun.* 67:6715-6715.
8. Asaka, M., A. R. Sepulveda, T. Sugiyama, and D. Y. Graham. 2001. Gastric Cancer, p. 481-498. *In* H. L. T. Mobley, G. L. Mendz, and S. L. Hazell (ed.), *Helicobacter pylori*: physiology and genetics. American Society for Microbiology, Washington, D.C.
9. Azevedo, N. F., C. Almeida, L. Cerqueira, S. Dias, C. W. Keevil, and M. J. Vieira. 2007. Coccoid form of *Helicobacter pylori* as a morphological manifestation of cell adaptation to the environment. *Appl. Environ. Microbiol.* 73:3423-3427.
10. Azevedo, N. F., S. Giao, C. Almeida, I. Fernandes, C. W. Keevil, and M. J. Vieira. 2007. Relevance of heterotrophic biofilms on the agglomeration of

*Helicobacter pylori* in water environments: Implications for transmission. *Helicobacter* 12:392-392.

11. Azevedo, N. F., A. P. Pacheco, C. W. Keevil, and M. J. Vieira. 2006. Adhesion of water stressed *Helicobacter pylori* to abiotic surfaces. *J. Appl. Microbiol.* 101:718-724.
12. Azevedo, N. F., A. P. Pacheco, C. W. Keevil, and M. J. Vieira. 2004. Nutrient shock and incubation atmosphere influence recovery of culturable *Helicobacter pylori* from water. *Appl. Environ. Microbiol.* 70:490-493.
13. Azevedo, N. F., A. R. Pinto, N. M. Reis, M. J. Vieira, and C. W. Keevil. 2006. Shear stress, temperature, and inoculation concentration influence the adhesion of water-stressed *Helicobacter pylori* to stainless steel 304 and polypropylene. *Appl. Environ. Microbiol.* 72:2936-2941.
14. Azevedo, N. F., M. J. Vieira, and C. W. Keevil. 2003. Establishment of a continuous model system to study *Helicobacter pylori* survival in potable water biofilms. *Water Sci. Technol.* 47:155-160.
15. Baker, K. H., and J. P. Hegarty. 2001. Presence of *Helicobacter pylori* in drinking water is associated with clinical infection. *Scan. J. Infect. Dis.* 33:744-746.
16. Bellack, N. R., M. W. Koehoorn, Y. C. MacNab, and M. G. Morshed. 2006. A conceptual model of water's role as a reservoir in *Helicobacter pylori* transmission: a review of the evidence. *Epidemiol. Infect.* 134:439-449.
17. Benaissa, M., P. Babin, N. Quellard, L. Pezennec, Y. Cenatiempo, and J. L. Fauchere. 1996. Changes in *Helicobacter pylori* ultrastructure and antigens during conversion from the bacillary to the coccoid form. *Infect. Immun.* 64:2331-2335.
18. Bergman, M. P., G. Faller, M. M. D'Elis, G. D. Prete, C. M. J. E. Vandenbrouke-Grauls, and B. J. Appelmek. 2001. Gastric autoimmunity, p. 429-440. *In* H. L. T. Mobley, G. L. Mendz, and S. L. Hazell (ed.), *Helicobacter pylori* physiology and genetics. American Society for Microbiology, Wahington D. C.
19. Bode, G., F. Mauch, and P. Malfertheiner. 1993. The coccoid forms of *Helicobacter pylori*. Criteria for their viability. *Epidemiol. Infect.* 111:483-490.
20. Bovill, R. A., and B. M. Mackey. 1997. Resuscitation of 'non-culturable' cells from aged cultures of *Campylobacter jejuni*. *Microbiol.* 143:1575-1581.

21. Bumann, D., H. Habibi, B. Kan, M. Schmid, C. Goosmann, V. Brinkmann, T. F. Meyer, and P. R. Jungblut. 2004. Lack of stage-specific proteins in coccoid *Helicobacter pylori* cells. *Infect. Immun.* 72:6738-6742.
22. Bunn, J. E. G., W. G. MacKay, J. E. Thomas, D. C. Reid, and L. T. Weaver. 2002. Detection of *Helicobacter pylori* DNA in drinking water biofilms: implications for transmission in early life. *Lett. Appl. Microbiol.* 34:450-454.
23. Cao, P., M. S. McClain, M. H. Forsyth, and T. L. Cover. 1998. Extracellular release of antigenic proteins by *Helicobacter pylori*. *Infect. Immun.* 66:2984-2986.
24. Cellini, L., N. Allocati, D. Angelucci, T. Iezzi, E. Dicampoli, L. Marzio, and B. Dainelli. 1994. Coccoid *Helicobacter pylori* not culturable in vitro reverts in mice. *Microbiol. Immunol.* 38:843-850.
25. Chan, W. Y., P. K. Hui, K. M. Leung, J. Chow, F. Kwok, and C. S. Ng. 1994. Coccoid forms of *Helicobacter pylori* in the human stomach. *Amer. J. Clin. Pathol.* 102:503-507.
26. Citterio, B., A. Casaroli, L. Pierfelici, M. Battistelli, E. Falcieri, and W. Baffone. 2004. Morphological changes and outer membrane protein patterns in *Helicobacter pylori* during conversion from bacillary to coccoid form. *Microbiol.* 27:353-360.
27. Cole, S. P., D. Cirillo, M. F. Kagnoff, D. G. Guiney, and L. Eckmann. 1997. Coccoid and spiral *Helicobacter pylori* differ in their abilities to adhere to gastric epithelial cells and induce interleukin-8 secretion. *Infect. Immun.* 65:843-846.
28. Cole, S. P., J. Harwood, R. Lee, R. She, and D. G. Guiney. 2004. Characterization of monospecies biofilm formation by *Helicobacter pylori*. *J. Bacteriol.* 186:3124-3132.
29. Covacci, A., J. L. Telford, G. Del Giudice, J. Parsonnet, and R. Rappuoli. 1999. *Helicobacter pylori* virulence and genetic geography. *Science.* 284:1328-1333.
30. Cussac, V., R. L. Ferrero, and A. Labigne. 1992. Expression of *Helicobacter pylori* urease genes in *Escherichia coli* grown under nitrogen limiting conditions. *J. Bacteriol.* 174:2466-2473.
31. Danon, S. J., and L. Adrian. 2001. Other gastric *Helicobacters* and spiral organisms, p. 549-563. *In* H. L. T. Mobley, G. L. Mendz, and S. L. Hazell (ed.), *Helicobacter pylori* physiology and genetics. American Society for Microbiology, Washington D. C.

32. de Oliveira, A. M. R., G. A. Rocha, D. Queiroz, S. B. de Moura, and A. L. T. Rabello. 1999. Seroconversion for *Helicobacter pylori* in adults from Brazil. *Transact. Royal Soc. Trop. Med. and Hyg.* 93:261-263.
33. Degnan, A. J., W. C. Sonzogni, and J. H. Standridge. 2003. Development of a plating medium for selection of *Helicobacter pylori* from water samples. *Appl. Environ. Microbiol.* 69:2914-2918.
34. Dixon, M. F. 2001. Pathology of gastritis and peptic ulceration, p. 459-469. *In* H. L. T. Mobley, G. L. Mendz, and S. L. Hazell (ed.), *Helicobacter pylori* physiology and genetics. American Society for Microbiology, Washington D. C.
35. Donelli, G., P. Matarrese, C. Fiorentini, B. Dainelli, T. Taraborelli, E. Di Campi, S. Di Bartolomeo, and L. Cellini. 1998. The effect of oxygen on the growth and cell morphology of *Helicobacter pylori*. *FEMS Microbiol. Lett.* 168:9-15.
36. Dunn, B. E., G. P. Campbell, G. I. Perezperez, and M. J. Blaser. 1990. Purification and characterization of urease from *Helicobacter pylori*. *J. Biol. Chem.* 265:9464-9469.
37. Dunn, B. E., and S. H. Phadnis. 1998. Structure, function and localization of *Helicobacter pylori* urease. *Yale J. Biol. Med.* 71:63-73.
38. Dunn, B. E., N. B. Vakil, B. G. Schneider, M. M. Miller, J. B. Zitzer, T. Peutz, and S. H. Phadnis. 1997. Localization of *Helicobacter pylori* urease and heat shock protein in human gastric biopsies. *Infect. Immun.* 65:1181-1188.
39. Dye, B. A., D. Kruszon-Moran, and G. McQuillan. 2000. *Helicobacter pylori* infection and pathological periodontal conditions among adults in the United States. *Amer. J. Epidemiol.* 151:59.
40. Dye, B. A., D. Kruszon-Moran, and G. McQuillan. 2002. The relationship between periodontal disease attributes and *Helicobacter pylori* infection among adults in the United States. *Amer. J. Pub. Health.* 92:1809-1815.
41. Eaton, K. A., D. R. Morgan, and S. Krakowka. 1992. Motility as a factor in the colonization of gnotobiotic piglets by *Helicobacter pylori*. *J. Med. Microbiol.* 37:123-127.
42. Eaton, K. A., S. Suerbaum, C. Josenhans, and S. Krakowka. 1996. Colonization of gnotobiotic piglets by *Helicobacter pylori* deficient in two flagellin genes. *Infect. Immun.* 64:2445-2448.

43. Enroth, H., and L. Engstrand. 1995. Immunomagnetic separation and PCR for the detection of *Helicobacter pylori* in water and stool specimens. *J. Clin Microbiol.* 33:2162-2165.
44. Everhart, J. E., D. Kruszon-Moran, G. I. Perez-Perez, T. S. Tralka, and G. McQuillan. 2000. Seroprevalence and ethnic differences in *Helicobacter pylori* infection among adults in the United States. *J. Infect. Dis.* 181:1359-1363.
45. Gebara, E. C. E., C. Pannuti, C. M. Faria, L. Chehter, M. P. A. Mayer, and L. Lima. 2004. Prevalence of *Helicobacter pylori* detected by polymerase chain reaction in the oral cavity of periodontitis patients. *Oral Microbiol. Immunol.* 19:277-280.
46. Georgopoulos, S. D., A. F. Mentis, C. A. Spiliadis, L. S. Tzouvelekis, E. Tzelepi, A. Moshopoulos, and N. Skandalis. 1996. *Helicobacter pylori* infection in spouses of patients with duodenal ulcers and comparison of ribosomal RNA gene patterns. *Gut.* 39:634-638.
47. Giao, M. S., N. F. Azevedo, S. A. Wilks, M. J. Vieira, and C. W. Keevil. 2008. Persistence of *Helicobacter pylori* in heterotrophic drinking-water biofilms. *Appl. Environ. Microbiol.* 74:5898-5904.
48. Graham, D. Y., A. R. Opekun, M. S. Osato, H. M. T. El-Zimaity, C. K. Lee, Y. Yamaoka, W. A. Qureshi, M. Cadoz, and T. P. Monath. 2004. Challenge model for *Helicobacter pylori* infection in human volunteers. *Gut* 53:1235-1243.
49. Graham, D. Y., and M. S. Osato. 2000. *H. pylori* in the pathogenesis of duodenal ulcer: Interaction between duodenal acid load, bile, and *H. pylori*. *Amer. J. Gastroenterol.* 95:87-91.
50. Graham, D. Y., and Y. Yamaoka. 2000. Disease-specific *Helicobacter pylori* virulence factors: The unfulfilled promise. *Helicobacter* 5:S3-S9.
51. Han, Y. H., W. Z. Liu, Y. Z. Shi, L. Q. Lu, S. D. Xiao, and Q. H. Zhang. 2009. Gene expression profile of *Helicobacter pylori* in response to growth temperature variation. *J. Microbiol.* 47:455-465.
52. Ishihara, K., T. Miura, R. Kimizuka, Y. Ebihara, Y. Mizuno, and K. Okuda. 1997. Oral bacteria inhibit *Helicobacter pylori* growth. *FEMS Microbiol. Lett.* 152:355-361.
53. Joshua, G. W. P., C. Guthrie-Irons, A. V. Karlyshev, and B. W. Wren. 2006. Biofilm formation in *Campylobacter jejuni*. *Microbiol.-Sgm* 152:387-396.

54. Kelly, D. J., N. J. Hughes, and R. K. Poole. 2001. Microaerobic physiology: aerobic respiration, anaerobic respiration, and carbon dioxide metabolism, p. 113-124. In H. L. T. Mobley, G. L. Mendz, and S. L. Hazell (ed.), *Helicobacter pylori* physiology and genetics. American Society for Microbiology, Washington D.C.
55. Kersulyte, D., H. Chalkauskas, and D. E. Berg. 1999. Emergence of recombinant strains of *Helicobacter pylori* during human infection. *Mol. Microbiol.* 31:31-43.
56. Kikuchi, S., A. Ohgihara, A. Hasegawa, K. Miki, E. Kaneko, and H. Mizukoshi. 2004. Seroconversion and seroreversion of *Helicobacter pylori* antibodies over a 9-year period and related factors in Japanese adults. *Helicobacter.* 9:335-341.
57. Kitaeva, L. V., I. A. Mikhailova, D. M. Semov, S. N. Proshin, and V. Y. Kravtsov. 2008. Mucocytes with micronuclei and sowing with the coccoid forms of *Helicobacter pylori* in a mucous membrane of human stomach. *Cell Tiss. Biol.* 2:170-175.
58. Kivi, M., and Y. Tindberg. 2006. *Helicobacter pylori* occurrence and transmission: A family affair? *Scand. J. Infect. Dis.* 38:407-417.
59. Kurokawa, M., M. Nukina, and H. Nakanishi. 1999. Resuscitation from the viable but non culturable state of *Helicobacter pylori*. *Kan. Zasshi.* 73:15-19.
60. Kusters, J. G., M. M. Gerrits, J. A. G. VanStrijp, and C. VandenbrouckeGrauls. 1997. Coccoid forms of *Helicobacter pylori* are the morphologic manifestation of cell death. *Infect. Immun.* 65:3672-3679.
61. Kusters, J. G., A. H. M. van Vliet, and E. J. Kuipers. 2006. Pathogenesis of *Helicobacter pylori* infection. *Clin. Microbiol. Reviews* 19:449-490.
62. Li, C., P. R. Musich, T. Ha, D. A. Ferguson, N. R. Patel, D. S. Chi, and E. Thomas. 1995. High prevalence of *Helicobacter pylori* in saliva demonstrated by a novel PCR assay. *J. Clin. Pathol.* 48:662-666.
63. Liu, Z. F., C. Y. Chen, W. Tang, J. Y. Zhang, Y. Q. Gong, and J. H. Jia. 2006. Gene-expression profiles in gastric epithelial cells stimulated with spiral and coccoid *Helicobacter pylori*. *J. Med. Microbiol.* 55:1009-1015.
64. Malaty, H. M., A. El-Kasabany, D. Y. Graham, C. C. Miller, S. G. Reddy, S. R. Srinivasan, Y. Yamaoka, and G. S. Berenson. 2002. Age at acquisition of *Helicobacter pylori* infection: a follow-up study from infancy to adulthood. *Lancet* 359:931-935.

65. Mankoski, R., T. Hoepf, S. Krakowka, and K. A. Eaton. 1999. *flaA* mRNA transcription level correlates with *Helicobacter pylori* colonisation efficiency in gnotobiotic piglets. *J. Med. Microbiol.* 48:395-399.
66. Marshall, B. J. 1995. *Helicobacter pylori* - the etiologic agent for peptic ulcer. *J. Amer. Med. Assoc.* 274:1064-1066.
67. Matz, C., D. McDougald, A. M. Moreno, P. Y. Yung, F. H. Yildiz, and S. Kjelleberg. 2005. Biofilm formation and phenotypic variation enhance predation-driven persistence of *Vibrio cholerae*. *Proc. Nat. Acad. Sci.* 102:16819-16824.
68. Melius, E., R. Wierzba, S. Davis, J. Sobel, B. Gold, A. Henderson, and J. Cheek. 2005. Risk factors for *Helicobacter pylori* in a rural community. Unpublished data. Centers for Disease Control, 55<sup>th</sup> Annual epidemic intelligence service conference, April 24-28, 2006.
69. Merrell, D. S., M. L. Goodrich, G. Otto, L. S. Tompkins, and S. Falkow. 2003. pH-regulated gene expression of the gastric pathogen *Helicobacter pylori*. *Infect. Immun.* 71:3529-3539.
70. Mitchell, H. M. 2001. Epidemiology of infection, p. 7-18. *In* H. L. T. Mobley, G. L. Mendz, and S. L. Hazell (ed.), *Helicobacter pylori* physiology and genetics. American Society for Microbiology, Washington D. C.
71. Moss, S. F., and P. Malfertheiner. 2007. *Helicobacter* and gastric malignancies. *Helicobacter* 12:23-30.
72. Nakamura, H., H. Yoshiyama, H. Takeuchi, T. Mizote, K. Okita, and T. Nakazawa. 1998. Urease plays an important role in the chemotactic motility of *Helicobacter pylori* in a viscous environment. *Infect. Immun.* 66:4832-4837.
73. Nedenskov, P. 1994. Nutritional requirements for growth of *Helicobacter pylori*. *Appl. Environ. Microbiol.* 60:3450-3453.
74. Nilsson, H. O., J. Blom, W. Abu Al-Soud, A. Ljungh, L. P. Andersen, and T. Wadstrom. 2002. Effect of cold starvation, acid stress, and nutrients on metabolic activity of *Helicobacter pylori*. *Appl. Environ. Microbiol.* 68:11-19.
75. Pan, Z. J., R. W. M. van der Hulst, G. N. J. Tytgat, J. Dankert, and A. van der Ende. 1999. Relation between *vacA* subtypes, cytotoxin activity, and disease in *Helicobacter pylori*-infected patients from the Netherlands. *Amer. J. Gastroenterol.* 94:1517-1521.

76. Parsonnet, J., H. Shmueli, and T. Haggerty. 1999. Fecal and oral shedding of *Helicobacter pylori* from healthy infected adults. *J. Amer. Med. Assoc.* 282:2240-2245.
77. Perez, L. M., F. Codony, D. L. Leyton, M. Fittipaldi, B. Adrados, and J. Morato. 2010. Quantification of *Helicobacter pylori* levels in soil samples from public playgrounds in Spain. *J. Zhejiang Univ.-Science B* 11:27-29.
78. Perez-Perez, G. I., D. Rothenbacher, and H. Brenner. 2004. Epidemiology of *Helicobacter pylori* infection. *Helicobacter* 9:1-6.
79. Perez-Perez, G. I., R. B. Sack, R. Reid, M. Santosham, J. Croll, and M. J. Blaser. 2003. Transient and persistent *Helicobacter pylori* colonization in native American children. *J. Clin. Microbiol.* 41:2401-2407.
80. Phadnis, S. H., D. Ilver, L. Janzon, S. Normark, and T. U. Westblom. 1994. Pathological significance and molecular characterization of the vacuolating toxin gene of *Helicobacter pylori*. *Infect. Immun.* 62:1557-1565.
81. Queralt, N., R. Bartolome, and R. Araujo. 2005. Detection of *Helicobacter pylori* DNA in human faeces and water with different levels of faecal pollution in the north-east of Spain. *J. Appl. Microbiol.* 98:889-895.
82. Rudi, J., C. Kolb, M. Maiwald, D. Kuck, A. Sieg, P. R. Galle, and W. Stremmel. 1998. Diversity of *Helicobacter pylori vacA* and *cagA* genes and relationship to VacA and CagA protein expression, cytotoxin production, and associated diseases. *J. Clin. Microbiol.* 36:944-948.
83. Saito, N., K. Konishi, F. Sato, M. Kato, H. Takeda, T. Sugiyama, and M. Asaka. 2003. Plural transformation processes from spiral to coccoid *Helicobacter pylori* and its viability. *J. Infect.* 46:49-55.
84. Sasaki, K., Y. Tajiri, M. Sata, Y. Fujii, F. Matsubara, M. G. Zhao, S. Shimizu, A. Toyonaga, and K. Tanikawa. 1999. *Helicobacter pylori* in the natural environment. *Scand. J. Infect. Dis.* 31:275-280.
85. She, F. F., J. Y. Lin, J. Y. Liu, C. Huang, and D. H. Su. 2003. Virulence of water-induced coccoid *Helicobacter pylori* and its experimental infection in mice. *World J. Gastroenterol.* 9:516-520.
86. Shimomura, H., S. Hayashi, K. Yokota, K. Oguma, and Y. Hirai. 2004. Alteration in the composition of cholesteryl glucosides and other lipids in *Helicobacter pylori* undergoing morphological change from spiral to coccoid form. *FEMS Microbiol. Lett.* 237:407-413.

87. Skouloubris, S., J. M. Thiberge, A. Labigne, and H. De Reuse. 1998. The *Helicobacter pylori* UreI protein is not involved in urease activity but is essential for bacterial survival in vivo. *Infect. Immun.* 66:4517-21.
88. Solnick, J. V., L. M. Hansen, D. R. Canfield, and J. Parsonnet. 2001. Determination of the infectious dose of *Helicobacter pylori* during primary and secondary infection in rhesus monkeys (*Macaca mulatta*). *Infect. Immun.* 69:6887-6892.
89. Sorberg, M., M. Nilsson, H. Hanberger, and L. E. Nilsson. 1996. Morphologic conversion of *Helicobacter pylori* from bacillary to coccoid form. *Euro. J. Clin. Microbiol. Infect. Dis.* 15:216-219.
90. Souto, G. S. S., G. A. S. Pereira, A. C. Medeiros, M. C. C. Sampaio, and R. C. Barreto. 2003. Prevalence of the *Helicobacter pylori* in the dental biofilme, for the technique "nested pcr". *J. Dent. Res.* 82:284-284.
91. Souto, R., and A. P. V. Colombo. 2008. Detection of *Helicobacter pylori* by polymerase chain reaction in the subgingival Biofilm and saliva of non-dyspeptic periodontal patients. *J. Periodontol.* 79:97-103.
92. Spohn, G., and V. Scarlato. 2001. Motility, chemotaxis, and flagella, p. 239-248. *In* H. L. T. Mobley, G. L. Mendz, and S. L. Hazell (ed.), *Helicobacter pylori* physiology and genetics. American Society for Microbiology, Washington D. C.
93. Suerbaum, S., J. M. Smith, K. Bapumia, G. Morelli, N. H. Smith, E. Kunstmann, I. Dyrek, and M. Achtman. 1998. Free recombination within *Helicobacter pylori*. *Proc. Nat. Acad. Sci.* 95:12619-12624.
94. Suzuki, J., H. Muraoka, I. Kobayasi, T. Fujita, and T. Mine. 1999. Rare incidence of interspousal transmission of *Helicobacter pylori* in asymptomatic individuals in Japan. *J. Clin. Microbiol.* 37:4174-4176.
95. Testerman, T. L., P. B. Conn, H. L. T. Mobley, and D. J. McGee. 2006. Nutritional requirements and antibiotic resistance patterns of *Helicobacter* species in chemically defined media. *J. Clin. Microbiol.* 44:1650-1658.
96. Testerman, T. L., D. J. McGee, and H. L. T. Mobley. 2001. Adherence and colonization, p. 381-417. *In* H. L. T. Mobley, G. L. Mendz, and S. L. Hazell (ed.), *Helicobacter pylori* physiology and genetics. American Society for Microbiology, Wahington D.C.

97. Testerman, T. L., D. J. McGee, and H. L. T. Mobley. 2001. *Helicobacter pylori* growth and urease detection in the chemically defined medium Ham's F-12 nutrient mixture. *J. Clin. Microbiol.* 39:3842-3850.
98. Thomas, J. E., G. R. Gibson, M. K. Darboe, A. Dale, and L. T. Weaver. 1992. Isolation of *Helicobacter pylori* from human feces. *Lancet* 340:1194-1195.
99. Thompson, L. J., D. S. Merrell, B. A. Neilan, H. Mitchell, A. Lee, and S. Falkow. 2003. Gene expression profiling of *Helicobacter pylori* reveals a growth-phase-dependent switch in virulence gene expression. *Infect. Immun.* 71:2643-2655.
100. Vanzanten, S., P. T. Pollak, L. M. Best, G. S. Bezanson, and T. Marrie. 1994. Increasing prevalence of *Helicobacter pylori* infection with age - a continuous risk of infection in adults rather than cohort effect. *J. Infect. Dis.* 169:434-437.
101. Voytek, M. A., J. B. Ashen, L. R. Fogerty, J. D. Kirshtein, and E. R. Landa. 2005. Detection of *Helicobacter pylori* and fecal indicator bacteria in five North American rivers. *J. Water Health* 03:405-422.
102. Wai, S. N., T. Moriya, K. Kondo, H. Misumi, and K. Amako. 1996. Resuscitation of *Vibrio cholerae* O1 strain TSI-4 from a viable but nonculturable state by heat shock. *FEMS Microbiol. Lett.* 136:187-191.
103. Wang, J., T. G. Blanchard, and P. B. Ernst. 2001. Host inflammatory response to infection, p. 471-480. *In* H. L. T. Mobley, G. L. Mendz, and S. L. Hazell (ed.), *Helicobacter pylori* physiology and genetics. American society for microbiology, Washington D. C.
104. Wang, X., E. Sturegard, R. Rupa, H. O. Nilsson, P. A. Aleljung, B. Carlen, R. Willen, and T. Wadstrom. 1997. Infection of BALB/c mice by spiral and coccoid forms of *Helicobacter pylori*. *J. Med. Microbiol.* 46:657-663.
105. Watson, C. L., R. J. Owen, B. Said, S. Lai, J. V. Lee, S. Surman-Lee, and G. Nichols. 2004. Detection of *Helicobacter pylori* by PCR but not culture in water and biofilm samples from drinking water distribution systems in England. *J. Appl. Microbiol.* 97:690-698.
106. Worku, M. L., R. L. Sidebotham, M. M. Walker, T. Keshavarz, and Q. N. Karim. 1999. The relationship between *Helicobacter pylori* motility, morphology and phase of growth: implications for gastric colonization and pathology. *Microbiol.-Sgm* 145:2803-2811.
107. Wu, M. S., C. J. Chen, and J. T. Lin. 2005. Host-environment interactions: Their impact on progression from gastric inflammation to carcinogenesis and on

development of new approaches to prevent and treat gastric cancer. *Cancer Epidemiol. Biomark. Prevent.* 14:1878-1882.

108. Xia, H. H. X., and N. J. Talley. 1997. Natural acquisition and spontaneous elimination of *Helicobacter pylori* infection: Clinical implications. *Amer. J. Gastroenterol.* 92:1780-1787.
109. Yamaguchi, H., T. Osaki, N. Kurihara, H. Taguchi, T. Hanawa, T. Yamamoto, and S. Kamiya. 1997. Heat-shock protein 60 homologue of *Helicobacter pylori* is associated with adhesion of *H. pylori* to human gastric epithelial cells. *J. Med. Microbiol.* 46:825-831.
110. Yamaguchi, H., T. Osaki, H. Taguchi, T. Hanawa, T. Yamamoto, M. Fukuda, H. Kawakami, H. Hirano, and S. Kamiya. 1997. Growth inhibition of *Helicobacter pylori* by monoclonal antibody to heat-shock protein 60. *Microbiol. and Immunol.* 41:909-916.
111. Yamaguchi, H., T. Osaki, H. Taguchi, T. Hanawa, T. Yamamoto, and S. Kamiya. 1997. Production and characterisation of monoclonal antibodies to heat-shock protein 60 of *Helicobacter pylori*. *J. Med. Microbiol.* 46:819-824.
112. Yamaguchi, H., T. Osaki, H. Taguchi, T. Hanawa, T. Yamamoto, and S. Kamiya. 1998. Relationship between expression of HSP60, urease activity, production of vacuolating toxin, and adherence activity of *Helicobacter pylori*. *Journal of Gastroenterol.* 33:6-9.

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CHAPTER 3

**Detection of *Mycobacteria*, *Legionella*, and *Helicobacter* in Drinking Water and Associated Biofilms on the Crow Reservation, Montana, USA**

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## ABSTRACT

Public health has been shown to be directly related to water quality, and although drinking water quality has improved in much of the United States, rural areas typically have underserved water systems. Private residences in rural areas with water systems that are not adequately regulated, monitored, and updated could have drinking water that poses a health risk. To investigate water quality on the Crow Reservation in Montana, water and biofilm samples were collected from 57 public buildings and private residences served by both treated municipal and individual groundwater well systems. Three bacterial genera, with members that are potential drinking water pathogens, were chosen for investigation. *Mycobacteria*, *Legionella*, and *Helicobacter* were detected by PCR and/or standard culture techniques. Free and total chlorine, temperature, and pH were recorded at the time of sampling. Fecal coliform bacteria and heterotrophic plate count (HPC) bacteria were enumerated using m-Colibblue24<sup>®</sup> and R2A agar, respectively. All three target genera were detected in drinking water systems on the Crow Reservation. Species detected included the opportunistic and frank pathogens *Mycobacterium avium*, *M. gordonae*, *M. flavescens*, *Legionella pneumophila*, and *H. pylori*. There was no correlation between the presence of any genera and chlorine (free and total), temperature, pH or fecal coliforms. However, there was an association between HPC bacteria and the presence of *Mycobacteria* and *Legionella* but not the presence of *Helicobacter*. This research has shown that groundwater and municipal drinking water systems and associated biofilms may be reservoirs for *Mycobacteria*, *Legionella*, and *Helicobacter*.

## INTRODUCTION

In the United States over 15 million households rely on private ground water wells for their primary drinking water source (82), and in many rural areas private and community groundwater wells provide a major source of drinking water (12). Generally, most water obtained from private groundwater systems is considered safe to drink (23). However, in the United States from 1999-2002, 22% of water-borne illnesses were attributed to individual water systems and 36% were attributed to community systems (24). Private water systems are not routinely monitored for bacteriological water quality, thus little is known about the presence of bacterial pathogens in these systems.

Information regarding water quality on Indian Reservations in the United States is equally scant. However, it is known that American Indian populations have disproportionately high disease burdens compared to the overall population of the United States (59). This is due to many factors which include economics, geographic isolation, cultural barriers, and inadequate sewage disposal (5).

The United States Centers for Disease Control report that chronic lower respiratory disease, influenza and pneumonia are among the top ten causes of death among American Indian and Alaska Native populations (82). In Montana, cancer is included as a major cause of death for American Indian populations (21). Although it has been observed that the disease burden of these populations is greater than the overall population of the United States (59), very little research has been done to identify causes and potential routes of exposure to infectious agents and environmental carcinogens. In the present study, three bacterial genera with members that are potential drinking water

pathogens, *Mycobacteria*, *Legionella*, and *Helicobacter*, were chosen for investigation due to concerns expressed by Crow Tribal community members about poor drinking water quality and the relationship of these organisms to respiratory disease and stomach cancer (28).

*Mycobacteria* are common inhabitants of drinking water systems and are known to survive and proliferate in biofilms (29, 49). Several species of this genus cause respiratory disease in mainly immunocompromised humans. Species include members of the *Mycobacterium avium* complex, *M. gordonae*, *M. flavescens*, and others (18, 36, 54, 55). *Legionella* are ubiquitous throughout aquatic environments including ground and surface water, and manmade water reservoirs such as potable water systems and cooling towers (20, 53, 81). *Legionella pneumophila* is the main causative agent for respiratory disease in that genus, causing Legionellosis in the form of Legionnaire's disease and Pontiac fever (30). Legionellosis is thought to occur when *Legionella* are aerosolized and inhaled (62). However, it has been suggested that transmission of the different forms of Legionellosis, and the resultant severity of disease, may be related to an association with biofilms (42). *Helicobacter* are pathogens of the gastrointestinal tract of mammals but have been found in many environments such as well, river, and pond water, in addition to house flies, and cattle feces (68). *Helicobacter pylori* are the primary bacterial cause of gastritis, as well as peptic and duodenal ulcers in people around the world (63). Infection is known to increase the risk of the development of gastric mucosa-associated lymphoma and adenocarcinoma (62). Water is a short term reservoir, with the pathogen often

occurring sporadically in drinking water supplies that have been exposed to sewage, or have been contaminated by infected animals (10).

Drinking water samples and their associated biofilms were tested for heterotrophic and coliform bacteria by traditional culture methods. *Mycobacteria*, *Legionella*, and *Helicobacter* species were detected by culture and PCR. The aim of this study was to investigate whether these organisms are common inhabitants of drinking water systems on the Crow Reservation in southeast Montana.

## MATERIALS AND METHODS

**Study Area.** The Crow Indian Reservation, Montana, USA was the primary location for sample collection and analysis. Fifty-seven locations were sampled across the Crow Reservation (41 private residences and 14 public buildings) from March 2007 through July 2009. The Crow Reservation, the largest reservation in Montana, is rural with an average population density of 1.9 individuals per square mile (82). The Crow tribe has an enrolled membership of 11,357 and approximately 72% of members live on or near the Reservation (65). This Reservation has a diverse landscape spanning the Wolf, Big Horn and Pryor Mountain ranges, as well as the Big Horn and Little Big Horn River valleys. Land use is typical of rural areas in Montana with approximately 68% grazing rangeland, 12% dry cropland, 3% irrigated cropland, 15% forested areas, 1% wild land, and 1% developed areas (4). The Crow Reservation area receives approximately 12-18 total inches annual precipitation (4). The surface water in the area is dependent on precipitation, snowpack and groundwater for recharge while the aquifers on the

reservation rely on infiltration from rivers, streams, precipitation, stock ponds and reservoirs (38). The major township on the reservation, Crow Agency, has drinking water provided by treated surface water, while other townships utilize community and private groundwater wells and springs (33). The Crow Agency treatment facility performs reliably and adequately; however the distribution system in Crow Agency is nearly 100 years old and is vulnerable to cracks and leaks (27). Most of the residents outside of designated townships have privately maintained groundwater wells, often only drilled to first water.

**Sample Collection and Processing.** Samples were primarily collected from kitchen sinks in private residences and kitchen or restroom sinks in public buildings. Biofilm samples were collected first, before any flushing or sterilization of the tap. Biofilm samples were collected by systematically wiping the inside of the drinking water faucet with a sterile cotton swab. Three swabs were collected from one faucet at each residence or building and were placed in individual tubes containing sterile water for transport. To calculate surface area of the biofilm, the faucet dimensions (depth and width) were measured and recorded. After biofilm collection, the faucet was wiped with 95% ethanol to sanitize it before bulk water collection. After sanitization of the tap, one liter of water was collected without flushing and is denoted as “first flush”. First flush sample collection was added in 2008 (n=20) and thus this fraction was only analyzed for groundwater wells. After first flush collection, the water was run from the tap for two minutes minimum or until water temperature stabilized prior to parameter measurement. Physical and chemical characteristics were measured using standard methodologies. The

presence and quantity of free and total chlorine was measured using a colorimetric method (Hach kit model CN-70 chlorine test kit, Hach Co., Loveland, CO). The temperature and pH were measured using a multi-parameter probe (Oakton, Vernon Hills, IL). After a minimum of two minutes of flushing, “post flush” water was collected in three separate one liter sterile plastic bottles. All samples were placed on ice and transported to the laboratory and processed within 24 h. In the laboratory, tubes containing biofilm samples were vortexed for one minute and the cell suspensions from each swab were pooled and mixed. Pooled cell suspensions from the same source were used for all biofilm analysis. To concentrate water samples, 900 ml from each liter sample was filtered through a 0.45  $\mu\text{m}$  25mm diameter mixed cellulose ester filter (Pall Corp., Ann Arbor, MI). The filter was vortexed in 1.5 ml of PBS at maximum speed for one minute, and then removed and the cell suspension centrifuged at 13,000 x g for 10 minutes. The supernatant was removed and the cell pellet was resuspended in 100  $\mu\text{l}$  of sterile water. Pooled biofilm and both concentrated and un-concentrated water samples were used in DNA extractions. Pooled biofilm and un-concentrated water samples were used for genus specific culture methods.

**Quantification of Fecal Indicator Bacteria and Heterotrophic Bacteria.** Fecal indicator bacteria were quantified for first flush and post flush water samples using standard methodologies. The presence of fecal contamination was determined by growth on the selective and differential m-Colibblue24<sup>®</sup> broth (Hach Co., Loveland, CO). This medium was used to culture coliform bacteria and differentiates *Escherichia coli* by using an enzymatic indicator. One hundred ml of each water sample and appropriate dilutions

were filtered and the filter placed on a pad soaked with 2 ml of the Coliblu<sup>®</sup>24 broth. The filters were incubated at 37°C and growth was observed at 24 h. Post-flush water samples were collected in triplicate and each replicate was analyzed separately. Sterile water was filtered as a negative control, and sterile water was inoculated with environmental isolates of *Escherichia coli* and *Klebsiella pneumoniae* obtained from drinking water samples from New Haven, CT as a positive control. Heterotrophic bacteria were enumerated for biofilm, first flush and post flush samples. Each sample was diluted and plated on R2A agar followed by incubation at 30°C for 2 weeks.

**Control Bacterial Strains and Growth Conditions.** Representative species from each genus of interest were kept as frozen stocks at -70°C and used as positive controls in both PCR and culture methods. The *M. avium* W2001 strain used in this study was originally isolated from drinking water in the Boston area and has been classified as *M. avium* subsp. *hominisuis* based on the *hsp65* gene (80). The *M. avium* strain was cultured on Middlebrook 7H10 (Difco) and incubated at 37°C for 10 days. *L. pneumophila* strain 33153 was obtained from the American Type Culture Collection, cultured on *Legionella* agar (Difco) enriched with 0.7% L-cysteine and 0.3% ferric pyrophosphate (Difco) and incubated at 37°C for 7 days. The *H. pylori* strain 43504 was obtained from the American Type Culture Collection and was cultured on *H. pylori* specific HP medium (25). The *Helicobacter* cultures were placed in a BBL anaerobe jar with a BBL CampyPak Plus<sup>™</sup> sachet, which creates a microaerophilic atmosphere of 5-10% oxygen and 10% carbon dioxide, and incubated for one week at 37°C. All control strains were grown and sequentially transferred twice prior to use as positive controls.

**Culture of Drinking Water and Biofilm Samples.** The drinking water and biofilm samples were analyzed for the presence of *Mycobacteria*, *Legionella*, and *Helicobacter*, by organism appropriate culture techniques. Due to overgrowth by other microorganisms, specific selection methods were employed to target the organisms of interest. To select for members of the genus *Mycobacteria*, two hundred microliters of each unconcentrated water sample as well as the pooled biofilm suspension were treated with a final concentration of 0.005% cetyl pyridinium chloride (CPC) (Sigma, St. Louis, MO) for 30 minutes as previously described (70). Sterile tap water was inoculated with *M. avium* W2001 and treated with CPC as a positive control. The CPC treated cells were washed with phosphate buffered saline twice by centrifuging at 10,000 x g for 5 minutes. Subsequently, one hundred microliters were plated onto M7H10 agar (Difco), two replicates were plated for each sample and were incubated at 37°C for up to three weeks. To select for *Legionella* species, each sample was heated to 50°C for 30 minutes in a water bath (9). Subsequently, one hundred microliters were plated onto enriched *Legionella* agar (Difco), two replicates were plated for each sample and incubated at 37°C for one week. Sterile tap water was inoculated with *L. pneumophila* ATCC 33153 and treated with heat as a positive control. The samples were also cultured on *H. pylori* specific HP medium (25). *H. pylori* ATCC 43504 was inoculated into sterile water and plated as a positive control. The plates were placed in a BBL anaerobe jar with a BBL CampyPak Plus™ sachet, and incubated for one week at 37°C. All presumptive isolates were subcultured and subsequently identified by PCR and phylogenetic analysis.

**DNA Extraction from Biofilm and Water Samples.** Nucleic acids were extracted from the pooled biofilm suspensions and from concentrated and unconcentrated water samples. DNA was extracted within 48 h of sampling and the extracts were immediately frozen at  $-20^{\circ}\text{C}$ . Two ml of each biofilm sample was centrifuged at  $12,000 \times g$  for 15 minutes. Subsequently, all but  $100\mu\text{l}$  of the supernatant was removed, the pellet was mixed thoroughly into the liquid and the suspension was added to 2 ml plastic screw cap tubes with o-rings (Fisher) containing 0.4g of 0.1 mm sterile glass beads. Similarly,  $200\mu\text{l}$  of each concentrated and unconcentrated water sample was added to individual sterile bead tubes for DNA extraction. Two hundred microliters of lysis buffer consisting of 20 mM sodium acetate (Fisher Scientific, Fair Lawn, New Jersey), 0.5% sodium dodecyl sulfate (Fisher), and 1mM ethylenediamine-tetraacetic acid (Fisher) and  $500 \mu\text{l}$  phenol (pH 8.1) (Fisher) was also added to each 2 ml tube and the mixture was homogenized in a Fastprep<sup>®</sup> FP120 cell disrupter at speed 5.0 for 40 seconds. After homogenization, samples were placed on ice and allowed to rest for 10 minutes. The samples were then centrifuged at  $12,000 \times g$  for 10 minutes. The DNA was precipitated by transferring the supernatant to a fresh 2 ml tube containing an equal volume of chloroform: isoamylalcohol (24:1). The samples were vortexed for 30 seconds and then centrifuged at  $12,000 \times g$  for 5 minutes. The supernatant was transferred to another fresh tube containing an equal volume of isopropanol and 1/10 volume of 3M sodium acetate and held at  $-20^{\circ}\text{C}$  for 24 h. The nucleic acids were subsequently pelleted by centrifugation, washed once with 70% ethanol, air-dried, and finally resuspended in  $100\mu\text{l}$  of Tris-EDTA buffer (TE) consisting of 10mM Tris and 1mM EDTA (Fisher).

**PCR Amplification, Sequencing, and Phylogenetic Analysis.** The detection limit of each primer set was determined by amplification of a 10-fold dilution series of purified genomic DNA (10 ng - 0.0001 pg). The target genes, sequences, product sizes, and PCR conditions are listed in Table 3.1. To ensure that the PCR reaction was not inhibited by environmental contaminants, amplification of each sample was performed using eubacterial 16S rRNA primers as described by Voytek et al. (84). Amplification of the PCR products was done in a 25  $\mu$ l PCR mixture containing 1x PCR buffer II, 50-200ng template DNA, 200  $\mu$ M (each) deoxynucleoside triphosphates (Takara Bio Inc., Japan), 0.1  $\mu$ M (each) of primer (Integrated DNA Technologies, Coralville, IA), and 1U LA Taq polymerase (Takara). Aliquots of each PCR product were separated by electrophoresis in a 0.8% (w/v) agarose gel (Fisher) in TBE buffer consisting of 90 mM Tris-HCl (Fisher), 80 mM boric acid (Fisher), 2.5 mM EDTA (Fisher) and stained with ethidium bromide (0.5 $\mu$ g/ml). PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Automated sequencing from both strands of PCR products of positive samples was performed by the Molecular Research Core Facility at Idaho State University. DNA sequences were assessed for their similarity to published DNA sequences using the BLAST database (<http://www.ncbi.nlm.nih.gov/BLAST/>). Sequences were aligned with ClustalW (77). Phylogenetic trees were constructed with the neighbor-joining method (67) and the Jukes-Cantor distance model (41) with bootstrap values of 1,000 replicates within MEGA v4.0 (75). All sequences were deposited in GenBank, accession numbers HQ018935-HQ018989.

**Statistical Analysis.** All data were compiled and for all instances where plate count values had a value of zero indicating none detected, a substitution rule was used (83). An arbitrary value (0.25) was chosen to replace all zero plate counts so that log transformations could be performed. Multiple linear regression and logistic regression tests were performed in Minitab<sup>®</sup> to determine correlations between pH, temperature, drinking water and biofilm heterotrophic bacteria, total coliform bacteria, *Helicobacter*, *Legionella* and, *Mycobacteria* (44). Additionally, paired and Welch two sample t-tests were performed on heterotrophic and total coliform bacteria to determine if there were significant differences between first flush, post flush, and biofilm samples (44). Fisher's exact tests were performed to determine if the presence of *Mycobacteria*, *Legionella*, and/or *Helicobacter* had a relationship with each other (19). Fisher's exact tests were also done to determine if there was a relationship between *Mycobacteria*, *Legionella*, and *Helicobacter* and the source of the drinking water (treated municipal or groundwater well) (19). A Benjamini-Hochberg correction (10%) was applied to all analyses to minimize false discovery due to multiple comparisons (11).

## RESULTS

**Physical Characteristics of Sampled Drinking Water.** A total of 57 sites were sampled during this study. Sixteen samples were collected from public buildings and private residences that had drinking water supplied by treated municipal systems, while the 41 remaining systems were community and private groundwater wells. Total and free chlorine were quantified in drinking water sampled from municipal systems and ranged from none detected - 2.5 mg/L and none detected - 1.3 mg/L with means of 0.34 mg/L

and 0.27 mg/L, respectively. The pH of the drinking water ranged from 5.82-9.56 with a mean of 7.42. The temperature of the bulk water was recorded after flushing the tap and ranged from 8 - 33°C with a mean of 15.7°C and one outlier at 46°C. Treated municipal systems had a mean temperature of 21.5°C, while groundwater well systems had a mean temperature of 14.2°C. Simple linear regression and logistic regression were used to analyze relationships between the measured physical characteristics and HPC bacteria, total coliforms, *Legionella*, *Mycobacteria*, and *Helicobacter*. No significant statistical correlation was detected between the measured physical characteristics and any of the bacteria identified.

**Detection of Heterotrophic Bacteria and Fecal Indicator Bacteria.** Heterotrophic bacteria were enumerated to assess whether these organisms were associated with the presence of potential pathogens. Table 3.2 shows the range and arithmetic mean of HPC bacteria in first flush, biofilm and post flush drinking water samples. The significance from the statistical analyses of the interactions between HPC bacteria counts and response variables are shown in Tables 3.3 and 3.4. Differences in the mean HPC populations between the first flush and post flush fractions collected were evaluated using Minitab<sup>®</sup> (Table 3.3). There was a significant difference in mean HPC bacteria when first flush and bulk water samples were compared ( $P = 0.025$ , significant with Benjamini-Hochberg correction) with first flush samples having higher numbers of HPC bacteria on average. Differences in HPC bacteria between treated municipal and groundwater wells were also evaluated (Table 3.5). There was a difference between the mean HPC bacteria in biofilm samples and the drinking water source ( $P = 0.049$ ), however after applying the

Benjamini-Hochberg correction this relationship was not significant. Overall, biofilm samples collected from groundwater wells had higher HPC bacteria counts than biofilm samples collected from treated municipal systems. There was not a significant difference in HPC bacteria numbers between the source water types for the post flush water samples.

Total coliform bacteria were enumerated in first flush and post flush drinking water samples. Coliform bacteria were found in both treated municipal (37.5% or 6/16) and untreated groundwater wells (40% or 16/40) in post flush water samples.

*Escherichia coli* was not observed in treated municipal samples but was found in 10% or 4/40 of post flush groundwater well samples. Table 3.2 shows the range and arithmetic mean of coliform bacteria and *E. coli* in drinking water samples. Although there was a significant difference in HPC bacteria in first flush and post flush fractions, there was not a significant difference between mean coliform counts in first flush and post flush fractions ( $P > 0.13$ ). *E. coli* had a positive association with post flush HPC bacteria ( $P = 0.026$ , significant with correction) (Table 3.3).

**Presence of *Mycobacteria*, *Legionella*, and *Helicobacter*.** *Mycobacterium* species were detected in 35.1% or 20/57 of the locations sampled, with 15 found in the biofilm fraction, and 8 in the drinking water fraction. Three of these occurrences of *Mycobacteria* were found in both the drinking water and biofilm fractions. From the biofilm fractions, 7 of the 15 positive samples were identified by PCR alone and 5 were identified by culture alone while 3 were identified by both PCR and culture. From the drinking water fractions, 2 were identified by both PCR and culture while the remaining 6 were

identified by PCR only. Fig. 3.1 shows the phylogenetic relatedness of the PCR and culture isolates. The *Mycobacterium* species sequences detected were closely related to known species including *M. gilvum*, *M. mucogenicum*, *M. murale*, *M. flavescens*, *M. gordonae*, *M. manitobense* and members of the *Mycobacterium avium* complex (MAC) (>95% similarity).

To assess whether total coliforms or HPC bacteria influence the likelihood that *Mycobacteria* may be present, logistic regression was applied in Minitab<sup>®</sup>. Identical analyses were performed for all three genera tested in this study. The analysis showed a relationship between *Mycobacteria* and both post flush ( $P = 0.044$ , significant after correcting for multiple comparisons) and biofilm HPC bacteria ( $P = 0.01$ , not significant after corrections) (Tables 3 and 4 respectively). This showed that, in general, as HPC bacteria increased, the odds of encountering *Mycobacteria* increased as well.

*Mycobacteria* were detected when Coliforms were present in 50% or 10/20 of the locations sampled. Of the 20 locations that tested positive for *Mycobacteria*, 8 were treated municipal systems and 12 were groundwater well systems. There were no significant relationships between the presence of *Mycobacteria* and total coliforms (logistic regression) or the source type of the drinking water system (Fisher's exact test).

*Legionella* species were detected in 21% or 12 of the 57 locations sampled with 5 of those in the biofilm fraction, 8 in the drinking water fraction and only one occurrence of *Legionella* in both the biofilm and drinking water. Of the 5 positive biofilm samples, 3 were identified by PCR alone, 1 was identified by culture alone, and 1 was identified by both PCR and culture. From the 8 drinking water samples, 2 were identified by both

PCR and culture while the remaining 6 were identified by PCR alone. Fig. 3.2 shows the phylogenetic relatedness of *Legionella* detected by PCR directly and from culture isolates. The *Legionella* species detected include uncultured *Legionella* sp., *L. pneumophila*, *Legionella* sp., *L. fairfieldensis*, and *L. dresdeniensis* (sequence similarity >95%).

The results of the logistic regression showed a positive relationship between post flush HPC bacteria counts and *Legionella* in the system ( $P = 0.003$ , significant after correcting for multiple comparisons) (Table 3.3). In general, as post flush HPC bacteria increased, the odds of encountering *Legionella* increase as well. The greatest interaction occurred between *Legionella* detected in the biofilm fraction and post flush HPC bacteria ( $P = 0.001$ ). *Legionella* detected in the drinking water fraction did not have a significant interaction ( $P = 0.068$ ) with post flush fractions (Table 3.3). There was no significant relationship between the presence of *Legionella* (in either the biofilm or drinking water fractions) and biofilm HPC bacteria (Table 3.4). Coliforms were present in 6 of the 12 samples where *Legionella* were detected, but there was no significant relationship between the presence of *Legionella* and total coliforms ( $P = 0.679$ ). However, there was a significant association between the presence of *Legionella* and *E. coli* ( $P = 0.018$ ). Of the 12 samples positive for *Legionella*, 8 were at treated municipal sites and 4 were in groundwater. Unlike *Mycobacteria*, the source type of the drinking water system did have a relationship with the presence of *Legionella* ( $P = 0.002$ ) (Table 3.5).

*Helicobacter* species were detected in 7% or 4/57 of locations sampled, with 2 of those in the biofilm and 2 in the drinking water. There were no occurrences of

*Helicobacter* in the drinking water and biofilm concurrently. All of the positive samples were identified by PCR alone. Fig. 3.3 shows the phylogenetic relatedness of the *Helicobacter* sequences detected by PCR directly. The only *Helicobacter* species detected was *H. pylori*. Coliforms were found in 2 of the 4 samples where *Helicobacter* were detected. Logistic regression did not demonstrate any significant correlation between the presence of *Helicobacter* and any of the biological or physical parameters collected or the source type of the drinking water.

**Interactions Between Potentially Pathogenic Genera.** To determine whether there was a relationship between the presence of the three genera of interest a Fisher's exact test was performed. There was no statistically significant relationship between the three genera. Interestingly, 50% or 6/12 of locations positive for *Legionella* were also positive for *Mycobacteria* while only one location had both *Legionella* and *Helicobacter*. Conversely, there were 20 occurrences of *Mycobacteria* with six of these samples also positive for *Legionella* (28.5%) and two samples positive for *Helicobacter* (9.5%). *Helicobacter* was found alone in one location (25% or 1/4).

## DISCUSSION

The results of our study show that *Mycobacteria*, *Legionella*, and *Helicobacter* can be found in drinking water and associated biofilms on the Crow Reservation, in both treated municipal water and untreated well water. The data also indicated that the number of HPC bacteria correlated with the presence of *Mycobacteria* or *Legionella*.

**Fecal Coliforms and HPC Bacteria in Drinking Water.** Although the presence of coliform bacteria in drinking water is a potential indicator of fecal contamination and

may indicate the possible presence of harmful pathogens in drinking water (48), members of the coliform group are also common inhabitants of rural drinking water systems (45). This study found that 40% of community and private groundwater wells contained coliform bacteria while 37.5% of treated municipal samples were positive. This is in agreement with an Iowa statewide rural well water survey that found that 44% of private groundwater systems were contaminated with coliforms (37). In our study area, surface water municipal and groundwater systems are vulnerable to contamination, particularly during wet seasons that result in flooding events. These flooding events can drastically increase the turbidity of surface waters and hinder water treatment, potentially allowing coliform contamination of finished water. During March 2007, the largest treatment facility on the Reservation was required to shut down due to mud and debris that clogged the intake pipe after a flood (16). This particular event accounts for all of the coliform positive municipal system samples except one, which occurred shortly after this flood. The temporary closure of the treatment facility required a town-wide boil order and resulted in turbid water at the tap. Groundwater wells in rural areas are vulnerable to flooding, but are also susceptible to contamination from septic systems and inappropriate disposal of sewage effluents and sludges (12). During sample collection, we occasionally observed instances where well heads were completely inundated after precipitation, and water at the tap was turbid and/or odiferous.

Coliform detection has inherent limitations and high levels of background bacteria can interfere with the assays (17, 32, 48). Coliform bacteria often do not adequately predict the presence of pathogens, as has been demonstrated in waterborne outbreaks of

*Cryptosporidia*, *Giardia*, and *Salmonella* (43). The lack of concurrence between the detection of *Mycobacteria*, *Legionella*, and *Helicobacter* and coliforms indicates that fecal indicator bacteria have limited use in predicting the presence of these environmental pathogens. Our finding agrees with that of others who found no correlation between these organisms and fecal coliform bacteria (61, 72, 84, 86).

Heterotrophic plate count bacteria are the normal flora of drinking water and include a wide range of organisms including *Acinetobacter*, *Aeromonas*, *Bacillus*, *Corynebacterium*, *Pseudomonas*, *Mycobacteria*, and *Legionella* (2). *Helicobacter* also utilize organic nutrients for growth and thus fit the general definition of a heterotroph, but their microaerophilic lifestyle make them less suited for growth in the drinking water environment (1, 35). This study has shown that HPC bacteria can occur in numbers  $>10^6$  CFU/ml and that water that was stagnant in plumbing (first flush) had significantly greater numbers of HPC bacteria than water that has been collected after flushing ( $P = 0.025$ ). Water stagnation in drinking water pipes promotes bacterial accumulation and may compromise microbiological quality of drinking water when those organisms are flushed out (52). In this study, groundwater wells generally had higher levels of HPC bacteria than treated municipal water, which can be at least partially explained by the presence of chlorine residuals in municipal systems.

Heterotrophic plate count bacteria in biofilm and post flush drinking water fractions had significant relationships with the presence of both *Mycobacteria* and *Legionella*. Logistic regression showed that as the number of HPC bacteria increase, the odds of encountering *Mycobacteria* or *Legionella* increase as well. The presence of

*Mycobacteria* in the system had a stronger relationship with HPC bacteria in post flush drinking water fractions (odds ratio 1.68,  $P = 0.044$ ) than in biofilm fractions (odds ratio 0.63,  $P = 0.010$ ). The data showed a relationship between *Mycobacteria* identified in different fractions (biofilm and drinking water) and the number of HPC bacteria in the different fractions. The most significant relationship was the interaction between the presence of *Mycobacteria* in the drinking water and elevated HPC bacteria in post flush water (odds ratio 2.05,  $P = 0.030$ ). September et al. (72) found that water quality parameters do not provide any indication of the possible presence of *Mycobacteria* in drinking water biofilms, while another group found a relationship between elevated HPC counts and *Mycobacteria* in surface waters (39). The relationship between *Mycobacteria* and HPC in drinking water systems remains unclear and more research is required to elucidate all of the factors involved. The presence of *Legionella* in the system had a stronger relationship with HPC bacteria in post flush drinking water (odds ratio 2.75,  $P = 0.003$ ) than in biofilm samples (odds ratio 0.77,  $P = 0.185$ ). The relationship between *Legionella* identified in biofilm and drinking water and the number of HPC bacteria in the corresponding fractions was analyzed. Although the minority of *Legionella* sequences were found in biofilm samples (41.6%), they accounted for the significant interaction with elevated HPC bacteria in post flush water. Finding elevated levels of HPC bacteria in post flush samples significantly increased the odds of encountering *Legionella* in a biofilm (odds ratio 31.66,  $P = 0.001$ ). There is very little data regarding the usefulness of HPC counts for predicting the presence of *Legionella*. It has been shown that certain common HPC bacteria inhibit the growth of *Legionella* while others

stimulate it (79). Our data is in agreement with LeChevallier et al. (47) who concluded that HPC bacteria were useful for predicting the presence of opportunistic pathogens and provide insight into the overall quality of drinking water. There was no relationship between HPC bacteria and the presence of *Helicobacter* in any fraction.

**Sampling Strategy Influences Detection of *Mycobacteria*, *Legionella*, and**

***Helicobacter*.** This research has attempted to identify the presence of potential pathogens and identify factors that may play a role in where and when these organisms may be present. Because the residents on the Crow Reservation were concerned with overall drinking water quality, samples of drinking water and associated biofilms were taken at public buildings and private residences. This resulted in samples being collected from treated municipal and untreated groundwater systems. In this study, biofilm samples were collected in addition to drinking water samples according to the recommendations for *Legionella* (VAMC; Pittsburgh, Pa., CDC; Atlanta, Ga.). Although this recommendation specifically addresses *Legionella* detection, it is in agreement with many other findings that *Mycobacteria*, *Legionella*, and *Helicobacter* can be harbored and detected in drinking water biofilms (35, 69, 74). Finally, two methods for detecting the organisms of interest, PCR and culturing, were chosen. It is well documented that traditional culturing techniques underestimate the quantity and diversity of microorganisms in the environment (60). However, when looking at issues of public health it is also important to identify whether these organisms are viable and perhaps capable of infection. By combining molecular detection with traditional culturing methods, it is possible to increase the likelihood of detecting an organism of interest.

*Mycobacteria*, *Legionella*, and *Helicobacter* were found in both treated municipal water and untreated well water systems. *Mycobacteria* were found more often in groundwater systems than in treated municipal systems (61.9% and 38.1% of the 20 samples positive for *Mycobacteria*, respectively). Reports of the detection of *Mycobacteria* in groundwater have been sporadic, but generally have shown relative frequencies from not detected to up to 68% of locations testing positive (46, 71). *Mycobacteria* have been detected in treated systems with varying results as well (22, 46, 78). Overall, our results are consistent with other reports of *Mycobacteria* in treated municipal and groundwater systems.

Unlike *Mycobacteria*, *Legionella* had a statistically significant relationship with the source of the drinking water. *Legionella* were found more often in treated municipal systems (66.7%) than in groundwater systems (33.3%). In other studies, *Legionella* has been frequently found in municipal water systems and sporadically in groundwater (14, 20, 53, 87). It has been shown that the presence and diversity of *Legionella* varies spatially in drinking water distribution systems and in groundwater (87). It is possible that premise plumbing in buildings with light or sporadic use could promote the planktonic and/or necrotrophic growth of *Legionella* as described by others (51, 76). It is also likely that the overall warmer temperature of the treated municipal system is supportive for *Legionella* survival and growth.

*Helicobacter* were detected in 4 locations of our study area with 50% in treated municipal systems and 50% in untreated groundwater systems. One of the instances of *Helicobacter* occurred during the flood event that closed the water treatment facility for a

short period of time. Reports of *Helicobacter* detection in drinking water have been intermittent with most reports finding infrequent positive samples (15, 40, 85). Although the environmental reservoir of *Helicobacter* is unknown, it is possible that water distribution systems may be vulnerable to contamination through breaks or leaks in distribution pipes. Groundwater systems that are too shallow may be under the influence of surface water which could be contaminated by agricultural practices and inadequate sewage disposal (12).

*Mycobacteria*, *Legionella*, and *Helicobacter* were detected in both biofilm and drinking water samples. *Mycobacterium gilvum* and *M. avium* complex were most frequently identified (>95% sequence similarity) and were both found in biofilms more often than drinking water. *Legionella pneumophila* occurred more often in biofilm samples while sequences identified as *Legionella* sp. were more often identified in drinking water samples. Interestingly, both *Mycobacteria* and *Legionella* had greater rates of culture positive tests in biofilm samples. This could indicate that tap water biofilms are protective and supportive for these organisms. *H. pylori* sequences occurred in two drinking water samples and two biofilm samples. Although *H. pylori* were not detected in a large number of locations, biofilm sampling doubled the detection of this organism. These data are consistent with reports of others that indicate that all three of these genera can be found in both drinking water and associated biofilm samples (50, 53).

Consistent with other reports, molecular detection of all three genera was more successful than traditional culture methods (3, 49, 73, 85). The majority of detections were achieved by PCR, with only a small fraction of the samples positive for

*Mycobacteria* and *Legionella* culture isolates. While it is known that molecular techniques are important for detecting organisms that are injured, or viable but not culturable (1, 7), culture techniques provide valuable information as well. In this study, both *Mycobacteria* and *Legionella* were detected by culture methods and PCR. However, in a minority of cases, culture positive locations could not be identified by PCR performed directly on the samples. This has been documented by others as well and could be due to PCR inhibitors, such as heavy metals, intrinsic to the drinking water system (31, 66).

**Health Consequences of *Mycobacteria*, *Legionella*, and *Helicobacter* in Drinking**

**Water.** All of the *Mycobacteria* sequences detected in this study were of the nontuberculous Mycobacteria group (NTM). One important detected NTM are the slow-growing opportunistic pathogens in the *M. avium* complex (MAC), which includes the *M. avium* subsp. *avium*, *M. avium* subsp. *intracellulare*, *M. avium* subsp. *hominisuis*, and *M. avium* subsp. *paratuberculosis* (64). MAC accounts for over 70 percent of nontuberculous mycobacterial disease in the United States and for more than 95 percent of nontuberculous disease among persons infected with human immunodeficiency virus (HIV) (64). It has been shown that MAC isolates recovered from hospital water had a close relationship (large-restriction-fragment pattern analysis) with clinical isolates recovered from patients indicating that water could be the reservoir for infection (6). Other sequences identified in this study were of >95% similarity to *M. gordonae*, *M. flavescens*, and *M. mucogenicum*. These species are all known to be inhabitants of drinking water systems and have been implicated in adverse health consequences (36, 46,

54, 55). One interesting fast-growing *Mycobacterium* species that we encountered fairly frequently was *M. gilvum*. This bacterium is an environmental mycobacterium that has been isolated from soils in Montana (57), which is known to degrade polyaromatic hydrocarbons and has not been implicated in any health effects.

The *Legionella* species sequences detected in this study were mainly *L. pneumophila* and *Legionella* sp. but also included sequences similar to *L. fairfieldensis*, *L. dresdeniensis*, and *L. birminghamiensis*. *L. pneumophila* is a well-documented opportunistic pathogen that has a low infection rate (1-6%), but a mortality rate of 10-15%, and accounts for 1-4% of all pneumonia cases in the United States' general population (62). *L. pneumophila* is the primary disease causing species of this genus but there have been occasional cases of disease caused by other *Legionella* sp. (58). Other *Legionella* species such as *L. fairfieldensis* and *L. birminghamiensis* have been documented in drinking water systems (26), but are not implicated in health effects.

This study detected *H. pylori* sequences at four of the locations sampled. Recent research using the <sup>13</sup>C urea breath test has shown that the prevalence of *H. pylori* in one rural community of Montana is greater than 50% (56). Their research also indicated that the presence of *H. pylori* infection was associated with regular consumption of city water as indicated by questionnaire results (Unpublished data, USEPA) (56). Untreated well water has also been implicated in clinical infections in the United States (8, 10). Areas with poor water quality may be more likely to have higher rates of water-borne transmission of disease, especially in children (15, 85).

In conclusion, microbes such as *M. avium*, *L. pneumophila*, and *H. pylori* can be found in drinking water systems in rural underserved areas. Coliforms were shown to be inadequate indicators for all of these organisms, while HPC bacterial levels did have a relationship with the presence of *Mycobacteria* and *Legionella*. Both treated municipal water and groundwater fed systems can harbor these organisms, which can be found in both bulk water and associated biofilms. Consequently, it is impossible to rule out drinking water as a route of infection for pathogenic bacteria such as *M. avium*, *L. pneumophila*, and *H. pylori*. To address health disparities in underserved communities such as American Indian reservations it is important to determine potential reservoirs of infection. These results are pertinent to water utility managers, regulatory agencies, as well as epidemiologists interested in identifying disease causing agents in rural drinking water systems.

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#### REFERENCES

1. Adams, B. L., T. C. Bates, and J. D. Oliver. 2003. Survival of *Helicobacter pylori* in a natural freshwater environment. *Appl. Environ. Microbiol.* 69:7462-7466.
2. Allen, M. J., S. C. Edberg, and D. J. Reasoner. 2004. Heterotrophic plate count bacteria - what is their significance in drinking water? *Int. J. Food Microbiol.* 92:265-274.
3. Angenent, L. T., S. T. Kelley, A. St Amand, N. R. Pace, and M. T. Hernandez. 2005. Molecular identification of potential pathogens in water and air of a hospital therapy pool. *Proc. Natl. Acad. Sci. U.S.A.* 102:4860-4865.
4. Anonymous. 2002. Crow Indian Reservation Natural, Socio-Economic, and Cultural Resources Assessment and Conditions Report, Crow Tribe of Indians, Montana.
5. Anonymous. 1999. *The Health Care Challenge: Acknowledging Disparity, Confronting Discrimination, and Ensuring Equality*. United States National Archives and Records Administration, Washington D.C. 20402.
6. Aronson, T., A. Holtzman, N. Glover, M. Boian, S. Froman, O. G. W. Berlin, H. Hill, and G. Stelma. 1999. Comparison of large restriction fragments of

*Mycobacterium avium* isolates recovered from AIDS and non-AIDS patients with those of isolates from potable water. J. Clin. Microbiol. 37:1008-1012.

7. Atlas, R. M. 1999. *Legionella*: from environmental habitats to disease pathology, detection and control. Environ. Microbiol. 1:283-293.
8. Baker, K. H., and J. P. Hegarty. 2001. Presence of *Helicobacter pylori* in drinking water is associated with clinical infection. Scand. J. Infect. Dis. 33:744-746.
9. Bartie, C., S. N. Venter, and L. H. Nel. 2003. Identification methods for *Legionella* from environmental samples. Water Res. 37:1362-1370.
10. Bellack, N. R., M. W. Koehoorn, Y. C. MacNab, and M. G. Morshed. 2006. A conceptual model of water's role as a reservoir in *Helicobacter pylori* transmission: a review of the evidence. Epidemiol. Infect. 134:439-449.
11. Benjamini, Y., and Y. Hochberg. 1995. Controlling the false discovery rate - a practical and powerful approach to multiple testing. J. R. Stat. Soc. Series B Stat. Methodol. 57:289-300.
12. Bitton, G., and C. P. Gerba. 1984. Groundwater Pollution Microbiology: the Emerging Issue, p. 1-7. In G. Bitton and C. Gerba (ed.), Groundwater pollution microbiology. John Wiley & Sons, New York.
13. Boddington, B., T. Rogall, T. Flohr, H. Blocker, and E. C. Bottger. 1990. Detection and identification of *Mycobacteria* by Amplification of RNA. J. Clin. Microbiol. 28:1751-1759.
14. Brooks, T., R. A. Osicki, V. S. Springthorpe, S. A. Sattar, L. Filion, D. Abrial, and S. Riffard. 2004. Detection and identification of *Legionella* species from groundwaters. J. Toxicol. Environ. Health. 67:1845-1859.
15. Bunn, J. E. G., W. G. MacKay, J. E. Thomas, D. C. Reid, and L. T. Weaver. 2002. Detection of *Helicobacter pylori* DNA in drinking water biofilms: implications for transmission in early life. Lett. Appl. Microbiol. 34:450-454.
16. Burkland, B. 2010. Personal communication regarding treatment facility at Crow Agency, US Environmental Protection Agency. Helena, MT.
17. Burlingame, G. A., J. McElhaney, M. Bennett, and W. O. Pipes. 1984. Bacterial interference with coliform colony sheen production on membrane filters. Appl. Environ. Microbiol. 47:56-60.

18. Cassidy, P. M., K. Hedberg, A. Saulson, E. McNelly, and K. L. Winthrop. 2009. Nontuberculous mycobacterial disease prevalence and risk factors: A changing epidemiology. *Clin. Infect. Dis.* 49:E124-E129.
19. Conover, W. J. 1999. *Practical nonparametric statistics*, Third ed. Elm Street Publishing Services, Inc., New York.
20. Costa, J., I. Tiago, M. S. da Costa, and A. Verissimo. 2005. Presence and persistence of *Legionella* spp. in groundwater. *Appl. Environ. Microbiol.* 71:663-671.
21. Covers Up, T., R. Turnsplenty, D. Wetzel, and J. Giroux. 2005. 1990-2002 Montana and Wyoming American Indian top ten causes of death. Rocky Mountain Tribal Epidemiology Center.
22. Covert, T. C., M. R. Rodgers, A. L. Reyes, and G. N. Stelma. 1999. Occurrence of nontuberculous mycobacteria in environmental samples. *Appl. Environ. Microbiol.* 65:2492-2496.
23. Craun, G. F., R. L. Calderon, and T. J. Wade. 2006. Assessing waterborne risks: an introduction. *J. Water Health* 04. Suppl 2:3-18.
24. Craun, M. F., G. F. Craun, R. L. Calderon, and M. J. Beach. 2006. Waterborne outbreaks reported in the United States. *J. Water Health* 04. Suppl 2:19-30.
25. Degnan, A. J., W. C. Sonzogni, and J. H. Standridge. 2003. Development of a plating medium for selection of *Helicobacter pylori* from water samples. *Appl. Environ. Microbiol.* 69:2914-2918.
26. Diederens, B. M. W., C. M. A. de Jong, I. Aarts, M. F. Peeters, and A. van der Zee. 2007. Molecular evidence for the ubiquitous presence of *Legionella* species in Dutch tap water installations. *J. Water Health* 5:375-383.
27. Doyle, J. 2010. Personal communication regarding municipal distribution system at Crow Agency, Crow Environmental Health Steering Committee. Crow Agency, MT.
28. Doyle, J., L. Kindness, U. Bear Don't Walk, S. Young, and M. Lefthand. 2006. Personal communication regarding water quality on Crow Reservation, Crow Environmental Health Steering Committee. Crow Agency, MT.
29. Falkinham, J. O., C. D. Norton, and M. W. LeChevallier. 2001. Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*,

- and other mycobacteria in drinking water distribution systems. *Appl. Environ. Microbiol.* 67:1225-1231.
30. Fields, B. S., R. F. Benson, and R. E. Besser. 2002. *Legionella* and Legionnaires' disease: 25 years of investigation. *Clin. Microbiol. Rev.* 15:506-526.
  31. Fiume, L., M. A. Bucca Sabattini, and G. Poda. 2005. Detection of *Legionella pneumophila* in water samples by species-specific real-time and nested PCR assays. *Lett. Appl. Microbiol.* 41:470-475.
  32. Franzblau, S. G., B. J. Hinnebusch, L. M. Kelley, and N. A. Sinclair. 1984. Effect on noncoliforms on coliform detection in potable groundwater - improved recovery with an anaerobic membrane-filter technique. *Appl. Environ. Microbiol.* 48:142-148.
  33. Geach, J. 2007. Source Water Delineation and Assessment Report, p. 1-34. United States Environmental Protection Agency, Helena, MT.
  34. Germani, Y., C. Dauga, P. Duval, M. Huerre, M. Levy, G. Pialoux, P. Sansonetti, and P. A. D. Grimont. 1997. Strategy for the detection of *Helicobacter* species by amplification of 16S rRNA genes and identification of *H. felis* in a human gastric biopsy. *Res. Microbiol.* 148:315-326.
  35. Giao, M. S., N. F. Azevedo, S. A. Wilks, M. J. Vieira, and C. W. Keevil. 2008. Persistence of *Helicobacter pylori* in heterotrophic drinking-water biofilms. *Appl. Environ. Microbiol.* 74:5898-5904.
  36. Guillen, S. M., J. S. Hospital, E. G. Mampaso, A. G. Espejo, C. E. Baquedano, and A. O. Calderon. 1986. Gluteal abscess caused by *Mycobacterium flavescens*. *Tubercle.* 67:151-153.
  37. Hallberg, G. R., and B. C. Kross. 1990. Iowa statewide rural well water survey - summary of results. Iowa Department of Natural Resources Geological Survey Bureau and University of Iowa Center for Health Effects of Environmental Contamination, Iowa City, IA 52242.
  38. Heath, R. C. 1984. Ground-water regions of the United States, p. 1-78. U.S. Geological Survey Supply Paper 2242.
  39. Iivanainen, E. K., P. J. Martikainen, P. K. Vaananen, and M. L. Katila. 1993. Environmental factors affecting the occurrence of *Mycobacteria* in brook waters. *Appl. Environ. Microbiol.* 59:398-404.

40. Janzon, A., A. Sjoling, A. Lothigius, D. Ahmed, F. Qadri, and A. M. Svennerholm. 2009. Failure to detect *Helicobacter pylori* DNA in drinking and environmental water in Dhaka, Bangladesh, using highly sensitive real-time PCR assays. *Appl. Environ. Microbiol.* 75:3039-3044.
41. Jukes, T. H., and C. R. Cantor. 1969. Evolution of protein molecules, p. 21-132. *In* H. N. Munro (ed.), *Mammalian protein metabolism*. Academic Press, New York.
42. Kramer, M. H., and T. E. Ford. 1994. Legionellosis: ecological factors of an environmentally 'new' disease. *Zentralbl Hyg Umweltmed* 195:470-82.
43. Kramer, M. H., B. L. Herwaldt, R. L. Calderon, and D. D. Juranek. 1996. Surveillance for Waterborne-Disease Outbreaks -- United States, 1993-1994, p. 1-33, vol. 45. Centers for Disease Control, Washington D.C.
44. Kutner, M., C. Nachtsheim, J. Neter, and W. Li. 2004. *Applied linear statistical models*, Fifth ed. McGraw-Hill/Irwin, New York.
45. Lamka, K. G., M. W. LeChevallier, and R. J. Seidler. 1980. Bacterial contamination of drinking water supplies in a modern rural neighborhood. *Appl. Environ. Microbiol.* 39:734-738.
46. Le Dantec, C., J. P. Duguet, A. Montiel, N. Dumoutier, S. Dubrou, and V. Vincent. 2002. Occurrence of mycobacteria in water treatment lines and in water distribution systems. *Appl. Environ. Microbiol.* 68:5318-5325.
47. LeChevallier, M. W., R. J. Seidler, and T. M. Evans. 1980. Enumeration and characterization of standard plate-count bacteria in chlorinated and raw water supplies. *Appl. Environ. Microbiol.* 40:922-930.
48. Leclerc, H., D. A. A. Mossel, S. C. Edberg, and C. B. Struijk. 2001. Advances in the bacteriology of the coliform group: their suitability as markers of microbial water safety. *Annu. Rev. Microbiol.* 55:201-234.
49. Lehtola, M. J., E. Torvinen, J. Kusnetsov, T. Pitkanen, L. Maunula, C. H. von Bonsdorff, P. J. Martikainen, S. A. Wilks, C. W. Keevil, and I. T. Miettinen. 2007. Survival of *Mycobacterium avium*, *Legionella pneumophila*, *Escherichia coli*, and caliciviruses in drinking water-associated biofilms grown under high-shear turbulent flow. *Appl. Environ. Microbiol.* 73:2854-2859.
50. Mackay, W. G., L. T. Gribbon, M. R. Barer, and D. C. Reid. 1998. Biofilms in drinking water systems - A possible reservoir for *Helicobacter pylori*. *Water Sci. Technol.* 38:181-185.

51. Mampel, J., T. Spirig, S. S. Weber, J. A. J. Haagensen, S. Molin, and H. Hilbi. 2006. Planktonic replication is essential for biofilm formation by *Legionella pneumophila* in a complex medium under static and dynamic flow conditions. *Appl. Environ. Microbiol.* 72:2885-2895.
52. Manuel, C. M., O. C. Nunes, and L. F. Melo. 2010. Unsteady state flow and stagnation in distribution systems affect the biological stability of drinking water. *Biofouling* 26:129-139.
53. Marciano-Cabral, F., M. Jamerson, and E. S. Kaneshiro. 2010. Free-living amoebae, *Legionella* and *Mycobacterium* in tap water supplied by a municipal drinking water facility. *J. Water Health* 08:71-82.
54. Marshall, C., J. Samuel, A. Galloway, and S. Pedler. 2008. *Mycobacterium mucogenicum* from the Hickman line of an immunocompromised patient. *J. Clin. Pathol.* 61:140-141.
55. Mazumder, S. A., A. Hicks, and J. Norwood. 2010. *Mycobacterium gordonae* pulmonary infection in an immunocompetent adult. *N. A. J. Med. Sci.* 2:205-207.
56. Melius, E., R. Wierzba, S. Davis, J. Sobel, B. Gold, A. Henderson, and J. Cheek. 2005. Risk factors for *Helicobacter pylori* in a rural community.
57. Miller, C. D., R. Child, J. E. Hughes, M. Benscai, J. P. Der, R. C. Sims, and A. J. Anderson. 2007. Diversity of soil mycobacterium isolates from three sites that degrade polycyclic aromatic hydrocarbons. *J. Appl. Microbiol.* 102:1612-1624.
58. Muder, R. R., and V. L. Yu. 2002. Infection due to *Legionella* species other than *L. pneumophila*. *Clin. Infect. Dis.* 35:990-998.
59. National Center for Health Statistics. 2010. Health, United States, 2009: with special feature on medical technology., Hyattsville, MD.
60. Pace, N. R. 1997. A molecular view of microbial diversity and the biosphere. *Science.* 276:734-740.
61. Palmer, C. J., G. F. Bonilla, B. Roll, C. Paszkokolva, L. R. Sangermano, and R. S. Fujioka. 1995. Detection of *Legionella* species in reclaimed water and air with the ENVIROAMP *Legionella* PCR kit and direct fluorescent-antibody staining. *Appl. Environ. Microbiol.* 61:407-412.

62. Percival, S., R. Chalmers, M. Embrey, P. Hunter, J. Sellwood, and P. Wyn-Jones. 2004. *Microbiology of Waterborne Diseases*. Elsevier Academic Press, California.
63. Perez-Perez, G. I., D. Rothenbacher, and H. Brenner. 2004. Epidemiology of *Helicobacter pylori* infection. *Helicobacter*. 9:1-6.
64. Reed, C., C. F. von Reyn, S. Chamblee, T. V. Ellerbrock, J. W. Johnson, B. J. Marsh, L. S. Johnson, R. J. Trenchel, and C. R. Horsburgh. 2006. Environmental risk factors for infection with *Mycobacterium avium* complex. *Am. J. Epidemiol.* 164:32-40.
65. Research and Analysis Bureau. 2008. Demographic and Economic Information for the Crow Reservation, p. 1-8. Montana Department of Labor and Industry, Helena, MT 59624.
66. Riffard, S., S. Douglass, T. Brooks, S. Springthorpe, L. G. Filion, and S. A. Sattar. 2001. Occurrence of *Legionella* in groundwater: an ecological study. *Water Sci. Technol.* 43:99-102.
67. Saitou, N., and M. Nei. 1987. The neighbor-joining method - a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406-425.
68. Sasaki, K., Y. Tajiri, M. Sata, Y. Fujii, F. Matsubara, M. G. Zhao, S. Shimizu, A. Toyonaga, and K. Tanikawa. 1999. *Helicobacter pylori* in the natural environment. *Scand. J. Infect. Dis.* 31:275-280.
69. Schulze-Robbeke, R., B. Janning, and R. Fischeder. 1992. Occurrence of mycobacteria in biofilm samples. *Tuber. Lung Dis.* 73:141-144.
70. Schulze-Robbeke, R., A. Weber, and R. Fischeder. 1991. Comparison of decontamination methods for the isolation of mycobacteria from drinking water samples. *J. Microbiol. Methods* 14:177-183.
71. Schwartz, T., S. Kalmbach, S. Hoffmann, U. Szewzyk, and U. Obst. 1998. PCR-based detection of mycobacteria in biofilms from a drinking water distribution system. *J. Microbiol. Methods* 34:113-123.
72. September, S. M., V. S. Brozel, and S. N. Venter. 2004. Diversity of nontuberculous *Mycobacterium* species in biofilms of urban and semiurban drinking water distribution systems. *Appl. Environ. Microbiol.* 70:7571-7573.

73. Springer, B., L. Stockman, K. Teschner, G. D. Roberts, and E. C. Bottger. 1996. Two-laboratory collaborative study on identification of mycobacteria: Molecular versus phenotypic methods. *J. Clin. Microbiol.* 34:296-303.
74. Storey, M. V., J. Langmark, N. J. Ashbolt, and T. A. Stenstrom. 2004. The fate of legionellae within distribution pipe biofilms: measurement of their persistence, inactivation and detachment. *Water Sci. Technol.* 49:269-275.
75. Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24:1596-1599.
76. Temmerman, R., H. Vervaeren, B. Nosedá, N. Boon, and W. Verstraete. 2006. Necrotrophic growth of *Legionella pneumophila*. *Appl. Environ. Microbiol.* 72:4323-4328.
77. Thompson, J. D., T. J. Gibson, and D. G. Higgins. 2002. Multiple sequence alignment using ClustalW and ClustalX. *Curr Protoc Bioinformatics Chapter 2:Unit 2.3.*
78. Torvinen, E., S. Suomalainen, M. J. Lehtola, I. T. Miettinen, O. Zacheus, L. Paulin, M. L. Katila, and P. J. Martikainen. 2004. *Mycobacteria* in water and loose deposits of drinking water distribution systems in Finland. *Appl. Environ. Microbiol.* 70:1973-1981.
79. Toze, S., L. I. Sly, I. C. Macrae, and J. A. Fuerst. 1990. Inhibition of growth of *Legionella* species by heterotrophic plate-count bacteria isolated from chlorinated drinking water. *Curr. Microbiol.* 21:139-143.
80. Turenne, C. Y., M. Semret, D. V. Cousins, D. M. Collins, and M. A. Behr. 2006. Sequencing of hsp65 distinguishes among subsets of the *Mycobacterium avium* complex. *J. Clin. Microbiol.* 44:433-440.
81. Turetgen, I., E. I. Sungur, and A. Cotuk. 2005. Enumeration of *Legionella pneumophila* in cooling tower water systems. *Environ. Monit. Assess.* 100:53-58.
82. United States Census Bureau. 2007. Current Housing Reports, Series H150/07, p. 1-642, American Housing Survey for the United States: 2007. U.S. Government Printing Office, Washington D.C.
83. United States Environmental Protection Agency. 1998. Guidance for data quality assessment-Practical methods for data analysis. Office of Research and Development, Washington, D.C.

84. Voytek, M. A., J. B. Ashen, L. R. Fogerty, J. D. Kirshtein, and E. R. Landa. 2005. Detection of *Helicobacter pylori* and fecal indicator bacteria in five North American rivers. *J. Water Health* 03:405-422.
85. Watson, C. L., R. J. Owen, B. Said, S. Lai, J. V. Lee, S. Surman-Lee, and G. Nichols. 2004. Detection of *Helicobacter pylori* by PCR but not culture in water and biofilm samples from drinking water distribution systems in England. *J. Appl. Microbiol.* 97:690-698.
86. Whan, L., H. J. Ball, I. R. Grant, and M. T. Rowe. 2005. Occurrence of *Mycobacterium avium* subsp *paratuberculosis* in untreated water in Northern Ireland. *Appl. Environ. Microbiol.* 71:7107-7112.
87. Wullings, B. A., and D. van der Kooij. 2006. Occurrence and genetic diversity of uncultured *Legionella* spp. in drinking water treated at temperatures below 15 degrees C. *Appl. Environ. Microbiol.* 72:157-166.

Table 3.1. Primer Sequences, References and PCR Conditions.

Target (Reference)	Sequence	Product size	PCR Conditions
16S RNA gene <i>Legionella</i> spp. (87)	LEG-225 5' AAGATTAGC CTGCGTCCGAT; LEG-858 5' GTCAACTTATCGCGTT TGCT	656 bp	94°C 2 min (1 cycle); 94°C 20 sec, 60°C 30 sec, 72°C 40 sec (40 cycles); 72°C 5 min (1 cycle)
16S RNA gene <i>Mycobacterium</i> spp. (13)	MycgenF 5' AGAGTTTG ATCCTGGCTCAG; MycgenR 5' TGCACACAGG CCACAAGGGA	1,030 bp	95°C 2 min (1 cycle); 93°C 1 min, 60°C 1 min, 72°C 1 min (35 cycles); 72°C 5 min (1 cycle)
16S RNA gene <i>Helicobacter</i> spp. (34)	HS1 5' AACGATGAAGCTTCT AGCTTGCTAG; HS2 5' GTGCTTATTCGTTAGATA CCGTCAT	400 bp	94°C 5 min (1 cycle); 94°C 1 min, 65°C 1 min, 72°C 1 min (35 cycles); 72°C 5 min (1 cycle)
16S RNA gene Eubacteria (84)	46f 5' GCYTAACACATGCA AGTCGA; 519r 5' GTATTACCG CGGCKGCTG	490 bp	95°C 5 min (1 cycle); 94°C 0.5 min, 56°C 0.5 min, 72°C 1.5 min (30 cycles); 72°C 7 min (1 cycle)

Table 3.2. Range and Arithmetic Mean of HPC Bacteria, Total Coliforms, and *E. coli*

Bacteria	Measurement	Source	
		Treated Municipal (n=16)	Groundwater Well (n=41)
Heterotrophic plate counts			
In first flush (CFU/ml)	Range	*	$1.5 \times 10^0 - 5.12 \times 10^7$
	Arithmetic Mean	*	$2.81 \times 10^6$
In water (CFU/ml)	Range	$3.57 \times 10^2 - 5.15 \times 10^5$	$2.0 \times 10^0 - 9.23 \times 10^5$
	Arithmetic Mean	$9.02 \times 10^4$	$5.7 \times 10^4$
In biofilm (CFU/mm <sup>2</sup> )	Range	$<1 - 1.24 \times 10^5$	$<1 - 3.22 \times 10^5$
	Arithmetic Mean	$1.79 \times 10^4$	$4.29 \times 10^4$
Total Coliforms			
In first flush (CFU/100ml)	Range	*	$<1 - 1.19 \times 10^3$
	Arithmetic Mean	*	$1.17 \times 10^2$
In water (CFU/100ml)	Range	$<1 - 2.63 \times 10^1$	$<1 - 2.96 \times 10^3$
	Arithmetic Mean	$2.71 \times 10^0$	$1.07 \times 10^2$
<i>Escherichia coli</i>			
In first flush (CFU/100ml)	Range	*	$<1$
	Arithmetic Mean	*	$<1$
In water (CFU/100ml)	Range	$<1$	$<1 - 2.22 \times 10^2$
	Arithmetic Mean	$<1$	$5.68 \times 10^0$

\* No samples were taken in this category.

Table 3.3 Statistical Analysis of the Interactions between HPC Bacteria in Drinking Water and Response Variables

Response Variable	Model	Significance and FDR	
		<i>P</i>	<i>P</i> <sub>(12)</sub>
<i>Helicobacter</i> (BF)	Binary Logistic Regression	0.95	0.100
<i>Mycobacteria</i> (BF)	Binary Logistic Regression	0.778	0.092
<i>Helicobacter</i> (System)	Binary Logistic Regression	0.628	0.083
Total coliforms (DW)	Simple Linear Regression	0.463	0.075
<i>Helicobacter</i> (DW)	Binary Logistic Regression	0.457	0.067
<i>Legionella</i> (DW)	Binary Logistic Regression	0.068	0.058
<i>Mycobacteria</i> (System)	Binary Logistic Regression	0.044*	0.050
<i>Mycobacteria</i> (DW)	Binary Logistic Regression	0.03*	0.042
<i>Escherichia coli</i> (DW)	Simple Linear Regression	0.026*	0.033
HPC bacteria (FF)	Paired t-test	0.025*	0.023
<i>Legionella</i> (System)	Binary Logistic Regression	0.003*	0.017
<i>Legionella</i> (BF)	Binary Logistic Regression	0.001*	0.008

\* Denotes P-value with statistical significance.

(FDR) false discovery rate, (FF) first flush, (BF) biofilm, (DW) drinking water, (System) combines all sample fractions.

Table 3.4. Statistical Analysis of the Interactions between HPC Bacteria in Biofilms and Response Variables

Response Variable	Model	Significance and FDR	
		<i>P</i>	<i>P</i> <sub>(10)</sub>
<i>Helicobacter</i> (BF)	Binary Logistic Regression	0.872	0.100
<i>Helicobacter</i> (DW)	Binary Logistic Regression	0.746	0.082
<i>Helicobacter</i> (System)	Binary Logistic Regression	0.726	0.073
Total coliforms (DW)	Simple Linear Regression	0.606	0.064
<i>Mycobacteria</i> (DW)	Binary Logistic Regression	0.509	0.055
<i>Legionella</i> (BF)	Binary Logistic Regression	0.204	0.045
<i>Legionella</i> (DW)	Binary Logistic Regression	0.208	0.036
<i>Legionella</i> (System)	Binary Logistic Regression	0.185	0.027
<i>Mycobacteria</i> (BF)	Binary Logistic Regression	0.051	0.018
<i>Mycobacteria</i> (System)	Binary Logistic Regression	0.01	0.009

(FDR) false discovery rate, (BF) biofilm, (DW) drinking water, (System) combines all sample fractions.

Table 3.5. Statistical Analysis of the Interactions between Drinking Water Source (Treated Municipal or Groundwater Well) and Response Variables

Variable	Model	Significance and FDR	
		<i>P</i>	<i>P</i> <sub>(7)</sub>
<i>Escherichia coli</i> (DW)	Two sample t-test	0.418	0.100
<i>Helicobacter</i> (System)	Fisher's exact test	0.393	0.086
<i>Mycobacteria</i> (System)	Fisher's exact test	0.216	0.071
HPC bacteria (DW)	Two sample t-test	0.13	0.057
Total coliforms (DW)	Two sample t-test	0.128	0.043
HPC bacteria (BF)	Two sample t-test	0.049	0.029
<i>Legionella</i> (System)	Fisher's exact test	0.003*	0.014

\* Denotes P-value with statistical significance.

(FDR) false discovery rate, (BF) biofilm, (DW) drinking water, (System) combines all sample fractions.

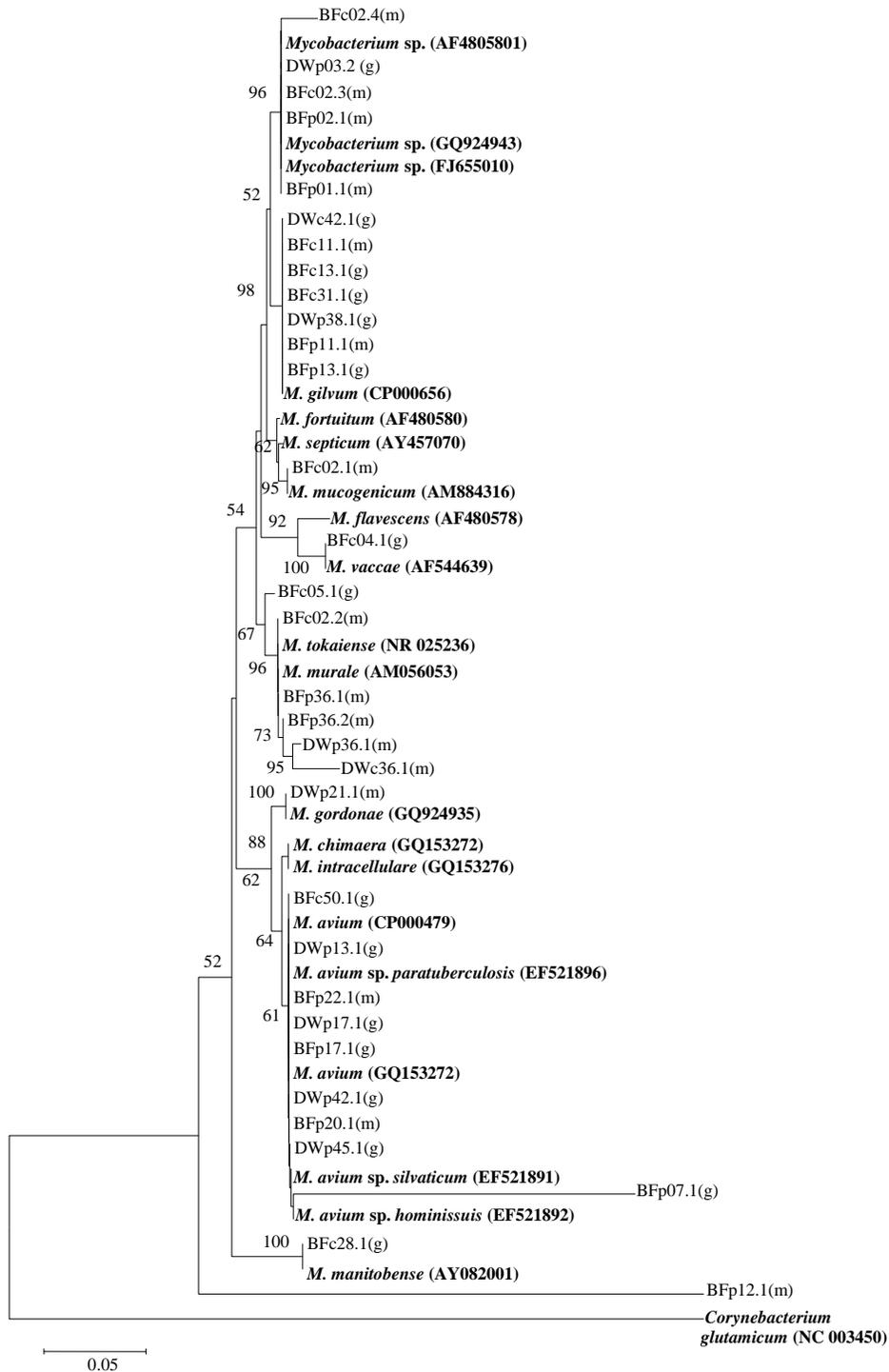


Figure 3.1. Phylogenetic relationship of 16s rRNA gene amplified with *Mycobacterium* genus-specific primers with *Corynebacterium glutamicum* as the out-group. Reference sequences are in bold with accession numbers in parentheses. Sequences from this study are indicated by code as follows. (DW) drinking water, (BF) biofilm, (p) PCR, (c) culture, (g) groundwater, and (m) municipal.

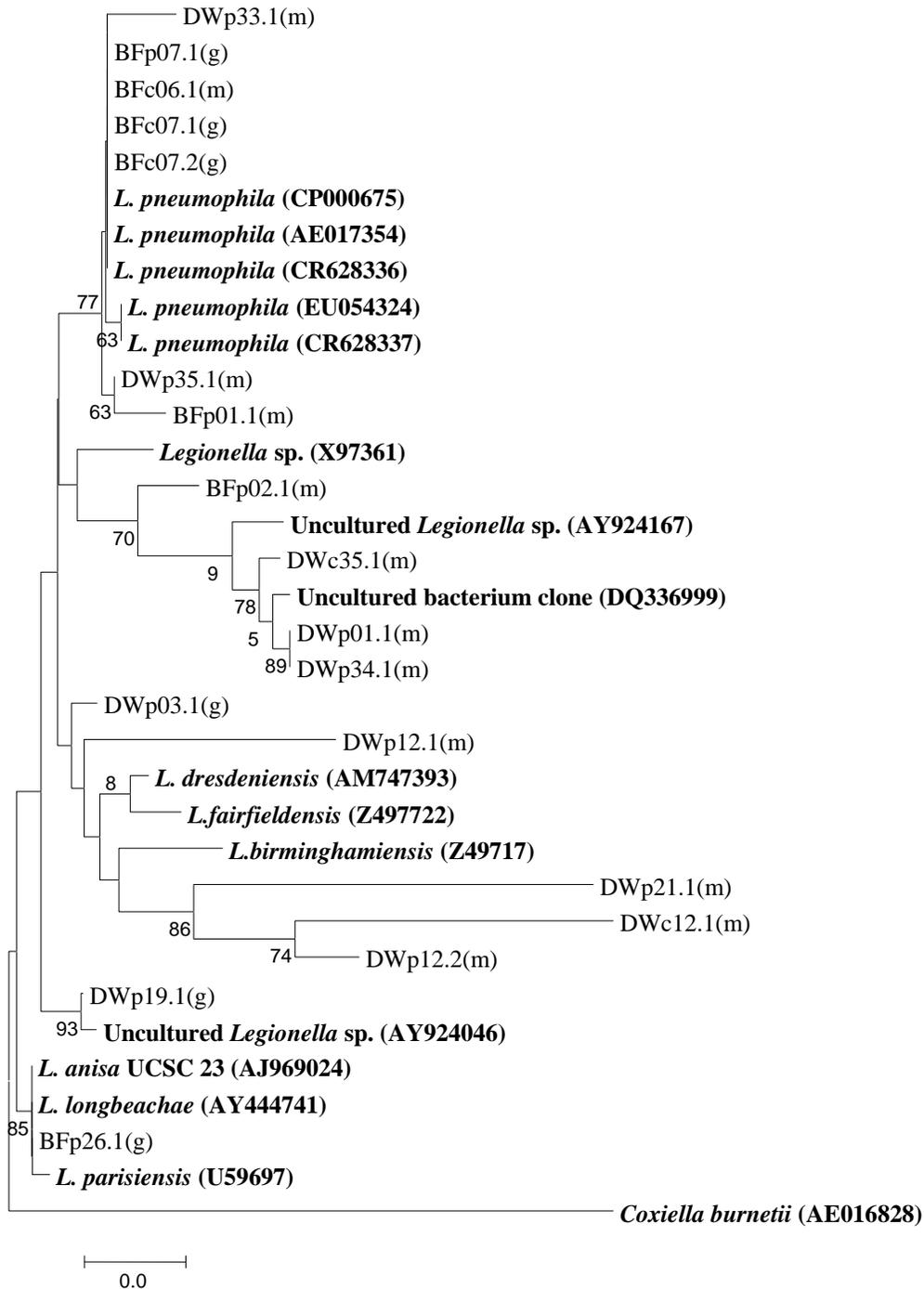


Figure 3.2. Phylogenetic relationship of 16s rRNA gene amplified with *Legionella* genus-specific primers with *Coxiella burnetii* as the out-group. Reference sequences are in bold with accession numbers in parentheses. Sequences from this study are indicated by code as follows. (DW) drinking water, (BF) biofilm, (p) PCR, (c) culture, (g) groundwater, and (m) municipal.

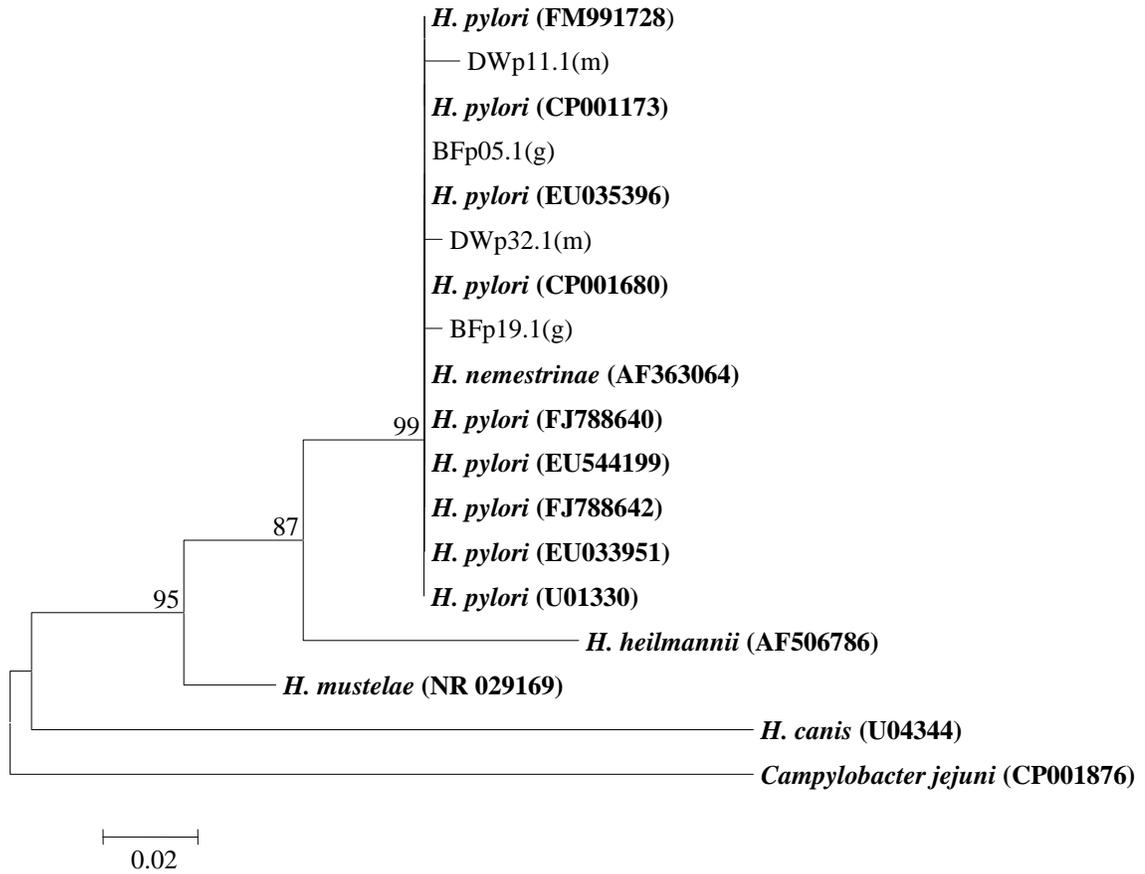


Figure 3.3. Phylogenetic relationship of 16S rRNA gene amplified with *Helicobacter* genus-specific primers with *Campylobacter jejuni* as the out-group. Reference sequences are in bold with accession numbers in parentheses. Sequences from this study are indicated by code as follows. (DW) drinking water, (BF) biofilm, (p) PCR, (c) culture, (g) groundwater, and (m) municipal.

CONTRIBUTION OF AUTHORS AND CO-AUTHORS

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CHAPTER 4

**Multiple Processes Govern Switch to Nonculturable State in *H. pylori***

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## ABSTRACT

The route of transmission of the human pathogen *H. pylori* has not been well understood due to the conversion of the organism into a nonculturable state in most environments outside the stomach. The organisms are thought to survive adverse conditions by entering into a dormant state, which renders them nonculturable *in vitro*, but the resulting changes in gene transcription have not been characterized. Two stress conditions that cause a nonculturable state, oxygen stress and nutrient deprivation (incubated under atmospheric levels of oxygen), were examined for changes in cell morphology and gene transcription. The two treatments caused a differential response regarding the morphological conversion of the cells; oxygen stress promoted the formation of coccoid cells while nutrient deprivation did not (< 45% coccoid after 2 weeks). Microarray analysis of the two treatments showed that the oxygen stressed cells quickly up expressed a large number of genes (24 hours of stress exposure) in all functional categories while nutrient deprived cells did not drastically alter transcription until 168 hours of stress exposure. Of interest was the down regulation of virulence genes and up regulation of genes that encode for cell envelope functions such as outer membrane proteins. After exposure to stress, cells were resuscitated in a nutrient rich broth and the transcription of two virulence factors, *ureA* and *vacA* was assessed. After resuscitation, the recovered cells showed differential expression of these virulence genes. Transcription levels of *ureA* were compared to the intracellular urease activity. Increases and decreases in transcription were related to biological activity of the enzyme. This

research has shown that stressed *H. pylori* cells use multiple mechanisms, specific for the type of stress encountered, to adapt to environmental changes.

Key words: *Helicobacter pylori*, culturability, microarray, transcriptome, virulence

## INTRODUCTION

The gastrointestinal pathogen *Helicobacter pylori* infects approximately 50% of the world wide population, with infection increasing the risk of developing gastric and duodenal ulcers and gastric cancer (41). The exact route of transmission remains poorly understood with oral-oral and environmental routes being implicated (31, 39).

Elucidation of the mode of transmission has been hindered for several reasons. First, *H. pylori* is extremely variable in its culturability and converts to a viable but not culturable state (VBNC) in most environments outside the human stomach (1, 7, 55).

Microbiologists have traditionally relied on culturing an organism to prove the organism is viable and capable of infection, but due to the loss in culturability the mode of transmission for this organism is difficult to pinpoint.

Second, due to the potential for serious health consequences such as cancer, there have been very few human trials that adequately measure the infectious dose or investigate potential transmission sources directly. One study utilizing human subjects found that a dose of  $10^4$  CFU was adequate to cause infection (32). Other studies have utilized primate or mouse models to study *H. pylori* transmission and infection (59, 63). Coccoid *H. pylori* in a nonculturable state are capable of infecting mice and producing ulcer and inflammatory cell infiltration, although with decreased efficacy compared to healthy cells (19, 22, 59, 72).

Investigations of *H. pylori* outside the human stomach have revealed the presence of the organism in a variety of environments (8, 27, 30, 38, 40, 51, 65, 67). It has been consistently identified, primarily using molecular methods, from vomitus, saliva, and dental plaque, and occasionally identified in fecal samples (28, 51, 65, 67). *H. pylori* adheres to gastric epithelial cells and achieves this with numerous outer membrane proteins, phospholipids, glycolipids, and other adhesions. This ability to adhere may allow stressed *H. pylori* to persist outside the body and provide an environmental reservoir in plumbing, potable water systems, and even drinking water receptacles (14, 40). Indeed, nonculturable *H. pylori* have been detected in drinking water sources and associated biofilms, and in some cases an association between infection and drinking water sources has been demonstrated (8, 14, 30, 73). The presence of nonculturable *H. pylori* in these environments suggests a potential exposure risk.

Nonculturable cells will not grow on traditional bacteriological media *in vitro* (50). In the case of *H. pylori*, they are thought to be dormant forms that retain minimal metabolic activities and are postulated to be an adaptive response to environmental change (6, 49). Non-culturable *H. pylori* have been characterized by many researchers (6, 15, 35, 37). The loss in culturability is often coincident with the morphological conversion of the cells from a spiral, flagellated form to a coccoid, aflagellated form (11, 37, 55, 64). The conversion to a coccoid form is associated with aging *H. pylori* cells in the stationary phase of growth (37). Environmental stress also induces the coccoid, nonculturable state and the proportion of cells converting to a coccoid morphology

changes with variations in the environment such as nutrient deprivation, oxygen stress, temperature, and antimicrobial agents (1, 26, 37).

The mechanism associated with the conversion to a coccoid morphology is not well understood and conflicting hypotheses regarding the nature of this conversion have been presented (6, 37). Nonculturable *H. pylori* cells maintain their cell membrane (1), produce ATP (at lower yields than healthy cells) (29), produce mRNA (47), and retain oxidative metabolism (33). However, it is unknown if the dormant forms contribute to an overall low RNA and ATP yield, or if a few viable cells retain full energy supplies, because measurements are often averaged observations from batch cultures (29).

RNA techniques have been used for studying *H. pylori* in a nonculturable state and have often focused on pathogenicity genes. Many factors contribute to virulence in *H. pylori*, such as the *cag* pathogenicity island, vacuolating cytotoxin, and the urease enzyme, as well numerous adhesion proteins that promote adherence to gastric epithelial cells (54, 70, 75). Detectable levels of *ureA* (urease enzyme alpha subunit), *cagA* (cytotoxin associated gene), *vacA* (vacuolating cytotoxin gene), and *sodB* (superoxide dismutase) mRNA have been observed in nonculturable, coccoid cells (44, 61) indicating a potential for virulence.

Microarray technology also allows the analysis of transcriptome level gene expression in response to experimental conditions (23). There have been several published transcriptomes for *H. pylori* including the primary transcriptome, as well as those that examined growth phase, pH, and temperature (34, 42, 58, 68). However,

changes in gene transcription that allow *H. pylori* to survive the transition from host to host remains poorly understood.

To understand the survival of dormant, nonculturable *H. pylori*, we investigated changes in cell morphology and gene transcription associated with the loss in culturability seen under oxygen stress and nutrient deprivation. Previous research by our group found that a portion of nonculturable oxygen stressed and nutrient deprived *H. pylori* cells could be resuscitated under appropriate culture conditions (53). That work led to the hypothesis that the transition of *H. pylori* to a nonculturable form is caused by the active conversion of the cells to the coccoid morphology. This study examined the morphological conversion and gene transcription profiles associated with the transition to a nonculturable form under stress by microarray analysis. Microarray results were confirmed by qRT-PCR and a urease activity assay. After conversion to the nonculturable state, the cells were stimulated with a *H. pylori* specific resuscitation medium and gene expression of two important virulence factors, *ureA* and *vacA*, were reassessed. We present data highlighting the difference in cell morphology and gene transcription observed under oxygen exposure and nutrient deprivation.

## MATERIALS AND METHODS

**Bacterial strain and culturing conditions.** *Helicobacter pylori* 26695 was obtained from the American Type Culture Collection (ATCC) and was routinely grown from frozen stocks on tryptic soy agar (TSA) (Difco, Milwaukee, WI) with 5% sheep blood (Quad Five, Montana) and the antibiotics vancomycin (Sigma, St. Louis, MO) 10 µg/mL,

trimethoprim (MP Biomedical, Solon, OH) 0.5 µg/mL, cefsulodin (Sigma) 0.5 µg/mL, polymixin B (Sigma) 3.5 U/mL and amphotericin B (Sigma) 7.5 µg/mL. Initial cultivation on antibiotics helped inhibit contamination of cultures after subculturing onto media without antibiotics. The plates were incubated in a microaerophilic atmosphere using GasPak™ EZ Campy sachets (Becton Dickinson and Co., Franklin Lakes, NJ) in a BBL anaerobe jar for 48h at 37°C. Cells were removed from the blood agar using sterile swabs and were inoculated into tryptic soy broth (TSB) (Difco) with 5% bovine calf serum (Thermo Scientific Hyclone, Logan UT) followed by inoculation into biphasic slants at the desired concentration. A biphasic slant consists of a 50 ml culture tube (Fisher Scientific, Fair Lawn, NJ) containing 25 ml TSA with 5% sheep blood (blood agar slant) with no antibiotics, solidified at an angle with a pool of 5-10 ml TSB in the bottom of the slant. *H. pylori* cells were added to a blood agar slant at ca.  $1 \times 10^7$  CFU/ml and incubated for 24 hours in a microaerophilic atmosphere at 37°C. Slants were covered with 0.2 µm breathable vented caps (Becton Dickinson and Co.) which allow atmospheric exchange while excluding microbial contaminants. After incubation, the slants were either used directly (healthy cells) or were subjected to two different stress treatments, oxygen exposure or nutrient deprivation in the presence of atmospheric oxygen.

**Stress exposure and enumeration of total and viable cells.** To compare *H. pylori* cells that are ageing naturally to those given the stress treatments, growth curves were performed on cultures allowed to age in a microaerophilic atmosphere. Aliquots from ageing cultures were removed for up to 3 weeks and enumerated for total and viable cells

as described below. Slants that were given the oxygen stress treatment were removed from the microaerophilic atmosphere and placed in a 37°C incubator with atmospheric oxygen levels. *H. pylori* cells subjected to nutrient deprivation were decanted from the biphasic slant and centrifuged at 13,000 x g for 3 minutes. The cells were washed two times and resuspended in 10 ml ultra pure sterile water followed by incubation at 37°C with atmospheric levels of oxygen. Both treatments were sufficient to inhibit growth on TSA agar with 5% sheep blood within 24 hours.

Viable and total cell counts were performed on the oxygen stressed and nutrient deprived *H. pylori* cells. For total cells, aliquots of *H. pylori* cells at each time interval and treatment were fixed in 4% formaldehyde and stained with 100X SYBR Green (Invitrogen, Carlsbad, CA) at room temperature for up to an hour. Cells were diluted, filtered by a vacuum pump onto a 0.22 µm polycarbonate filter (Whatman, Maidstone, England), and visualized on a Zeiss Axioskop epifluorescence microscope (Carl Zeiss, Inc., Maple Grove, MN) at 1000X total magnification under oil immersion. The microscope utilized the Chroma set #41001 filter for visualization of FITC with excitation at 480/40 and emission at 535/50. A total of 20 fields or 400 cells were counted per filter and duplicate membrane filters were counted for each biological replicate. Viable cell counts were done by diluting, if appropriate, and plating, in duplicate, on tryptic soy agar with 5% sheep blood with and without antibiotics to check for contamination.

**RNA isolation, microarray hybridization and analysis.** RNA was extracted from healthy *H. pylori* at time zero of stress treatment and from stressed cells at 24, 72, and

168 hours. The cells from each treatment were pelleted at 4°C for five minutes at 13,000 x g and the supernatant was removed. Trizol<sup>®</sup> (Invitrogen) was heated to 65°C and added to the cell pellet followed by brisk pipetting of the cells to remove all clumps. The Trizol<sup>®</sup>/cell mixture was incubated at 65°C for 5 minutes with vortexing on maximum every minute for 10 seconds, and subsequent incubation at room temperature for 5 minutes. One hundred microliters of ice-cold chloroform was added to the reaction and the tubes were shaken for 15 seconds followed by incubation at room temperature for 2-3 minutes. The top aqueous layer was added to 250µl 100% ethanol (Sigma), applied directly to an RNeasy column (Qiagen, Valencia, CA) and treated according to the manufacturer's instructions. RNA samples were subsequently treated to remove DNA contamination using the commercially available TURBO DNase treatment (Ambion, Austin, TX) according to the manufacturer's instructions. The rigorous DNase treatment was used with an extra round of incubation with DNase (total of 3µl DNase added). RNA samples were subsequently stored at -80°C.

Although RNA could be successfully extracted from all time points, the yields from oxygen stressed *H. pylori* were too low after 72 hours to enable adequate microarray hybridization. Accordingly, RNA extracted at 24 hours of oxygen stress and RNA extracted at 24, 72 and 168 hours of nutrient deprivation treatment were used as templates for cDNA synthesis and subsequent microarray hybridization for a total of four distinct biological groups. RNA extracted from 72 and 168 hours of oxygen stress was also used in quantitative reverse transcriptase PCR for analysis of targeted genes.

RNA was reverse transcribed into cDNA, indirectly labeled, and cleaned up according to SOP#: M007 released by Pathogen Functional Group Research Center (PFGRC), J. Craig Venter Institute (JCVI) (<http://pfgrc.jcvi.org/index.php/microarray/protocols.html>). Briefly, Superscript II (Invitrogen, CA) was incubated with 1X first strand synthesis buffer, 1.3 mM DL-dithiothreitol (DTT) (Invitrogen), 3-5 µg total RNA, 6 µg random hexamers (Invitrogen), 40 U RNaseOUT™ (Invitrogen) for 18 hours at 37°C. The reaction was halted, the RNA was hydrolyzed by the addition of sodium hydroxide (1N, 10 µl per reaction) and the pH neutralized with hydrochloric acid (1N, 10 µl per reaction). The QIAGEN MinElute PCR purification kit (Qiagen) was used for the removal of unincorporated aa-dUTP and free amines according to the manufacturer's instruction with the following modifications. A 5 mM pH 8.5 phosphate wash buffer with 80% ethanol was substituted for the kit wash buffer and nuclease-free water was substituted for the elution buffer. After elution from the column, the cDNA was concentrated and lyophilized for 35 minutes at 45°C in a vacuum concentrator (Jouan, VA). Dried aminoallyl-labeled cDNA was resuspended in 0.1 M sodium carbonate buffer (pH 9.3) and 4.5 µL of either cy3 (untreated control, early log phase *H. pylori*) or cy5 (experimental treatment, oxygen stress or nutrient deprivation) was added, and the reaction was incubated for 3 hours in the dark. After coupling was complete, 35 µL 4.5 M sodium acetate pH 5.2 (Ambion) was added and the reaction cleaned on a QIAGEN MinElute PCR purification kit (Qiagen) utilizing the same modifications as the first cDNA cleanup. Following cleanup, the samples were quantified on the Nanodrop ND-1000 (Thermo Scientific) and labeled reference (cy3) and treated (cy5) cDNA was

combined in equal concentrations. Mixed probes were dried again and resuspended in 5X SSC (Ambion), 0.1% sodium dodecyl sulfate (Fisher), and 0.1 DTT (Sigma). To inhibit non-specific binding, 10 µg sheared salmon sperm was added to the mixture and the probes were heated to 95°C for 10 minutes before addition to the array.

Hybridization was performed using 70-mer spotted *H. pylori* version 1 aminosilane coated glass microarrays from the PFGRC, JCVI. These microarrays were designed based on the two published genome sequences of *H. pylori* strains 26695 and J99 (69). A total of 2,572 70-mer oligonucleotides were designed for each array and each oligonucleotide was printed in triplicate. Probes were hybridized to arrays according to SOP#:008 (<http://pfgrc.jcvi.org/index.php/microarray/protocols.html>) with the following modifications. Arrays were prehybridized for 3 hours to minimize background fluorescence. The arrays were hybridized at 37°C for 19 hours and washed according SOP#:008. To ensure reproducibility of results, three independent biological replicates were performed for each time point examined by microarray. Additionally, each gene was spotted on each array in triplicate. The hybridized slides were scanned and analyzed using an GenePix<sup>®</sup> 4000B dual wavelength scanner (Molecular devices, CA). Spots showing obvious abnormalities were excluded from analysis and local background subtraction was applied.

Data normalization was performed with MIDAS (TIGR) and included Lowess normalization, standard deviation regularization, and in-slide replicate analysis which combined technical replicates on each slide. After normalization, a total of 2,564 genes were included in the analysis. A one-way ANOVA with four groups (each group

composed of three biological replicates) was applied in MeV (TIGR) to compare the mean expression levels of the test and reference samples. The ratio of the red (test) to green (reference) for each spot was expressed as the R/G ratio and was  $\log_2$  transformed. Genes were considered significant if the  $\log_2$  intensity ratio was significantly different from zero, ( $P < 0.05$ ). Because of the disparate transcription levels between the treatment groups, an arbitrary  $\log_2$  fold change of 1.0 was applied to screen for significant genes. A  $\log_2$  fold change of 1.0 indicates a doubling of the expression (R/G ratio) of that particular gene. Functional categories were assigned to genes using the JCVI Comprehensive Microbial Resource, gene attribute download available at <http://cmr.jcvi.org/cgi-bin/CMR/shared/MakeFrontPages.cgi?page=geneattribute>.

**Assessment of transcriptional changes of nonculturable cells after incubation in a regrowth medium.** To determine if incubation of stressed, nonculturable cells, in a *H. pylori* specific growth medium containing lysed human erythrocytes and human serum restored transcription to levels seen in early log phase cells, quantitative RT-PCR was utilized. The resuscitation media consisted of Brucella broth (Difco) with 10mM HEPES (Sigma), at a pH of 7.8, 0.2 $\mu$ M CuSO<sub>4</sub> (Sigma), 0.2  $\mu$ M MnSO<sub>4</sub> (Sigma), 3  $\mu$ M ZnSO<sub>4</sub> (Sigma), with 50  $\mu$ M FeSO<sub>4</sub> (Sigma), 250  $\mu$ M MgCl<sub>2</sub> (Fisher scientific), 250 mg/l sodium pyruvate (Fisher Scientific), 2% human blood serum, and 2% human blood lysate added after autoclaving. Five milliliters of cells from each stress treatment was centrifuged at 13,000 x g for 3 minutes, followed by washing in phosphate buffered saline (PBS) with 10 mM ammonium sulfate (Sigma) two times and resuspension into 500  $\mu$ l PBS with ammonium sulfate. Phosphate buffered saline was composed of 12 g/l

sodium phosphate monobasic (Fisher Scientific), 2.2 g/l sodium phosphate dibasic (J. T. Baker Chemical Co., Phillipsburg, NJ), and 85 g/l sodium chloride (Fisher Scientific) and diluted 1:10 to obtain a working stock solution. Stressed *H. pylori* cells were heat shocked (37°C for 10min, 45°C for 30 sec. and cooled to 4°C), added to regrowth medium and incubated in a microaerophilic atmosphere for 48 hours at 37°C. All treatments were repeated in triplicate. RNA was extracted after resuscitation as previously described.

Quantitative RT-PCR was achieved using a one step QuantiTect SYBR Green kit (Qiagen) in a 25 µl total volume. The mixture contained 1.25 µl (0.5 mmol) forward and reverse primer (Integrated DNA Technologies, Coralville IA), 12.5 µl master mix, 0.25µl RT mix, 5µl RNA, and 4.75 µl water. The assay was carried out at 50°C for 30 minutes and denaturation at 95°C for 15 minutes. Thirty cycles were run at 94°C for 15s, 60°C for 30s, and 72 °C for 30s followed by a 30 minute extension step at 72°C. Standard curves were prepared using serially diluted gene specific PCR products generated from randomly primed reference cDNA. Two technical replicates were performed on each biological replicate to control for variation in the assay. A negative control without reverse transcriptase was included for every RNA sample to detect contamination by DNA and RNA-free controls were routinely run with reagents and each primer set to detect RNA contamination.

**Reliability and validity of array data.** To further validate the gene expression data obtained from the array experiments four genes, HP0073, HP0875, HP0887, and HP1444, were used to evaluate the microarray data by qRT-PCR. Two virulence factors,

HP0073 and HP0887, were chosen due to our interest in the pathogenicity of stressed *H. pylori*. These genes encode functional components of the urease enzyme and vacuolating cytotoxin, respectively. *ureA* and *vacA* transcription levels were evaluated quantitatively before and after the addition of the regrowth medium to the stressed cells. Two other genes of interest were analyzed as well, HP0875 which encodes the catalase enzyme and HP1444 which encodes the smp protein. The gene encoding the catalase enzyme was chosen because of the differential expression levels seen between the two stress treatments. The gene encoding the smp protein was chosen as a control because it was relatively unchanged across all treatments. The primer sets are as follows: Ure-R 5' AAAAATGTTGGCGACAGACC 3', Ure-L 5' TTACCGCCAATGTCAATCAA 3' (182 BP); VacA-R 5' CCCAGCCTCCATCAATCTTA 3', VacA-L 5' GCTGCTGTAGGAACGGTCTC 3' (187 BP); Cat-R 5' ATTCAGTCCGGCTAATGTCG 3' Cat-L 5' AGAGTGGGAATGGGCATCTTG 3' (154 BP); smp-R 5' AGACGCTTCAATCTGCCATT 3'; TGAAGGCTTTGAGGCAAAC 3' (195 bp).

**Urease activity assay.** To assess whether transcriptional changes reflected biological activities, a urease activity assay was employed. The activity of the intracellular urease enzyme was measured for both oxygen stress and nutrient deprivation, before and after resuscitation, at 24, 72, and 168 hours of exposure to stress treatments. Two ml of each stress culture (at each time point indicated) was centrifuged at 13,000 x g to pellet the cells, followed by rinsing twice in 0.9% NaCl, and resuspension in water. Samples were then sonicated with a Vibra cell sonicator (Sonics and materials, CT) for 30 second

bursts, followed by cooling on ice for 30 seconds and repeated for a total of two minutes of sonication. The samples were centrifuged at 5,000 x g to remove cell debris. Total protein was determined using the BCA total protein kit (Thermo Scientific, IL) according to the manufacturer's instructions. Briefly, 100 µl of cell lysate was added to 2 ml BCA reagent, incubated at 50°C for 60 min, and finally measured on the Genesys™ 10UV spectrophotometer (Thermo Scientific) at 562nm. Standard curves using a dilution series of bovine serum albumin (Fisher) were utilized to determine total protein in mg. Five hundred microliters of cell lysate was added to 3 ml urea R broth, (Difco) at pH 6.7, and incubated at 37°C for 30 min. Production of ammonia was measured by the color change (560 nm) associated with the increase in pH. Standard curves using a dilution series of ammonium chloride allowed the determination of urea utilized/min. One molecule of urea was assumed to be equivalent to two molecules of ammonia.

## RESULTS

**Effect of aging, oxygen stress, and nutrient deprivation on the conversion to coccoid and culturability of *H. pylori*.** There were different effects on cell morphology associated with the aging of *H. pylori* and stress treatments (Fig. 4.1). The aging of *H. pylori* caused a conversion from a spiral shape (Fig. 4.2A) to a mixture of spiral and coccoid cells (Fig. 4.2B) that approached 95% coccoid after 3 weeks. The switch in cell morphology occurred at the onset of stationary phase and was concomitant with a decrease in culturability, as indicated by decreasing CFU counts (Figure 4.1A).

The two different stress treatments, oxygen stress and nutrient deprivation, had different rates of conversion to coccoid. Oxygen stress in the presence of nutrients at 37°C caused the nearly complete conversion to a coccoid morphology by 72 hours (Figs. 4.1B and 4.2C). This treatment was sufficient to inhibit growth on blood agar within 24 hours of exposure and the total number of cells did not change drastically over time. Nutrient deprivation at 37°C resulted in a slower conversion to a coccoid cell morphology (<45% after 2 weeks of exposure) and a faster loss in culturability (within 4 hours of exposure) (Fig. 4.1C). Over time, the nutrient deprivation treatment resulted in  $\leq 45\%$  coccoid cells with a majority of the cells maintaining their spiral morphology (Fig. 4.2D). Similar to the oxygen stress treatment, the total number of cells did not vary much over time (Fig. 4.1C.).

RNA yields of *H. pylori*, after RNeasy purification and DNase treatment, varied significantly depending on the stress treatment. RNA extracted from reference cells (*H. pylori* grown to early log phase, 24 hours), gave the highest yields of RNA with a mean and standard deviation of  $53.5 \pm 7.3$   $\mu\text{g}$  total RNA. RNA extracted from oxygen stressed cells decreased substantially over time to  $9.1 \pm 0.7$   $\mu\text{g}$  total RNA at 24 hours,  $2.3 \pm 0.24$   $\mu\text{g}$  total RNA at 72 hours, and  $1.18 \pm 0.35$   $\mu\text{g}$  total RNA at 168 hours. Attempts to combine several technical replicates of oxygen stressed cells to obtain enough cDNA for array hybridization were unsuccessful. RNA extracted from nutrient deprived cells decreased more gradually;  $7.4 \pm 1.3$   $\mu\text{g}$  total RNA at 24 hours,  $9.7 \pm 2.2$   $\mu\text{g}$  total RNA at 72 hours, and  $3.4 \pm 0.81$   $\mu\text{g}$  total RNA at 168 hours.

**Global gene expression analysis.** The microarray experiments showed a large number of up and down expressed genes. Of the 2,564 genes that were included in the analysis, 654 were considered significantly differentially expressed ( $P \leq 0.05$ ) after analysis in MeV (Fig. 4.4). Further analysis applied a  $\log_2$  fold change of 1.0 as biologically significant and the gene was included in the analysis. After screening for biological significance, the total number of genes with a  $\log_2$  fold change above 1.0 in at least one of the groups was 200, while the number of genes that showed a fold change of -1.0 in at least one of the groups was 196. Table 4.1 shows selected up expressed genes. The complete set of up expressed and down expressed genes is listed in Appendix C.

Up expressed genes include hypothetical protein genes (80/200, 40.2%), substance metabolism genes (including protein, nucleic acid, cofactors and carriers; fatty acid and phospholipid biosynthesis; central intermediary metabolism) (46/200, 23.2%), energy metabolism (10/200, 5.0%), cellular envelope genes (21/200, 10.5%), cellular processes (11/200, 5.0%), protein fate (5/200, 2.5%), purines, pyrimidines, nucleosides, and nucleotides (4/200, 2.0%), regulatory functions (3/200, 1.5%), transcription (2/200, 1.0%), transport and binding proteins (10/199, 5.0%), mobile and extrachromosomal element functions (1/200, 0.5%), and unknown/unclassified (7/200, 3.5%). Down expressed genes include hypothetical protein genes (60/196, 31.0%), substance metabolism genes (including protein, nucleic acid, cofactors and carriers; fatty acid and phospholipid biosynthesis; central intermediary metabolism) (44/196, 22.5%), energy metabolism (36/196, 18.4%), cellular envelope genes (9/196, 4.6%), cellular processes (17/196, 8.7%), protein fate (7/196, 3.6%), purines, pyrimidines, nucleosides, and

nucleotides (3/196, 1.5%), regulatory functions (3/196, 1.5%), transcription (1/196, 0.5%), transport and binding proteins (10/196, 5.1%), and unknown/unclassified (7/196, 3.6%).

### **Oxygen stress alone causes a faster transcriptional response than nutrient**

**deprivation.** The transcriptional changes that occur at the onset of the nonculturable state were of interest in this study. Therefore, *H. pylori* cells exposed to oxygen stress or nutrient deprivation for 24 hours were analyzed by microarray hybridization. Figure 4.3 shows the significant  $\log_2$  fold changes in transcription at this time point. The transcriptional profiles of the two treatments at 24 hours were strikingly different. The oxygen stressed cells had a large variety of genes that were significantly up and down expressed. However, after 24 hours of exposure, the nutrient deprived cells had only 3 genes with a mean  $\log_2$  fold increase of 1.0 or greater; HP1529, a chromosomal replication initiator protein gene, and 2 hypothetical proteins (Table 4.1). There were 6 genes significantly down expressed; 4 hypothetical proteins, diacylglycerol kinase (HP0700), and indole ferredoxin oxidoreductase (jhp1035) (Appendix C).

Due to the high abundance of RNA in nutrient deprived cells, time points 72 and 168 hours were also analyzed by microarray (Fig. 4.3.). After 72 hours of nutrient deprivation, the cells begin to show a wider variety of up and down expressed genes, with 17 significantly up expressed genes in the cell envelope, energy metabolism, hypothetical protein, ribo-sugar synthesis, cellular processes (chemotaxis and motility) and regulatory function categories. There were 23 down expressed genes in the functional categories of substance metabolism, cellular processes (detoxification, chemotaxis and motility),

hypothetical proteins, energy metabolism, and protein fate. The trend continued with 65 up expressed genes and 55 down expressed genes representing every functional category after 168 hours of stress exposure.

Validation of the transcriptome data by qRT-PCR showed consistency with the microarray results (Table 4.2). Although there were quantitative differences in the fold change of, *ureA* (HP0073), *vacA* (HP0887), *catA* (HP0875) and *smp* (HP1444) transcription, the overall trends observed by microarray analysis were confirmed with qRT-PCR. The minor differences seen in transcription could be attributed to the greater sensitivity of qRT-PCR.

**Virulence gene transcription was affected by stress treatments.** Several important virulence factors were down expressed in response to stressful conditions. Both types of stress showed decreased expression of genes found in the *cag* pathogenicity island (PAI); HP0543, HP0545, HP0546, and HP0547 (Appendix C). Oxygen stressed cells decreased expression of all four of these genes; nutrient deprived cells down expressed HP0543 and HP0545. One *cag* PAI gene, HP0535, was significantly up expressed in oxygen stressed cells (1.9 log fold increase) but remained unchanged at 24 and 72 hours of nutrient deprivation (Table 4.1). After 168 hours of nutrient deprivation, HP0535 was down expressed in nutrient deprived cells (-1.5 fold change) to a level similar to the other down regulated *cag* pathogenicity island genes.

Genes responsible for survival in acidic conditions *in vivo*, the urease protein and urease accessory proteins, were down expressed at all conditions and time points evaluated, (HP0067, HP0068, HP0070, HP0071, HP0073, Appendix C). This included

the gene that encodes the inner membrane protein ureI, which is known to allow the import of urea and export of NH<sub>3</sub> across the cell membrane. Another important *H. pylori* virulence factor, vacuolating cytotoxin *vacA*, (HP0087), was down expressed at all conditions and time points although noticeably more repressed in oxygen stressed cells.

**Stressed cells exhibited different levels of virulence gene transcription when presented with favorable growth conditions.** Because the stress treatments caused a decrease in transcription of virulence factors, it seemed valuable to examine how stressed *H. pylori* cells respond when presented with subsequent favorable growth conditions. A regrowth medium designed specifically for *H. pylori* and intended to simulate exposure to a human host was used to resuscitate stressed cells. Oxygen stressed and nutrient deprived cells were harvested at 24, 72, and 168 hours of exposure to stressor, heat shocked and incubated in a *H. pylori* specific regrowth medium as previously described (53).

Conventional qRT-PCR, using primers specific for *ureA* and *vacA*, was performed on positive control *H. pylori* (early log phase) and both oxygen and nutrient stress treatments (all 3 time points for both treatments) before and after transfer to *H. pylori* specific culture medium containing lysed human erythrocytes and serum. All reactions were negative if the RT or template was omitted, indicating that DNA was not present and the reagents were not contaminated. A paired t-test showed the incubation of culturable, healthy cells in a regrowth medium did not increase the transcription of *ureA* ( $P = 0.757$ ) (Fig. 4.4, time zero). After regrowth treatment, transcription of the *vacA*

gene in control cells slightly increased but not significantly ( $P = 0.262$ ) (Fig. 4.5, time zero).

A comparison of healthy, stressed, and resuscitated cell *ureA* expression and urease enzyme activity are shown in Figure 4.4. Based on qRT-PCR results, transcription of the *ureA* gene decreased in oxygen stress treatments compared to the positive control, consistent with array results (Table 4.2). Compared to the reference cells, the oxygen stressed cells showed a decreased expression of *ureA* (Fig. 4.4A). Cells exposed to atmospheric oxygen for 24, 72, and 168 hours showed an increase in *ureA* transcription after the addition of regrowth medium at 24 ( $P = 0.001$ ) and 72 hours ( $P = 0.002$ ) but not after 168 hours of exposure to stress ( $P = 0.211$ ) (Fig. 4.4A). Transcription of *vacA* followed a trend similar to the transcription of *ureA* and was also consistent with microarray results (Table 4.2). Compared to the healthy reference cells, the oxygen stressed cells had significantly less expression of *vacA* at all three time points examined (Fig. 4.5A). After incubation of oxygen stressed cells in the regrowth medium, transcription of *vacA* was variable depending on the length of time the cells were exposed to oxygen. After the regrowth treatment, *vacA* expression increased at 24 hours of stress exposure ( $P = 0.008$ ), but after 72 hours and 168 hours of oxygen stress there was not a significant increase ( $P = 0.635$  and  $P = 0.742$ , respectively) (Fig. 4.5A).

Transcription of the *ureA* gene during nutrient deprivation differed from the levels seen during oxygen stress (Fig. 4.4C). Compared to the reference cells, the nutrient deprived cells had less expression of *ureA* at 24 hours, consistent with microarray results (Table 4.2). After incubation in the regrowth medium, cells that were nutrient deprived

for 24 hours showed a slight increase in mean *ureA* transcription ( $P = 0.502$ ). Nutrient deprived cells exposed for 72 and 168 hours and then given the regrowth treatment had significantly decreased *ureA* transcription ( $P = 0.000$  and  $P = 0.065$ , respectively). The transcription of *vacA* in nutrient deprived cells followed a pattern similar to the *ureA* gene, with slightly decreased transcription compared to healthy cells which declined further after resuscitation (Fig. 4.5B).

To corroborate gene transcription levels, a urease activity assay was employed to assess whether the *ureA* transcriptional activities were physiologically relevant. The urease enzyme in healthy, reference cells showed no significant change in activity when presented with the regrowth treatment (Fig. 4.4, time zero). Despite decreased transcription of the *ureA* gene after 24 hours of exposure to atmospheric oxygen, the intracellular urease enzyme activity stayed at a level similar to the reference cells and slowly decreased over time (Fig. 4.4B). The transfer of 24 hour oxygen stressed cells into the regrowth medium increased urease activity ( $P = 0.024$ ) (Fig. 4.4B). However, after 72 and 168 hours of oxygen stress, urease activity was decreased in the regrowth treatment ( $P = 0.039$  and  $P = 0.588$ , respectively) (Fig. 4.4B). Nutrient deprived cells had decreased urease activity compared to the healthy, reference cells at 24, 72, and 168 hours of stress treatment, and those levels were further decreased by the addition of nutrients at both subsequent time points examined (Fig. 4.4D).

## DISCUSSION

The conversion of *H. pylori* to a coccoid morphology and the associated physiology has been extensively studied (11, 12, 16, 18, 22, 26, 55, 59, 64). Coccoid

forms are associated with the loss in culturability seen during stationary phase and under stress. (6, 11, 37, 55) Although the conversion to coccoid was readily observed in this study, spiral forms can be dominant when a batch of *H. pylori* cells loses culturability. The filamentous spiral forms observed after prolonged incubation in sterile water at 37°C have been observed in other studies as well (6, 47, 57). Because a slightly different growth medium was used in this study, it was important to compare current results with observations by others of the ageing process (2, 37, 74). Consistent with other reports, a mixture of cell morphologies occurred at the onset of stationary phase, later followed by the nearly complete conversion of all cells to coccoid forms concomitant with a decrease in CFU (55, 57).

The stress treatments used in this research were carefully chosen to simplify the variables involved in the inducement of the viable but nonculturable state, as it is known that transcription can be greatly affected by many factors such as the age of cells and temperature (34, 43, 68). Oxygen stress was chosen because *H. pylori* will likely encounter oxygen levels outside its optimal growth range during the transition from host to host or during extended time in the environment. Nutrient deprivation (under atmospheric oxygen levels) was included because of the desire to understand how the organism survives and persists in a low nutrient environment such as drinking water, a possible reservoir for transmission. The oxygen and nutrient deprivation treatments showed that the cell morphology conversion and loss of culturability were consistent with other reports (37, 57). To avoid introducing other variables, the temperature was

maintained at 37°C and early logarithmic phase *H. pylori* were used for the microarray reference and stock cells for stress treatments.

In *H. pylori*, nonculturable cells maintain minimal metabolic activity for several months (11, 33, 47). This has led to conflicting conclusions about the nature of this process and whether these cells are truly viable and capable of infection (6, 37). Although there were quantitative differences in the observed amounts of total RNA, these results generally agree with those of others that have shown that coccoid *H. pylori* have drastically decreased RNA content (46, 47). RNA was more abundant in nutrient deprived cells than oxygen stressed cells throughout the study period. Nilsson et al. (47) observed that compared to late stationary phase broth cultured cells, cold-starved cells retained a greater proportion of spiral cells, maintained levels of RNA similar to healthy cells for up to 3 months, had significant amounts extracted after 28 months, and retained higher levels of ATP than aging cells. This highlights the different physiological processes that may govern the survival and persistence of *H. pylori* under varied types of stress.

Outer membrane, antigenic and lipid profiles change upon conversion of the cell to a coccoid form, however, the complete mechanism and genes involved have previously been unidentified (10, 21, 60). Initially, we were interested in the mechanism related to the conversion of the cells to a coccoid morphology, and hypothesized that this switch in morphology was controlled at the transcriptional level. Therefore, the expression of genes related to cell envelope and cell membrane processes were of interest. These genes may also play a role in the adherence of nonculturable *H. pylori* to abiotic and biotic

surfaces (48, 66). Microarray analysis showed that the oxygen stressed cells were up and down expressing more cell envelope related genes than cells that maintained the spiral morphology under nutrient deprivation stress. In fact, there were only 4/21 genes that were up expressed in both oxygen stressed and nutrient deprived cells. Outer membrane proteins appear to play an important role in the stress response of *H. pylori* and seem to be differentially regulated in response to specific signals. One outer membrane protein of interest, HopZ (HP0009), has been shown to play a role in adhesion, and was significantly up expressed in oxygen stressed cells (52). Peck et al. (52) demonstrated that mutants deficient in HopZ had markedly decreased adhesion to human gastric epithelial cells. This protein could be involved in the formation of clusters in coccoid cultures, observed by this group and others, and could be beneficial to a coccoid cell attempting to colonize the gastric epithelia (26). Many of the outer membrane proteins that appear to be highly regulated have not yet been characterized experimentally.

In contrast, nutrient deprived cells had many fewer up expressed cell envelope related genes. One gene of interest encodes cyclopropane fatty acyl phospholipid synthase (jhp0968) and was up expressed at 168 hours of nutrient deprivation. Cyclopropane fatty acyl synthases (CFA synthases) are enzymes that catalyze the addition of a methylene group across *cis* double bonds of monounsaturated fatty acyl chains in lipids (9). Although the physiological roles of CFAs in bacteria are not well understood (24) it has been demonstrated that cyclopropane-containing lipids protect bacteria from stressful conditions such as starvation, acidity and desiccation (9, 20).

More research will be required to fully elucidate the roles of these genes in biological changes that occur under stress.

Change in the antigenic profile of *H. pylori* has been postulated to be an active mechanism facilitated, at least in part, by fucosyltransferase and may be associated with an adaptive process possibly related to Lewis antigen mimicry (3, 4, 10). Fucosyltransferase was up expressed in both stress treatments and has been shown to play a role in lipopolysaccharide phase variation (3). Another enzyme involved in the generation of cell wall polysaccharides, undecaprenyl diphosphatase synthase (HP 1221), was upregulated in oxygen stressed cells only. This enzyme is involved in the generation of the glycosyl lipid precursor involved in the biosynthesis of bacterial cell wall polysaccharide components such as peptidoglycan and lipopolysaccharide (69). Both enzymes may be involved in the difference seen in the antigenic properties of healthy, spiral *H. pylori* and their coccoid counterparts (10, 17, 28, 45) and will require more investigation.

Protein synthesis genes made up a large portion of the up and down regulated substance metabolism genes (Appendix C). The majority of the up expressed genes observed in this category occurred in the oxygen stressed cells. This treatment induced the up expression of many ribosomal proteins, the translation initiation factors *infB* and *infC*, and the threonyl-tRNA synthetase *thrS* (Table 4.1). Of those, only *infB* was up expressed in nutrient deprived cells after 168 hours and *infC* was significantly repressed at this time point in these cells. Nutrient deprived cells showed constitutive or slightly decreased expression of nearly all the protein synthesis genes that were significantly up

or down expressed in oxygen stressed cells. This difference in protein synthesis related gene transcription may be related to the conversion to a coccoid cell morphology.

Bumann et al. (2004) found a lack of stage specific proteins in coccoid *H. pylori* and inferred that differences seen in protein profiles are due strictly to posttranslational modifications (13). While post-translational modification is a likely explanation for differences seen in protein profiles, these data show that *H. pylori* cells that were at the onset of the conversion to the coccoid form were up and down expressing genes related to protein synthesis, while the cells that maintain a majority of spiral cells did not drastically alter expression of these genes.

There is limited evidence regarding the infective dose and factors involved in transmission of *H. pylori* due to the severe outcomes seen in some infections (56). Although direct studies involving environmentally stressed *H. pylori* infection in humans are rare, indirect evidence suggests that less fit *H. pylori* may still be capable of infection. It has been shown that coccoid cultures of *H. pylori* caused infection in mice with a decreased level of host immune response compared to healthy cells (19, 72). This study has provided evidence that stressed *H. pylori* cells, nonculturable *in vitro*, are likely less virulent than unstressed cells. Virulence factors generally showed decreased expression in oxygen stressed cells while nutrient deprived cells decreased transcription at a slower rate. Functional components of the urease enzyme including the gene that codes for the inner membrane protein ureI that is known to allow the import of urea and export of NH<sub>3</sub> across the cell membrane enzyme was down regulated under stress (62). Other pathogenicity genes such as vacuolating cytotoxin, and components of the cytotoxin

associated gene (CAG) pathogenicity island were down regulated as well. The addition of nutrients to stressed cells caused differential expression of two virulence factors suggesting that different adaptations occur depending on the nature of the stress.

Transcription levels of the *ureA* and *vacA* genes down expressed in the microarray analysis were confirmed by qRT-PCR analysis. Oxygen stressed cells showed significantly decreased expression of *vacA* and *ureA* compared to reference cells. The vacuolating cytotoxin protein causes epithelial cell vacuolation *in vitro*, and strains that express it are more commonly associated with diseases (5, 71). The urease enzyme has dual purposes allowing the organism to survive the low pH of the gastric environment as well as providing nitrogen for the synthesis of amino acids (25). The transcriptional activity of *ureA* observed in this study was corroborated by a urease activity assay. In general, urease activity was decreased in oxygen stressed cells compared to the time zero reference cells. Nutrient deprived cells maintained their urease activity to levels similar to the reference cells, consistent with qRT-PCR and microarray results. When cells from these two treatments were presented with an undefined nutrient broth containing lysed human erythrocytes and serum, they exhibited markedly different responses. *H. pylori* stressed by oxygen exposure for 24 and 72 hours increased transcription of *ureA* and *vacA* after incubation in the regrowth medium. After 168 hours of oxygen exposure, the cells were unaffected by regrowth treatment. Interestingly, cells stressed by nutrient deprivation did not increase transcription of virulence factors after incubation in the regrowth medium. Water stressed *H. pylori* undergo nutrient shock when presented with nutrients after starvation (7). Nilsson et al. (47) showed that ATP production increased

dramatically in cold starved cells when incubated with human erythrocytes. While there is some evidence that the VBNC state can be reversed by incubating the cells with human blood derivatives (2, 36), the regrowth of this organism is variable depending on the length of time and type of stress the organism has been subjected to (manuscript in submission, Chapter 5). Nutrient shock is a possible explanation for the decreased transcription of virulence factors seen in nutrient deprived cells after incubation in a nutrient rich medium. Oxygen stressed cells had access to nutrients during their stress treatment and would be less likely to undergo nutrient shock during the regrowth treatment.

In conclusion, we have presented transcriptomic profiles of nonculturable *H. pylori* under two different stress conditions. *H. pylori* cells that convert to a coccoid morphology (oxygen stress) utilize transcriptome level changes to adapt to their environment. We also found that the majority of cells under harsh conditions (i.e. nutrient deprivation at 37°C) did not have a majority converting to a coccoid. These cells appear to be transcriptionally active and slowly modify their transcription to adapt to environmental conditions. Although this report is a simplified model of how *H. pylori* copes with environmental stresses, it does shed light on the different mechanisms *H. pylori* uses to survive changing conditions. Additionally, there is evidence that cells stressed by different factors will respond in varying ways when challenged with nutrients. This observation led to the conclusion that oxygen stressed and nutrient deprived cells are transcriptionally active but react differently to *in vitro* culture condition.

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## REFERENCES

1. Adams, B. L., T. C. Bates, and J. D. Oliver. 2003. Survival of *Helicobacter pylori* in a natural freshwater environment. *Appl. Environ. Microbiol.* 69:7462-7466.
2. Andersen, A. P., D. A. Elliott, M. Lawson, P. Barland, V. B. Hatcher, and E. G. Puszkin. 1997. Growth and morphological transformations of *Helicobacter pylori* in broth media. *J. Clin. Microbiol.* 35:2918-22.
3. Appelmelk, B. J., S. L. Martin, M. A. Monteiro, C. A. Clayton, A. A. McColm, P. Y. Zheng, T. Verboom, J. J. Maaskant, D. H. van den Eijnden, C. H. Hokke, M. B. Perry, C. Vandenbroucke-Grauls, and J. G. Kusters. 1999. Phase variation in *Helicobacter pylori* lipopolysaccharide due to changes in the lengths of poly(C) tracts in alpha 3-fucosyltransferase genes (vol 67, pg 5361, 1999). *Infect. Immun.* 67:6715-6715.

4. Appelmek, B. J., M. A. Monteiro, S. L. Martin, A. P. Moran, and C. Vandembrouck-Grauls. 2000. Why *Helicobacter pylori* has Lewis antigens. *Trends Microbiol.* 8:565-570.
5. Atherton, J. C., T. L. Cover, E. Papini, and J. L. Teleford. 2001. Vacuolating Cytotoxin, p. 97-110. *In* H. L. T. Mobley, G. L. Mendz, and S. L. Hazell (ed.), *Helicobacter pylori* physiology and genetics. American Society for Microbiology, Washington D. C.
6. Azevedo, N. F., C. Almeida, L. Cerqueira, S. Dias, C. W. Keevil, and M. J. Vieira. 2007. Coccoid form of *Helicobacter pylori* as a morphological manifestation of cell adaptation to the environment. *Appl. Environ. Microbiol.* 73:3423-3427.
7. Azevedo, N. F., A. P. Pacheco, C. W. Keevil, and M. J. Vieira. 2004. Nutrient shock and incubation atmosphere influence recovery of culturable *Helicobacter pylori* from water. *Appl. Environ. Microbiol.* 70:490-493.
8. Baker, K. H., and J. P. Hegarty. 2001. Presence of *Helicobacter pylori* in drinking water is associated with clinical infection. *Scan. J. Infect. Dis.* 33:744-746.
9. Basconcillo, L. S., R. Zaheer, T. M. Finan, and B. E. McCarry. 2009. Cyclopropane fatty acyl synthase in *Sinorhizobium meliloti*. *Microbiol.* 155:373-385.
10. Benaissa, M., P. Babin, N. Quellard, L. Pezennec, Y. Cenatiempo, and J. L. Fauchere. 1996. Changes in *Helicobacter pylori* ultrastructure and antigens during conversion from the bacillary to the coccoid form. *Infect. Immun.* 64:2331-2335.
11. Bode, G., F. Mauch, and P. Malfertheiner. 1993. The coccoid forms of *Helicobacter pylori*. Criteria for their viability. *Epidemiol. Infect.* 111:483-490.
12. Brenciaglia, M. I., A. M. Fornara, M. M. Scaltrito, and F. Dubini. 2000. *Helicobacter pylori*: cultivability and antibiotic susceptibility of coccoid forms. *Int. J. Antimicrob. Agents.* 13:237-241.
13. Bumann, D., H. Habibi, B. Kan, M. Schmid, C. Goosmann, V. Brinkmann, T. F. Meyer, and P. R. Jungblut. 2004. Lack of stage-specific proteins in coccoid *Helicobacter pylori* cells. *Infect. Immun.* 72:6738-6742.
14. Bunn, J. E. G., W. G. MacKay, J. E. Thomas, D. C. Reid, and L. T. Weaver. 2002. Detection of *Helicobacter pylori* DNA in drinking water biofilms: implications for transmission in early life. *Lett. Appl. Microbiol.* 34:450-454.

15. Byrd, J. 2000. Morphological changes leading to the nonculturable state, p. 7-18. In R. R. Colwell and J. D. Grimes (ed.), *Nonculturable microorganisms in the environment*. American Society for Microbiology, Washington, D. C.
16. Can, F., C. Karahan, I. Dolapci, M. Demirbilek, A. Tekeli, and H. Arslan. 2008. Urease activity and urea gene sequencing of coccoid forms of *H. pylori* induced by different factors. *Curr. Microbiol.* 56:150-155.
17. Cao, P., M. S. McClain, M. H. Forsyth, and T. L. Cover. 1998. Extracellular release of antigenic proteins by *Helicobacter pylori*. *Infect. Immun.* 66:2984-2986.
18. Catrenich, C. E., and K. M. Makin. 1991. Characterization of the morphological conversion of *Helicobacter pylori* from bacillary to coccoid forms. *Scan. J. Gastroenterol.* 26:58-64.
19. Cellini, L., N. Allocati, D. Angelucci, T. Iezzi, E. Dicampoli, L. Marzio, and B. Dainelli. 1994. Coccoid *Helicobacter pylori* not culturable in vitro reverts in mice. *Microbiol. Immunol.* 38:843-850.
20. Chang, Y.-y., and J. E. Cronan. 2002. Membrane cyclopropane fatty acid content is a major factor in acid resistance of *Escherichia coli*. *Mol. Microbiol.* 33:249-259.
21. Citterio, B., A. Casaroli, L. Pierfelici, M. Battistelli, E. Falcieri, and W. Baffone. 2004. Morphological changes and outer membrane protein patterns in *Helicobacter pylori* during conversion from bacillary to coccoid form. *Microbiologica* 27:353-360.
22. Cole, S. P., D. Cirillo, M. F. Kagnoff, D. G. Guiney, and L. Eckmann. 1997. Coccoid and spiral *Helicobacter pylori* differ in their abilities to adhere to gastric epithelial cells and induce interleukin-8 secretion. *Infect. Immun.* 65:843-846.
23. Conway, T., and G. K. Schoolnik. 2003. Microarray expression profiling: capturing a genome-wide portrait of the transcriptome. *Mol. Microbiol.* 47:879-889.
24. Cronan Jr., J. E. Phospholipid modifications in bacteria. *Curr. Opin. Microbiol.* 5:202-205.
25. De Reuse, H., and S. Skouloubris. 2001. Nitrogen metabolism, p. 125-133. In H. L. T. Mobley, G. L. Mendz, and S. L. Hazell (ed.), *Helicobacter pylori* physiology and genetics. American Society for Microbiology, Washington D.C.

26. Donelli, G., P. Matarrese, C. Fiorentini, B. Dainelli, T. Taraborelli, E. Di Campli, S. Di Bartolomeo, and L. Cellini. 1998. The effect of oxygen on the growth and cell morphology of *Helicobacter pylori*. FEMS Microbiol. Lett. 168:9-15.
27. Dye, B. A., D. Kruszon-Moran, and G. McQuillan. 2002. The relationship between periodontal disease attributes and *Helicobacter pylori* infection among adults in the United States. Amer. J. Pub. Health. 92:1809-1815.
28. Enroth, H., and L. Engstrand. 1995. Immunomagnetic separation and PCR for the detection of *Helicobacter pylori* in water and stool specimens. J. Clin. Microbiol. 33:2162-2165.
29. Enroth, H., K. Wreiber, R. Rigo, D. Risberg, A. Uribe, and L. Engstrand. 1999. In vitro aging of *Helicobacter pylori*: Changes in morphology, intracellular composition and surface properties. Helicobacter 4:7-16.
30. Giao, M. S., N. F. Azevedo, S. A. Wilks, M. J. Vieira, and C. W. Keevil. 2008. Persistence of *Helicobacter pylori* in heterotrophic drinking-water biofilms. Appl. Environ. Microbiol. 74:5898-5904.
31. Goodman, K. J., and P. Correa. 1995. The transmission of *Helicobacter pylori* - a critical review of the evidence. Int. J. Epidemiol. 24:875-887.
32. Graham, D. Y., A. R. Opekun, M. S. Osato, H. M. T. El-Zimaity, C. K. Lee, Y. Yamaoka, W. A. Qureshi, M. Cadoz, and T. P. Monath. 2004. Challenge model for *Helicobacter pylori* infection in human volunteers. Gut 53:1235-1243.
33. Gribbon, L. T., and M. R. Barer. 1995. Oxidative metabolism in nonculturable *Helicobacter pylori* and *Vibrio vulnificus* cells studied by substrate enhanced tetrazolium reduction and digital image processing. Appl. Environ. Microbiol. 61:3379-3384.
34. Han, Y. H., W. Z. Liu, Y. Z. Shi, L. Q. Lu, S. D. Xiao, and Q. H. Zhang. 2009. Gene expression profile of *Helicobacter pylori* in response to growth temperature variation. J. Microbiol. 47:455-465.
35. Knight, I. T. 2000. Molecular genetic methods for detection and identification of viable but nonculturable microorganisms, p. 77-85. In R. R. Colwell and D. J. Grimes (ed.), Nonculturable microorganisms in the environment. American Society for Microbiology, Washington D. C.
36. Kurokawa, M., M. Nukina, and H. Nakanishi. 1999. Resuscitation from the viable but non culturable state of *Helicobacter pylori*. Kan. Zasshi 73:15-19.

37. Kusters, J. G., M. M. Gerrits, J. A. G. Van Strijp, and C. Vandenbroucke Grauls. 1997. Coccoid forms of *Helicobacter pylori* are the morphologic manifestation of cell death. *Infect. Immun.* 65:3672-3679.
38. Li, C., P. R. Musich, T. Ha, D. A. Ferguson, N. R. Patel, D. S. Chi, and E. Thomas. 1995. High prevalence of *Helicobacter pylori* in saliva demonstrated by a novel PCR assay. *J. Clin. Pathol.* 48:662-666.
39. Ma, J. L., W. C. You, M. H. Gail, L. Zhang, W. J. Blot, Y. S. Chang, J. Jiang, W. D. Liu, Y. R. Hu, L. M. Brown, G. W. Xu, and J. F. Fraumeni. 1998. *Helicobacter pylori* infection and mode of transmission in a population at high risk of stomach cancer. *Int. J. Epidemiol.* 27:570-573.
40. Mackay, W. G., L. T. Gribbon, M. R. Barer, and D. C. Reid. 1998. Biofilms in drinking water systems - A possible reservoir for *Helicobacter pylori*. *Water Sci. Technol.* 38:181-185.
41. Marshall, B. J. 1995. *Helicobacter pylori* - the etiologic agent for peptic ulcer. *J. Amer. Med. Assoc.* 274:1064-1066.
42. Merrell, D. S., M. L. Goodrich, G. Otto, L. S. Tompkins, and S. Falkow. 2003. pH-regulated gene expression of the gastric pathogen *Helicobacter pylori*. *Infect. Immun.* 71:3529-3539.
43. Merrell, D. S., L. J. Thompson, C. C. Kim, H. Mitchell, L. S. Tompkins, A. Lee, and S. Falkow. 2003. Growth phase-dependent response of *Helicobacter pylori* to iron starvation. *Infect. Immun.* 71:6510-6525.
44. Monstein, H. J., and J. Jonasson. 2001. Differential virulence-gene mRNA expression in coccoid forms of *Helicobacter pylori*. *Biochem. Biophys. Res. Comm.* 285:530-536.
45. Moran, A. P. 2008. Relevance of fucosylation and Lewis antigen expression in the bacterial gastroduodenal pathogen *Helicobacter pylori*. *Carbohydr. Res.* 343:1952-1965.
46. Narikawa, S., S. Kawai, H. Aoshima, O. Kawamata, R. Kawaguchi, K. Hikiji, M. Kato, S. Iino, and Y. Mizushima. 1997. Comparison of the nucleic acids of helical and coccoid forms of *Helicobacter pylori*. *Clin. Diagn. Lab. Immunol.* 4:285-290.
47. Nilsson, H. O., J. Blom, W. Abu Al-Soud, A. Ljungh, L. P. Andersen, and T. Wadstrom. 2002. Effect of cold starvation, acid stress, and nutrients on metabolic activity of *Helicobacter pylori*. *Appl. Environ. Microbiol.* 68:11-19.

48. Oliver, J. D. 2010. Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol. Rev.* 34:415-425.
49. Oliver, J. D. 2000. The public health significance of viable but not culturable bacteria, p. 277-300. *In* R. R. Colwell and D. J. Grimes (ed.), *Nonculturable microorganisms in the environment*. American Society for Microbiology, Washington, D. C.
50. Parsonnet, J., H. Shmuelly, and T. Haggerty. 1999. Fecal and oral shedding of *Helicobacter pylori* from healthy infected adults. *J. Amer. Med. Assoc.* 282:2240-2245.
51. Peck, B., M. Ortkamp, K. D. Diehl, E. Hundt, and B. Knapp. 1999. Conservation, localization and expression of HopZ, a protein involved in adhesion of *Helicobacter pylori*. *Nucleic Acids Res.* 27:3325-3333.
53. Richards, C. L., B. J. Buchholz, T. E. Ford, S. C. Broadaway, B. H. Pyle, and A. K. Camper. 2010. Optimizing the Growth of Stressed *Helicobacter pylori*. Manuscript in Submission, *Journal of Microbiological Methods*.
54. Rudi, J., C. Kolb, M. Maiwald, D. Kuck, A. Sieg, P. R. Galle, and W. Stremmel. 1998. Diversity of *Helicobacter pylori* vacA and cagA genes and relationship to VacA and CagA protein expression, cytotoxin production, and associated diseases. *J. Clin. Microbiol.* 36:944-948.
55. Saito, N., K. Konishi, F. Sato, M. Kato, H. Takeda, T. Sugiyama, and M. Asaka. 2003. Plural transformation-processes from spiral to coccoid *Helicobacter pylori* and its viability. *J. Infect.* 46:49-55.
56. Salama, N. R., G. Otto, L. Tompkins, and S. Falkow. 2001. Vacuolating cytotoxin of *Helicobacter pylori* plays a role during colonization in a mouse model of infection. *Infect. Immun.* 69:730-736.
57. Shahamat, M., U. Mai, C. PaszkoKolva, M. Kessel, and R. R. Colwell. 1993. Use of autoradiography to assess viability of *Helicobacter pylori* in water. *Appl. Environ. Microbiol.* 59:1231-1235.
58. Sharma, C. M., S. Hoffmann, F. Darfeuille, J. Reignier, S. Findeiss, A. Sittka, S. Chabas, K. Reiche, J. Hackermuller, R. Reinhardt, P. F. Stadler, and J. Vogel. 2010. The primary transcriptome of the major human pathogen *Helicobacter pylori*. *Nature* 464:250-255.

59. She, F. F., J. Y. Lin, J. Y. Liu, C. Huang, and D. H. Su. 2003. Virulence of water-induced coccoid *Helicobacter pylori* and its experimental infection in mice. *World J. Gastroenterol.* 9:516-520.
60. Shimomura, H., S. Hayashi, K. Yokota, K. Oguma, and Y. Hirai. 2004. Alteration in the composition of cholesteryl glucosides and other lipids in *Helicobacter pylori* undergoing morphological change from spiral to coccoid form. *FEMS Microbiol. Lett.* 237:407-413.
61. Sisto, F., M. I. Brenciaglia, M. M. Scaltrito, and F. Dubini. 2000. *Helicobacter pylori*: ureA, cagA and vacA expression during conversion to the coccoid form. *Int. J. Antimicrob. Agents.* 15:277-282.
62. Skouloubris, S., J. M. Thiberge, A. Labigne, and H. De Reuse. 1998. The *Helicobacter pylori* UreI protein is not involved in urease activity but is essential for bacterial survival in vivo. *Infect. Immun.* 66:4517-21.
63. Solnick, J. V., L. M. Hansen, D. R. Canfield, and J. Parsonnet. 2001. Determination of the infectious dose of *Helicobacter pylori* during primary and secondary infection in rhesus monkeys (*Macaca mulatta*). *Infect. Immun.* 69:6887-6892.
64. Sorberg, M., M. Nilsson, H. Hanberger, and L. E. Nilsson. 1996. Morphologic conversion of *Helicobacter pylori* from bacillary to coccoid form. *Eur. J. Clin. Microbiol. Infect. Dis.* 15:216-219.
65. Souto, R., and A. P. V. Colombo. 2008. Detection of *Helicobacter pylori* by polymerase chain reaction in the subgingival biofilm and saliva of non-dyspeptic periodontal patients. *J. Periodontol.* 79:97-103.
66. Testerman, T. L., D. J. McGee, and H. L. T. Mobley. 2001. Adherence and colonization, p. 381-417. *In* H. L. T. Mobley, G. L. Mendz, and S. L. Hazell (ed.), *Helicobacter pylori* physiology and genetics. American Society for Microbiology, Washington D.C.
67. Thomas, J. E., G. R. Gibson, M. K. Darboe, A. Dale, and L. T. Weaver. 1992. Isolation of *Helicobacter pylori* from human feces. *Lancet* 340:1194-1195.
68. Thompson, L. J., D. S. Merrell, B. A. Neilan, H. Mitchell, A. Lee, and S. Falkow. 2003. Gene expression profiling of *Helicobacter pylori* reveals a growth-phase-dependent switch in virulence gene expression. *Infect. Immun.* 71:2643-2655.
69. Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J.

- Quackenbush, L. X. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzegerald, N. Lee, M. D. Adams, E. K. Hickey, D. E. Berg, J. D. Gocayne, T. R. Utterback, J. D. Peterson, J. M. Kelley, M. D. Cotton, J. M. Weldman, C. Fujii, C. Bowman, L. Wathey, E. Wallin, W. S. Hayes, J. M. Weidman, M. Borodovsky, P. D. Karp, H. O. Smith, C. M. Fraser, and J. C. Venter. 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* 388:539-547.
70. Umeda, M., N. Murata-Kamiya, Y. Saito, Y. Ohba, M. Takahashi, and M. Hatakeyama. 2009. *Helicobacter pylori* CagA causes mitotic impairment and induces chromosomal instability. *J. Biol. Chem.* 284:22166-22172.
71. van Amsterdam, K., A. H. M. van Vliet, J. G. Kusters, M. Feller, J. Dankert, and A. van der Ende. 2003. Induced *Helicobacter pylori* vacuolating cytotoxin VacA expression after initial colonisation of human gastric epithelial cells. *FEMS Immunol. Med. Microbiol.* 39:251-256.
72. Wang, X., E. Sturegard, R. Rupar, H. O. Nilsson, P. A. Aleljung, B. Carlen, R. Willen, and T. Wadstrom. 1997. Infection of BALB/c mice by spiral and coccoid forms of *Helicobacter pylori*. *J. Med. Microbiol.* 46:657-663.
73. Watson, C. L., R. J. Owen, B. Said, S. Lai, J. V. Lee, S. Surman-Lee, and G. Nichols. 2004. Detection of *Helicobacter pylori* by PCR but not culture in water and biofilm samples from drinking water distribution systems in England. *J. Appl. Microbiol.* 97:690-698.
74. Worku, M. L., R. L. Sidebotham, M. M. Walker, T. Keshavarz, and Q. N. Karim. 1999. The relationship between *Helicobacter pylori* motility, morphology and phase of growth: implications for gastric colonization and pathology. *Microbiol.-Sgm* 145:2803-2811.
75. Yamaguchi, H., T. Osaki, H. Taguchi, T. Hanawa, T. Yamamoto, and S. Kamiya. 1998. Relationship between expression of HSP60, urease activity, production of vacuolating toxin, and adherence activity of *Helicobacter pylori*. *J. Gastroenterol.* 33:6-9.

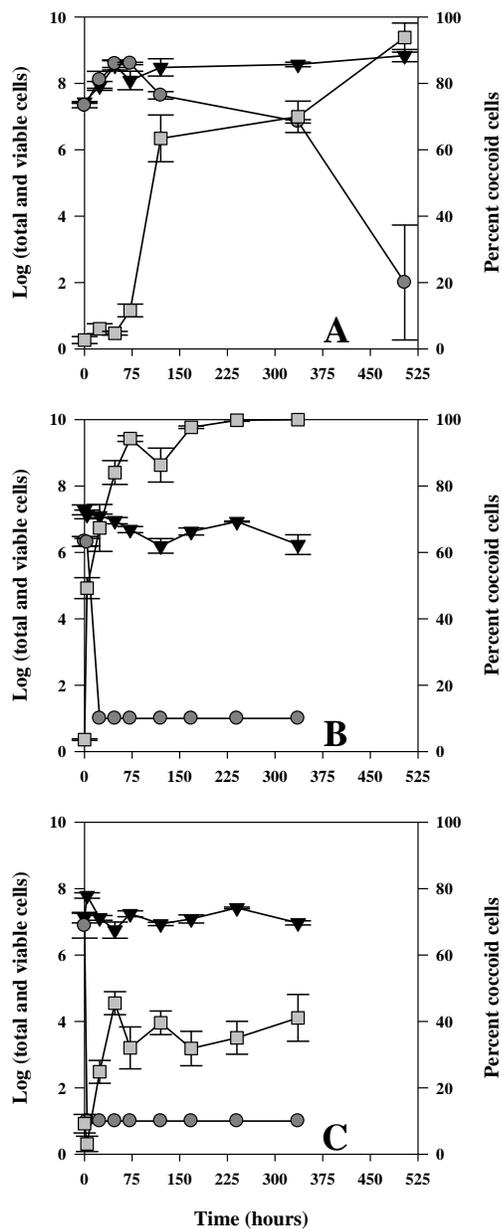


Figure 4.1. Relationships between the morphologic characteristic of the cell and viability of *H. pylori* under the following conditions: aging (A), oxygen stress in the presence of nutrients (B), nutrient deprivation in sterile MQ water (C). The black triangles represent the total number of cells counted microscopically (represent cells/ml), the dark gray circles represent the viable number of cells counted on plate counts (CFU/ml), and the light gray squares represent the percentage of coccoid cells. Data are means ( $\pm$  standard deviation) with three independent biological replicates for each time point. Viable cell counts had no variation after 24 hours of stress, no colony forming units were detected.

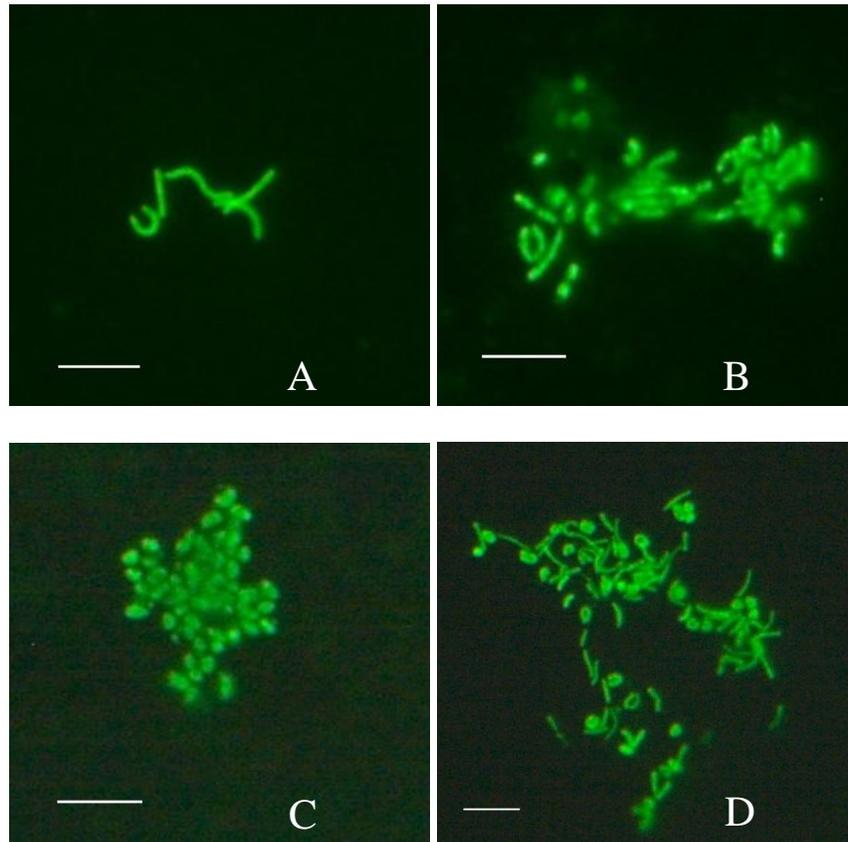


Figure 4.2. Fluorescence micrographs showing *H. pylori* in various stages of transformation to coccoid cell morphology. (A) *H. pylori* cells in early log phase, 24 hours of growth in a microaerophilic atmosphere. Bar, 2  $\mu\text{m}$ . (B) *H. pylori* cells in early stationary phase, 168 hours of growth in a microaerophilic atmosphere. (C) Typical coccoid cell cluster, 72 hours of incubation in atmospheric oxygen. (D) Spiral cells seen after 120 hours of nutrient deprivation.

Table 4.1. Oxygen stress and nutrient deprivation stress regulated genes of *H. pylori*.

Gene ID	Gene function and classification	Oxygen stress (24h)	Nutrient stress (24h)	Nutrient stress (72h)	Nutrient stress (168h)
Amino acid biosynthesis					
HP0652	phosphoserine phosphatase, <i>serB</i>	3.4	-0.4	-0.7	-0.9
jhp1150	aspartate kinase	1.8	0.2	0.0	1.3
HP1210	serine acetyltransferase, <i>cysE</i>	1.5	0.6	0.7	1.7
HP1050	homoserine kinase, <i>thrB</i>	1.4	0.2	0.5	0.7
HP0330	ketol-acid reductoisomerase, <i>ilvC</i>	1.1	0.4	0.4	0.8
HP1468	branched-chain-amino-acid aminotransferase, <i>ilvE</i>	1.1	0.0	0.0	1.3
HP0672	solute-binding signature and mitochondrial signature protein, <i>aspB</i>	1.0	-0.2	-0.3	-0.3
HP0290	diaminopimelate decarboxylase, <i>lysA</i>	0.7	0.4	0.6	1.4
jhp1294	Prephenate dehydrogenase	0.5	0.4	-0.1	2.0
Biosynthesis of cofactors, prosthetic groups, and carriers					
HP1221	undecaprenyl diphosphate synthase	2.8	0.0	0.1	-1.0
HP0239	glutamyl-tRNA reductase, <i>hemA</i>	1.3	0.5	-1.1	-0.2
HP0798	molybdenum cofactor biosynthesis protein C, <i>moaC</i>	1.3	0.0	0.8	1.0
HP0843	thiamin phosphate pyrophosphorylase/hydroxyethyl thiazole kinase, <i>thiB</i>	1.0	0.3	0.0	2.1
HP0804	GTP cyclohydrolase II/3,4-dihydroxy-2-butanone 4-phosphate synthase, <i>ribAB</i>	1.0	-0.1	0.5	1.4
HP1140	biotin operon repressor/biotin acetyl coenzyme A carboxylase synthetase, <i>birA</i>	0.9	0.1	0.8	1.5
Cell envelope					
HP0725	outer membrane protein, <i>omp17</i>	3.8	0.2	0.0	0.6
jhp0424	outer membrane protein	3.4	0.6	0.2	0.3
jhp1002	fucosyltransferase	3.1	0.7	0.6	2.9

**Table 4.1 continued**

HP0009	outer membrane protein, <i>omp1</i>	2.7	0.9	0.2	0.7
HP1052	UDP-3-0-acyl N-acetylglucosamine deacetylase, <i>envA</i>	2.7	0.3	-0.1	-0.4
HP0472	outer membrane protein, <i>omp11</i>	2.6	0.2	-0.4	0.6
HP0655	protective surface antigen D15	2.2	0.4	-0.2	-0.4
HP0122	lipoprotein, putative	2.1	0.3	0.1	-0.6
HP0805	lipooligosaccharide 5G8 epitope biosynthesis-associated protein, <i>lex2B</i>	1.9	-0.2	-0.2	-0.8
HP0638	outer membrane protein, <i>omp13</i>	1.8	0.2	0.1	0.4
HP0087	lipoprotein, putative	1.6	-0.1	0.0	-0.3
jhp0593	UDP-N-acetylglucosamine 1-carboxyvinyltransferase, <i>murA</i>	1.3	0.2	0.9	2.6
HP0018	lipoprotein, putative	1.3	0.8	0.1	1.1
HP0289	toxin-like outer membrane protein	1.2	0.3	0.2	1.0
HP1501	outer membrane protein, <i>omp32</i>	1.0	0.2	0.6	0.4
HP1373	rod shape-determining protein, <i>mreB</i>	0.7	0.3	0.5	1.1
jhp0007	outer membrane protein	0.5	-0.2	0.7	2.1
HP1157	outer membrane protein, <i>omp26</i>	-0.2	0.8	0.4	2.2
HP0648	UDP-N-acetylglucosamine enolpyruvyl transferase, <i>murZ</i>	-0.3	0.3	1.2	0.9
HP0859	ADP-L-glycero-D-mannoheptose-6-epimerase, <i>rfaD</i>	-0.6	0.3	1.4	0.0
HP0057	lipoprotein, putative	-2.0	0.2	1.3	0.0
Cellular processes-adaptation to atypical environments					
HP1228	invasion protein, <i>invA</i>	3.3	0.4	0.3	0.7
Cellular processes-cell division					
HP1556	cell division protein, <i>ftsI</i>	1.4	0.2	-0.5	-0.7
jhp1161	maf protein, <i>maf</i>	0.2	-0.3	-0.1	1.1
Cellular processes-chemotaxis and motility					
HP0584	flagellar switch protein, <i>fliN</i>	2.2	0.3	1.0	0.3

**Table 4.1 continued**

HP1558	flagellar basal-body rod protein (proximal rod protein), <i>flgC</i>	2.0	0.3	0.0	-0.2
HP0246	flagellar basal-body P-ring protein, <i>flgI</i>	1.9	0.5	-0.2	0.3
HP0325	flagellar basal-body L-ring protein, <i>flgH</i>	1.3	0.0	-2.1	-0.5
Cellular processes-detoxification					
jhp0252	chlorohydrolase	-0.6	0.0	0.4	2.9
HP1563	alkyl hydroperoxide reductase, <i>tsaA</i>	-1.9	0.2	1.0	0.4
Cellular processes-pathogenicity					
HP0535	cag pathogenicity island protein, <i>cagI4</i>	1.9	0.0	-0.5	-1.3
Cellular processes-toxin production and resistance					
HP1431	16S rRNA (adenosine-N6,N6-)-dimethyltransferase, <i>ksgA</i>	1.6	0.3	0.0	0.1
Central intermediary metabolism					
jhp0976	probable aminotransferase	1.2	-0.8	-1.2	0.0
DNA metabolism					
HP1208	ulcer associated adenine specific DNA methyltransferase	3.8	-0.5	0.3	1.8
HP0092	type II restriction enzyme M protein, <i>hsdM</i>	3.4	0.2	0.2	1.5
HP0012	DNA primase, <i>dnaG</i>	2.0	0.4	0.0	0.7
HP0091	type II restriction enzyme R protein, <i>hsdR</i>	1.6	0.2	0.2	0.9
HP1209	ulcer-associated gene restriction endonuclease, <i>iceA</i>	1.3	-0.2	0.1	1.6
jhp0435	cytosine-specific DNA methyltransferase	1.2	0.0	-0.1	0.8
HP0883	Holliday junction DNA helicase, <i>ruvA</i>	1.0	0.1	0.8	1.3
HP1529	chromosomal replication initiator protein, <i>dnaA</i>	0.1	1.0	0.1	0.8

**Table 4.1 continued**

## Energy metabolism

HP1261	NADH-ubiquinone oxidoreductase, NQO6 subunit	1.8	0.4	0.3	0.5
HP1212	ATP synthase F0, subunit c, <i>atpE</i>	1.5	0.4	0.6	1.0
HP1227	cytochrome c553	1.4	0.2	0.7	-0.4
HP1386	D-ribulose-5-phosphate 3 epimerase, <i>rpe</i>	1.4	0.3	0.1	0.1
jhp0661	l-asparaginase	1.2	0.3	0.6	1.6
jhp0908	phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent, <i>gpmA</i>	0.2	0.3	0.6	2.0
jhp1281	Carbohydrate kinase family	0.1	0.8	0.9	4.6
HP0277	ferredoxin	-0.4	0.0	1.1	-0.2
jhp0126	iron-sulfur cluster binding protein	-0.6	-0.4	0.0	1.6
HP0378	cytochrome c biogenesis protein, <i>ycf5</i>	-1.7	0.4	1.5	0.5

## Fatty acid and phospholipid metabolism

HP0808	4'-phosphopantetheinyl transferase, <i>acpS</i>	1.7	-0.7	-0.6	-0.6
jhp0968	cyclopropane-fatty-acyl-phospholipid synthase	-0.1	-0.2	0.0	1.4

## Protein fate

HP1549	protein-export membrane protein, <i>secF</i>	2.1	0.1	0.4	0.1
HP0033	ATP-dependent Clp protease, <i>clpA</i>	1.9	0.2	-0.5	-0.7
HP1332	co-chaperone and heat shock protein, <i>dnaJ</i>	1.8	-0.2	0.4	0.1
HP1550	protein-export membrane protein, <i>secD</i>	1.3	0.4	0.4	0.4
HP0657	processing protease, <i>ymxG</i>	1.2	0.2	0.2	0.2

## Protein synthesis

HP0123	threonyl-tRNA synthetase, <i>thrS</i>	2.6	0.3	0.3	0.2
HP1201	ribosomal protein L1, <i>rpL1</i>	1.8	0.2	0.2	-0.1
jhp1236	ribosomal protein L2, <i>rplB</i>	1.7	-0.3	0.3	-0.2
HP1319	ribosomal protein L3, <i>rpl3</i>	1.7	0.2	0.4	0.6

**Table 4.1 continued**

HP1318	ribosomal protein L4, <i>rpl4</i>	1.6	-0.4	-0.8	-0.3
HP1048	translation initiation factor IF-2, <i>infB</i>	1.6	0.5	0.6	1.5
HP0084	ribosomal protein L13, <i>rpl13</i>	1.3	-0.1	-0.1	-0.4
HP1315	ribosomal protein S19, <i>rps19</i>	1.3	-0.2	-0.2	-0.7
HP1295	ribosomal protein S11, <i>rps11</i>	1.2	0.1	0.2	0.0
HP0124	translation initiation factor IF-3, <i>infC</i>	1.2	0.2	-1.0	-2.7
HP1310	ribosomal protein S17, <i>rps17</i>	1.2	-0.3	-0.5	-1.2
HP0083	ribosomal protein S9, <i>rps9</i>	1.2	0.1	0.6	0.1
HP1316	ribosomal protein L2, <i>rpl2</i>	1.1	-0.1	0.3	0.1
HP1311	ribosomal protein L29, <i>rpl29</i>	1.1	-0.1	-0.1	-0.6
HP1147	ribosomal protein L19, <i>rpl19</i>	1.0	-0.1	0.4	-0.9
HP1294	ribosomal protein S4, <i>rps4</i>	1.0	0.2	0.5	0.2
HP1497	peptidyl-tRNA hydrolase, <i>pth</i>	0.7	-0.5	0.1	1.7
HP0281	tRNA-guanine transglycosylase, <i>tgt</i>	0.6	0.3	0.1	4.1
HP0402	phenylalanyl-tRNA synthetase, beta subunit, <i>pheT</i>	0.2	0.4	0.4	1.3
jhp0170	lysyl-tRNA synthetase, <i>lysS</i>	-0.1	0.7	0.8	1.4
Mobile and extrachromosomal element functions					
HP1000	PARA protein	2.1	0.3	0.2	-0.5
Purines, pyrimidines, nucleosides, and nucleotides					
HP1218	glycinamide ribonucleotide synthetase, <i>purD</i>	1.9	0.4	0.9	0.6
HP0409	GMP synthase, <i>guaA</i>	1.8	-0.3	0.2	-0.6
HP0919	carbamoyl-phosphate synthase (glutamine-hydrolysing), <i>pyrAb</i>	0.0	-0.3	1.2	-0.3
HP0198	nucleoside diphosphate kinase, <i>ndk</i>	0.0	0.6	1.1	1.1
Regulatory functions					
HP0088	RNA polymerase sigma-70 factor, <i>rpoD</i>	1.2	0.0	-0.3	-0.4
jhp1180	transcriptional regulator, Sir2 family family	0.4	0.3	1.1	1.5
jhp1282	histidine kinase sensor protein	0.4	0.1	0.5	2.4

**Table 4.1 continued**

Transcription					
HP1514	transcription termination factor, <i>nusA</i>	2.0	0.6	0.6	0.7
HP1448	ribonuclease P, protein component, <i>rnpA</i>	1.7	-0.1	-0.5	-0.4
Transport and binding proteins					
HP1220	ABC transporter, ATP-binding protein, <i>yhcG</i>	3.4	0.2	0.2	-0.3
HP0471	glutathione-regulated potassium-efflux system protein, <i>kefB</i>	3.0	-0.4	-0.5	-0.1
HP1491	phosphate permease	2.9	0.5	-0.4	0.5
HP1503	cation-transporting ATPase, P-type, <i>copA</i>	2.3	0.1	0.2	0.4
HP0251	oligopeptide ABC transporter, permease protein, <i>oppC</i>	1.9	0.2	0.2	0.2
HP1180	pyrimidine nucleoside transport protein, <i>nupC</i>	1.5	0.3	0.3	0.3
HP0300	dipeptide ABC transporter, permease protein, <i>dppC</i>	1.4	0.5	0.3	0.0
HP0301	dipeptide ABC transporter, ATP-binding protein, <i>dppD</i>	1.4	0.2	-0.2	-0.2
HP0299	dipeptide ABC transporter, permease protein, <i>dppB</i>	1.2	0.5	-0.5	-0.6
HP1577	ABC transporter, permease protein, <i>yae</i>	0.7	0.3	0.4	1.3
Unknown function					
HP0405	nifS-like protein	2.3	0.5	-0.6	0.0
jhp0376	Protein of unknown function (DUF448) superfamily	1.9	0.0	0.5	1.1
HP0431	protein phosphatase 2C homolog, <i>ptc1</i>	1.8	0.3	-0.4	0.4
jhp1123	50s ribosomal protein L10	1.8	-0.4	-0.5	-1.2
jhp0432	GTP-binding protein, <i>typA</i>	0.6	0.1	0.3	2.4
jhp0395	Domain of unknown function (DUF386) superfamily	0.4	0.1	0.7	1.9
HP0207	ATP-binding protein, <i>mpr</i>	0.1	0.3	0.3	1.1

Table 4.2. Compares the Log<sub>2</sub> fold changes in gene expression associated with microarrays and qRT-PCR.

Gene symbol	Gene name		Microarray log <sub>2</sub> fold change		qRT-PCR log <sub>2</sub> fold change	
HP0073	<i>ureA</i>	Oxygen stress (24h)	-2.15	± 0.44	-1.15	± 0.50
		Nutrient deprivation (24h)	0.00	± 0.07	-0.35	± 0.20
		Nutrient deprivation (72h)	-0.27	± 0.14	-0.39	± 0.10
		Nutrient deprivation (168h)	-0.61	± 0.14	-0.81	± 0.31
HP0887	<i>vacA</i>	Oxygen stress (24h)	-1.67	± 0.35	-0.56	± 0.20
		Nutrient deprivation (24h)	-0.19	± 0.13	-0.36	± 0.20
		Nutrient deprivation (72h)	-0.54	± 0.40	-0.37	± 0.12
		Nutrient deprivation (168h)	-0.43	± 0.04	-0.83	± 0.36
HP0875	<i>catA</i>	Oxygen stress (24h)	-2.08	± 0.51	-0.99	± 0.19
		Nutrient deprivation (24h)	-0.60	± 0.15	-0.15	± 0.08
		Nutrient deprivation (72h)	-0.60	± 0.07	-0.19	± 0.03
		Nutrient deprivation (168h)	-0.75	± 0.43	-0.95	± 0.06
HP1444	<i>smp</i>	Oxygen stress (24h)	0.64	± 0.07	-0.32	± 0.03
		Nutrient deprivation (24h)	0.19	± 0.12	-0.08	± 0.12
		Nutrient deprivation (72h)	0.34	± 0.27	-0.19	± 0.03
		Nutrient deprivation (168h)	-0.31	± 0.46	-0.50	± 0.02

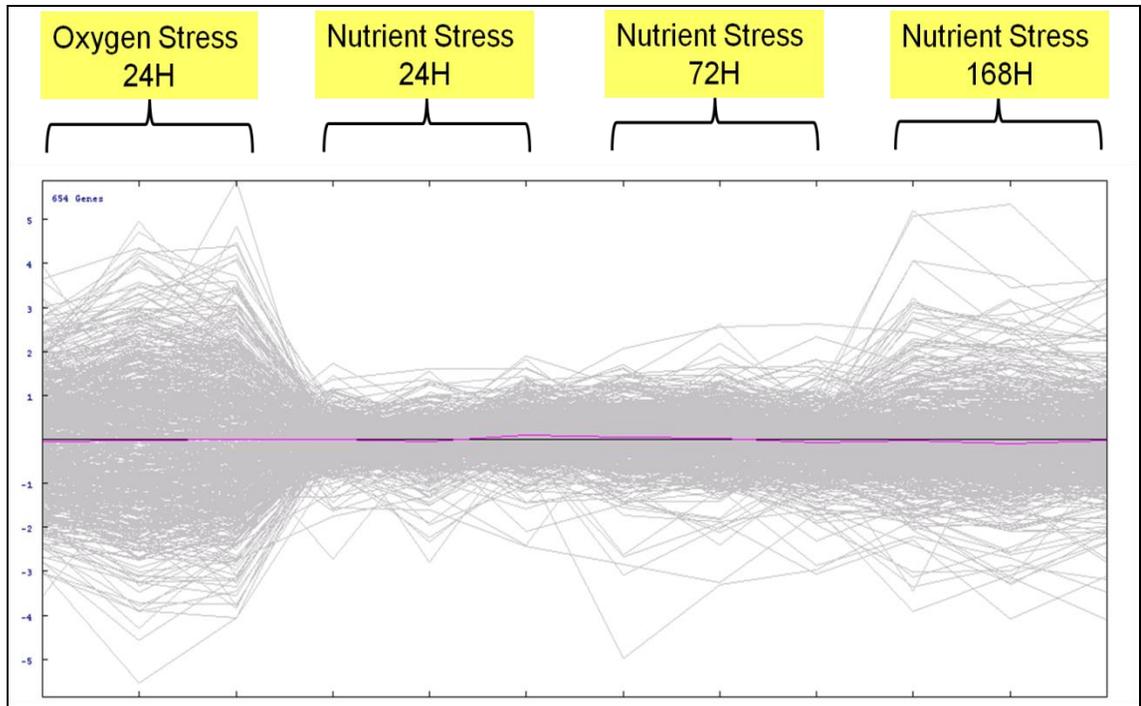


Figure 4.3. Expression analysis of statistically significant, differentially expressed genes in oxygen stressed and nutrient deprived *H. pylori*. Each treatment group, indicated at top, contains expression data from 3 biological replicates. The left axis indicates the Log<sub>2</sub> gene expression ratio of test to reference.

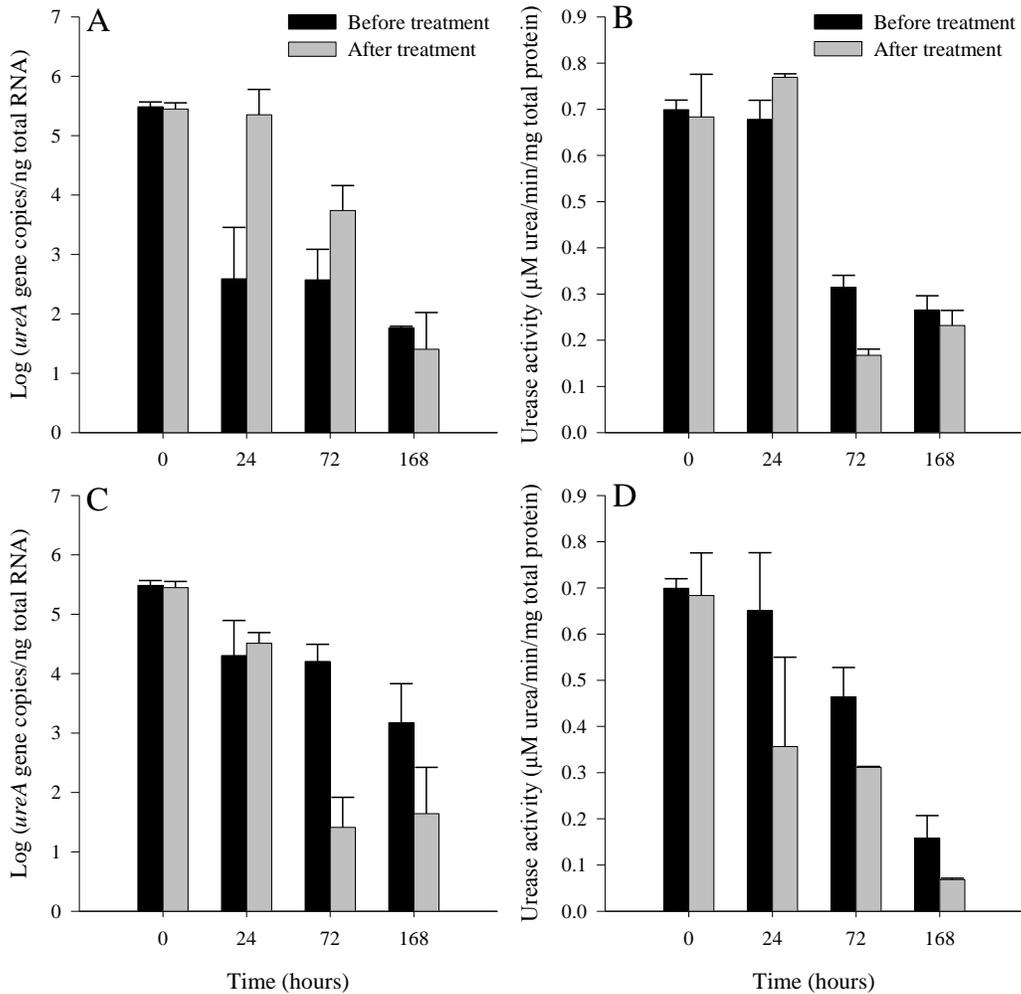


Figure 4.4. Transcription of *ureA* gene and urease activity before and after treatment with nutrients and human erythrocytes. The X-axis indicates the hours of stress exposure. Graphs represent mean measurements of three biological replicates and error bars represent  $\pm 1$  standard deviation. A) Transcription of *ureA* in oxygen stressed cells, B) urease activity of oxygen stressed cells, C) transcription of *ureA* in nutrient deprived cells, D) urease activity in nutrient deprived cells.

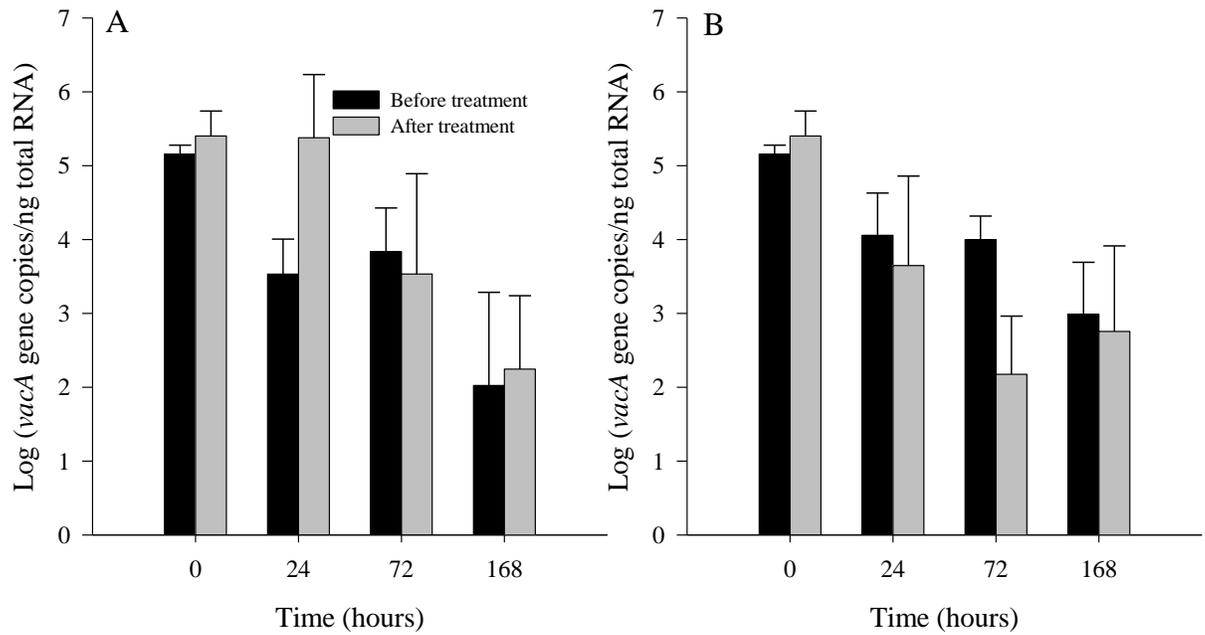


Figure 4.5. Transcription of the gene *vacA* in A) oxygen stressed and B) nutrient deprived *H. pylori* before and after treatment with nutrients including human erythrocytes. The X-axis indicates the hours of stress exposure. Graphs represent mean measurements of three biological replicates and error bars represent  $\pm 1$  standard deviation.

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CHAPTER 5

**Optimizing the Growth of Stressed *Helicobacter pylori***

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**Abstract**

*Helicobacter pylori* is a Gram-negative bacterium that colonizes the human stomach and is responsible for causing gastric ulcers. *H. pylori* is known to become stressed and nonculturable after exposure to unfavorable conditions. In this study, we enhanced resuscitation procedures that have been previously published, characterized conditions under which stressed *H. pylori* can be recovered, and formulated a selective and differential resuscitation medium. Results showed that a specialized broth supplemented with trace minerals and lysed human erythrocytes and serum is required for the recovery of nonculturable *H. pylori*. The type of stress was an important factor in the efficacy of resuscitation, with cells exposed to atmospheric oxygen more readily resuscitated than nutrient deprived cells. After resuscitation, culturable cells were recovered from previously nonculturable oxygen stressed cells (24 and 72 hours of exposure) and nonculturable nutrient deprived cells (24 hours of exposure). The length of time the cells were exposed to the stress was also an important factor in the recovery of stressed *H. pylori*. Additionally, the modification of the resuscitation broth into a selective and differential slant culture medium allowed the recovery of stressed *H. pylori*. The methods presented here highlight the benefits and limitations of using human blood products for recovering nonculturable *H. pylori*.

Key words: *H. pylori*, nonculturable, resuscitation, culturing

**1. Introduction**

*Helicobacter pylori* is a Gram negative, microaerophilic bacterium that colonizes the lining of the human stomach (Marshall, 1995, Veldhuyzen van Zanten and Sherman,

1994). This organism is responsible for causing gastric ulcers and increasing the risk of stomach cancer (Asaka et al., 2001, Moss and Malfertheiner, 2007). *H. pylori* is thought to infect approximately 50% of the human population worldwide and while the main route of transmission is likely fecal-oral (Kivi and Tindberg, 2006), it is possible that the organism may be contracted from the environment as well (Bellack et al., 2006, Sasaki et al., 1999). Culture of *H. pylori* is the most accurate way of assessing viability (Saito et al., 2003), and isolation of the organism can allow testing for pathogenicity, antibiotic susceptibility, and for analyzing differences between strains. Identifying *H. pylori* from the environment has long been problematic due to its oxygen sensitivity and ability to convert to a nonculturable form when stressed (Bode et al., 1993). Thus, culture of *H. pylori* recovered outside the human host is a rare occurrence and research has depended primarily on molecular methods for identification from environmental samples (Bellack et al., 2006, Bunn et al., 2002, Janzon et al., 2009).

Cells in a nonculturable state will not grow on the traditional bacteriological media used for healthy cells (Oliver, 2000). They are thought to be dormant forms that may retain the potential to become metabolically active (Azevedo et al., 2007, Oliver, 2010). Nonculturable *H. pylori* have been characterized by many researchers (Byrd, 2000, Knight, 2000), and are known to exhibit a characteristic transformation of cell shape from a rod to a coccoid form during the organism's life cycle and when exposed to stressful conditions (Bode et al. 1993). Resuscitation of *H. pylori* requires the restoration of culturability on routine bacteriological media (Oliver, 2000). Reports of resuscitation or regrowth of nonculturable *H. pylori* have shown variable results with limited testing

conditions (Andersen et al., 1997, Cellini et al., 1998, Kurokawa et al., 1999). Andersen and co-workers reported visual confirmation of the transformation from nonculturable coccoid to spiral morphology along with full recovery of urease activity when erythrocyte lysate was added to the culture medium (Andersen, et al., 1997). This report was followed by Kurokawa and co-workers (Kurokawa et al., 1999) who found that the recovery of culturable cells on solid agar depended on using human blood serum and lysate.

The aim of this study was to evaluate the potential for recovery of nonculturable *H. pylori* and characterize the conditions under which that recovery may occur. Because the recovery of stressed *H. pylori* has been attempted by several groups with mixed results, it was important to carefully characterize the efficacy of current resuscitation techniques. Results showed that reliable growth and recovery of stressed *H. pylori* in a previously developed broth (Kurokawa et al., 1999) required the addition of trace minerals, fresh lysed human erythrocytes and human serum. In the present study, we present data on the optimized composition of an *H. pylori* specific regrowth medium and the conditions under which recovery was successful. It was shown that resuscitation of cells from a nonculturable state varies depending on the type and exposure time of stress. This study also describes the preparation of a potentially selective and differential regrowth medium.

## **2. Materials and Methods**

### **2.1 Routine Culturing**

*Helicobacter pylori* 26695 was obtained from the American Type Culture Collection (ATCC 700392) and was routinely grown from frozen stocks on tryptic soy agar (TSA) (Difco, Milwaukee, WI) with 5% sheep blood (Quad V, Montana) and the antibiotics vancomycin (Sigma, St. Louis, MO) 10 µg/ml, trimethoprim (MP Biomedical, Solon, OH) 0.5 µg/ml, cefsulodin (Sigma) 0.5 µg/ml, polymixin B (Sigma) 3.5 U/ml and amphotericin B (Sigma) 7.5 µg/ml. This combination of antibiotics has been shown to have few deleterious effects on *H. pylori* growth (Degnan et al., 2003, Stevenson et al., 2000) and initial cultivation on antibiotics helped inhibit contamination of cultures after subculturing onto media without antibiotics. The plates were incubated in a microaerophilic atmosphere using GasPak™ EZ Campy sachets (Becton Dickinson and Co., Franklin Lakes, NJ) in a BBL anaerobe jar for 48 hours at 37°C. Cells were gently removed from agar using sterile swabs and were inoculated into tryptic soy broth (TSB) (Difco) with 5% bovine calf serum (Thermo Scientific Hyclone, Logan UT) followed by inoculation into biphasic slants.

## 2.2 Preliminary Testing

The growth media published by Kurokawa and co-workers (Kurokawa et al., 1999) was used initially to confirm the resuscitation of stressed *H. pylori*. Briefly, *H. pylori* was grown confluent (ca.  $1 \times 10^7$  CFU/ml) on TSA with 5% sheep blood at 37°C in a microaerophilic atmosphere and subsequently subjected to either oxygen stress or nutrient deprivation. *H. pylori* grown confluent on TSA with sheep blood for 48 hours were used as a positive control. Oxygen stress consisted of removing healthy 48 hour *H. pylori* cultures from the microaerophilic incubation chambers and placing them in a 37°C

incubator in the presence of atmospheric levels of oxygen. The nutrient deprivation treatment consisted of removing 48h *H. pylori* cultures from the microaerophilic incubation chambers and gently removing the cells with a sterile inoculation loop into sterile ultrapure water. The cells were rinsed by centrifugation at 13,000 x g for 3 minutes twice, resuspended in sterile ultrapure water, and incubated at 37°C under atmospheric conditions. The oxygen and nutrient stressed (24, 72, and 168 hours of exposure) *H. pylori* were then subjected to resuscitation as previously described (Kurokawa et al., 1999).

### 2.3 Biphasic Slant Culture and Stress Treatments

Initially, routine cultivation of *H. pylori* showed that growth in different broth media (tryptic soy broth, brain heart infusion broth, brucella broth, and Ham's F-12, all supplemented with 5% fetal calf serum) was variable and irreproducible. This observation and the desire to grow large quantities of cells in a liquid medium led to the investigation of different methods of cultivation for *H. pylori*. The blood agar biphasic slant growth system described by ATCC (Anonymous, 2005) was found to allow growth of large numbers of cells in a highly reproducible fashion. Slants were made in 50 ml culture tubes (Fisher Scientific) each containing 25 ml TSA and 5% sheep blood with no antibiotics, solidified at an angle with a pool of 5-10 ml of TSB placed at the bottom of the slant. *H. pylori* cells were grown as described in section 2.1, removed from the TSA with sheep blood and suspended in TSB (with 5% calf serum). The suspended cells were then added to a TSA slant at ca.  $1 \times 10^7$  CFU/ml and incubated for 24 hours in a microaerophilic atmosphere at 37°C. After incubation, cells from the slants were either

used directly (healthy cells for positive control) or were subjected to oxygen or nutrient deprivation.

To induce oxygen stress, inoculated slants were removed from the microaerophilic atmosphere and placed in a 37°C incubator with atmospheric oxygen levels. *H. pylori* cells subjected to nutrient deprivation were decanted from the biphasic slant and centrifuged at 13,000 x g for 3 minutes. The cells were washed twice with sterile ultrapure water, resuspended in sterile ultrapure water, and incubated in a 37°C incubator with atmospheric oxygen. Aliquots of stressed cells were plated onto TSA agar with 5% sheep blood at 24 hour intervals to assess culturability. Both stress treatments were sufficient to inhibit the formation of colonies on blood after 24 hours.

#### 2.4 Optimized Regrowth Medium (R broth)

The optimized regrowth media (R broth) developed in this study consisted of Brucella broth (Difco) with 10 mM Hepes (Sigma), at a pH of 7.8, 0.2 µM CuSO<sub>4</sub> (Sigma), 0.2 µM MnSO<sub>4</sub> (Sigma), 3 µM ZnSO<sub>4</sub> (Sigma), with 50 µM FeSO<sub>4</sub> (Sigma), 250 µM MgCl<sub>2</sub> (Fisher scientific, Fair Lawn NJ), 250 mg/l sodium pyruvate (Fisher Scientific), 2% human blood serum, and 2% human blood lysate added after autoclaving. The broth was based on studies performed by Andersen et al., (1997), Kurokawa et al., (1999), and Testerman et al., (2001, 2006). Differing concentrations of MgCl<sub>2</sub>, FeSO<sub>4</sub>, ZnSO<sub>4</sub> and sodium pyruvate were added to the base medium and evaluated to optimize the growth of healthy and stressed *H. pylori*. Qualitative assessments of the growth of the cells after varying the compounds were performed to check for inhibition and/or promotion of growth. Assessments were performed by spread plating aliquots of

inoculated broth formulations onto TSA with sheep blood and incubating the plates microaerophilically for up to 7 days while monitoring every 48 hours for growth. Human blood serum was produced by centrifugation of citrated whole blood (Innovative Research, Novi, MI) at 15,000 x g for 10 minutes, the supernatant was removed and added directly to R broth. Human blood lysate was produced by three freeze/thaw/sonicate cycles; blood cells were sonicated for 1 minute, frozen at -80°C for 15 minutes, and thawed at room temperature. Blood samples were examined microscopically to confirm lysis.

The *H. pylori* cells grown in biphasic slants and given the oxygen and nutrient deprivation stress treatments were used with the R broth optimization for the final experiments. Five milliliters of cells from each stress treatment were centrifuged at 13,000 x g for 3 minutes, washed twice in phosphate buffered saline (PBS) with 10 mM ammonium sulfate (Sigma) and resuspended into 500 µl working stock PBS with 10 mM ammonium sulfate. Phosphate buffered saline was composed of 12 g/l sodium phosphate monobasic (Fisher Scientific), 2.2 g/l sodium phosphate dibasic (J. T. Baker Chemical Co., Phillipsburg, NJ), and 85 g/l sodium chloride (Fisher Scientific) and diluted 1:10 to obtain a working stock solution. Because it has been shown to enhance resuscitation in both *H. pylori* and *Vibrio cholerae*, stressed *H. pylori* cells were heat shocked (37°C for 10min, 45°C for 30 sec. and cooled to 4°C) (Kurokawa et al., 1999, Wai et al., 1996). Heat shocked cells were added to R broth and incubated in a microaerophilic atmosphere for up to 7 days and monitored every 48 hours at 37°C. All treatments were repeated in triplicate.

## 2.5 Total and Culturable Cell Counts

For comparison and as enumeration controls, total and culturable cell counts were performed on oxygen and nutrient deprived *H. pylori* cells before and after resuscitation. To enumerate the total number of cells per sample, aliquots of *H. pylori* cells at each time interval and treatment were fixed in 4% formaldehyde and incubated with 100X SYBR Green (Invitrogen, Carlsbad, CA) stain at room temperature for up to an hour. Cells were diluted if appropriate, filtered by a vacuum pump onto a 0.22 polycarbonate  $\mu\text{m}$  filter (Whatman, Maidstone, England), and visualized on a Zeiss Axioskop epifluorescence microscope (Carl Zeiss, Inc., Maple Grove, MN) for enumeration of total cells. The microscope utilized the Chroma set #41001 filter with excitation at 480/40 and emission at 535/50. A total of 20 fields or 400 cells were counted per filter and duplicate filters were counted for each biological replicate. Because the gold standard for *in vitro* culturability is growth on solid agar, each resuscitation treatment was subcultured onto a widely accepted growth medium for *H. pylori*, TSA with sheep blood (prepared as described by ATCC) (Anonymous, 2005) after the 48 hour incubation period. Culturable cells were enumerated by diluting, if appropriate, and plating on TSA with sheep blood with and without antibiotics to check for contamination. Duplicate plates were counted for each biological replicate.

## 2.6 Selective and Differential Growth Medium

To assess if a resuscitation method based on the R broth could be modified to accommodate environmental samples, we created a selective and differential biphasic growth medium that allows selection using antibiotics and presumptive identification

using phenol red. Seven grams of Bacto™ agar (Becton Dickinson and Co.) and 50 mg of phenol red (Difco) were added to Brucella broth with 10 mM hepes pH7.8, 0.2  $\mu$ M  $\text{CuSO}_4$  (Sigma), 0.2  $\mu$ M  $\text{MnSO}_4$  (Sigma), and 3  $\mu$ M  $\text{ZnSO}_4$  (Sigma), prior to autoclaving. After autoclaving, 50  $\mu$ M  $\text{FeSO}_4$  (Sigma), 250  $\mu$ M  $\text{MgCl}_2$  (Fisher scientific), 250 mg/l sodium pyruvate (Fisher Scientific), 0.6 g/l urea (Sigma), 10  $\mu$ g/ml vancomycin, 0.5  $\mu$ g/ml trimethoprim, 0.5  $\mu$ g/ml cefsulodin, 3.5 U/ml polymyxin B and 7.5  $\mu$ g/ml amphotericin B were added to the cooled liquid and the final pH adjusted to 5.7. Urea was added as a substrate for the urease enzyme which converts urea to ammonia and bicarbonate. The activity of the urease enzyme and subsequent increase in pH associated with the production of ammonia and bicarbonate causes the phenol red indicator to change from a yellow-orange color to a bright red color. Twenty-five ml of the agar was added to a 50 ml culture tube and solidified at an angle and 5 ml of R broth (as previously described) was added to each slant. Five milliliters of cells from each stress treatment (24 and 72 hour time points were used) were centrifuged at 13,000 x g for 3 minutes, followed by washing twice in PBS with 10 mM ammonium sulfate and resuspension into 500  $\mu$ l PBS with 10 mM ammonium sulfate. Stressed *H. pylori* cells were heat shocked (37°C for 10min, 45°C for 30 sec. and cooled to 4°C). Heat shocked cells were added to R slant, incubated in a microaerophilic atmosphere for 48 hours at 37°C, and monitored for color change and growth on the slant surface. Aliquots of the R slant broth pool were plated on TSA with sheep blood to check for consistency with results seen in R broth experiments.

## 2.7 Statistical Analysis

All data were compiled and for all instances where plate count values had a value of zero indicating none detected, a substitution rule was used (USEPA, 1998). The detection limit of the plate culture technique used in this study was 10 CFU/ml and was chosen to replace all zero plate counts so that log transformations could be performed.

Accordingly, a log value of 1 was used for statistical analysis on all samples that had CFU recovery below the detection limit. To assess differences in mean total and viable cell counts before and after treatment with R broth, paired t-tests were applied in Minitab<sup>®</sup>. Welch's two-sample t-test was performed to assess significant differences in mean total and culturable cell counts between the stress groups, oxygen stress and nutrient deprivation (Kutner et al., 2004). A confidence level of 0.05 was used to determine statistical significance.

### **3. Results**

#### **3.1 Preliminary Results**

This report defines culturable as the ability of a cell to grow on a solid agar medium, forming a visible colony *in vitro* and is indicated by colony forming units (CFU). Initial experiments used *H. pylori* confluent grown on TSA with sheep blood and subjected to oxygen and nutrient deprivation stresses. The resuscitation broth tested initially was described by Kurokawa and co-workers (Kurokawa et al., 1999), and is a mixture of Brucella broth, Hepes, trace minerals, sodium pyruvate, human blood serum and lysed erythrocytes. To assess the usefulness of the resuscitation broth, a series of 10 biological replicates of each stress condition was performed over a period of three months. Stressed cells were incubated in the resuscitation medium for 48 hours and

subcultured onto TSA with sheep blood. Overall, 6 of 10 air stressed and 5 of 10 nutrient deprived *H. pylori* replicates showed regrowth after incubation in the resuscitation broth and subsequent subculture. Of note is that after approximately 45 days of storage, the human blood products (lysed erythrocytes and serum, stored at -20°C) could no longer support growth of the organism. When fresh blood products were obtained, the assay again allowed regrowth of stressed *H. pylori* on solid agar.

*H. pylori* is known to have specific nutritional requirements for in vitro growth and varying these compounds can affect growth (Testerman et al., 2001, 2006). To optimize regrowth, the concentrations of ferrous sulfate, magnesium chloride, and zinc sulfate were varied from starting concentrations of 2.5 µM, 200 µM, and 0.2 µM, respectively to final concentrations of 50 µM, 250 µM and 3 µM respectively. Aliquots of cells from each broth formulation were plated onto TSA with sheep blood and observations assessing results were qualitative, ranging from no growth, light, medium, and heavy growth. While the initial concentrations of trace minerals supported heavy growth of healthy cells and light growth of stressed *H. pylori*, increasing iron, magnesium, and zinc allowed heavier growth of stressed *H. pylori*. When any of the three metals were omitted from the broth, no growth was observed. The addition of trace minerals to culture media has been previously investigated by Testerman and co-workers (Testerman et al., 2006) and shown to greatly affect *in vitro* culture of *H. pylori*. Additionally, varying concentrations of sodium pyruvate were evaluated (50 mg/l, 110 mg/l, and 250 mg/l) with all concentrations supporting growth of healthy and stressed cells while growth was inhibited when it was omitted.

### 3.2 Culture of Stressed *H. pylori*

*H. pylori* cells were grown in TSA blood agar biphasic slants for 24 hours followed by either oxygen stress or nutrient deprivation by incubation in water, both at 37°C. Positive controls (healthy cells, presumed to be in early log phase) were grown for 24 hours on the bisphasic slants. The addition of healthy *H. pylori* cells to the optimized R broth medium did not significantly increase the total cell numbers ( $3.92 \times 10^7$  cells/ml before resuscitation to  $3.96 \times 10^7$  after resuscitation ( $P = 0.935$ )) (Time Zero, Figure 5.1). However, there was a statistically significant increase in culturable cells (CFU) after resuscitation of healthy cells from approximately  $1.98 \times 10^6$  CFU/ml before resuscitation to  $2.70 \times 10^7$  CFU/ml after resuscitation ( $P = 0.036$ ).

*H. pylori* cells grown in biphasic culture were subjected to oxygen stress for up to 168 hours (7 days) and were nonculturable when subcultured on TSA with sheep blood within 24 hours of exposure to stress treatment. Oxygen stress caused a switch in cell morphology from the typical spiral shape to a coccoid, with the cells nearing 100% coccoid after two weeks (data not shown). After resuscitation, there were statistically significant increases in both total (cells/ml) and culturable (CFU/ml) counts in samples that had been subjected to oxygen stress for 24 and 72 hours (Figure 5.1a and 5.1b). After 24 hours of exposure to atmospheric conditions and subsequent resuscitation treatment, the mean total number of cells increased from  $4.6 \times 10^6$  to  $7.2 \times 10^7$  cells/ml ( $P = 0.027$ ) and the mean culturable colony counts increased from none detected to  $1.1 \times 10^4$  CFU/ml ( $P = 0.001$ ). After 72 hours of oxygen stress and subsequent resuscitation treatment, the mean total number of cells increased from  $2.4 \times 10^6$  to  $1.4 \times 10^7$  cells/ml ( $P$

= 0.040) and the mean culturable colony counts increased from none detected to  $1.3 \times 10^4$  CFU/ml ( $P = 0.000$ ). After resuscitation, there was no recovery of culturable cells that had been exposed to atmospheric oxygen for 168 hours (Figure 5.1a).

*H. pylori* cells grown in biphasic culture were treated with nutrient deprivation for up to 168 hours and became nonculturable when subcultured on TSA with sheep blood within 24 hours of stress treatment. Nutrient deprived cells showed a much slower conversion of the cells to the coccoid morphology with approximately 45% of the cells coccoid after two weeks (data not shown). After 24 hours of nutrient deprivation, the mean total cell counts did not significantly change ( $2.8 \times 10^7$  cells/ml before resuscitation to  $3.1 \times 10^7$  cells/ml after resuscitation ( $P = 0.965$ )) however, there was an increase in culturable cell counts from none detected before treatment to ca.  $1 \times 10^3$  CFU/ml after treatment ( $P = 0.062$ ) (Figure 5.1d). *H. pylori* that were nutrient deprived for 72 hours or 168 hours showed no significant change in total cell counts or culturable cells (CFU) after the resuscitation treatment and subsequent subculture on TSA with sheep blood.

The total number of cells did not differ between the two types of stress treatments (Table 5.1) which indicates that neither stress condition caused lysis of the organisms over time. However, the cells responded differently to the two stresses. *H. pylori* exposed to oxygen stress for 24 hours recovered more colony forming units on TSA with sheep blood than nutrient deprived *H. pylori* at 24 hours, but both stress treatments had culturable cells (CFUs) after resuscitation. After 72 hours, only the oxygen stress treatment had culturable cells when subcultured after resuscitation. After 168 hours, neither stress treatment yielded culturable cells after incubation in R broth and

subsequent subculture on TSA with sheep blood. Both treatments showed that length of stress was a factor in the ability to be resuscitated, and suggests that optimization of recovery media should include investigations with a variety of stress conditions.

### 3.3 Selective and Differential Growth Medium

To assess whether the previously described resuscitation method could potentially be used to directly detect stressed *H. pylori* from environmental samples, we modified the R broth into a selective and differential R slant. Oxygen stressed and nutrient deprived *H. pylori* were heat shocked as previously described, added to the R slant, and incubated microaerophilically for 48 hours. To check for consistency with results obtained from the R broth, aliquots of the broth from the R slant were plated onto TSA with sheep blood after the 48 hour incubation period. The addition of antibiotics, urea, phenol red and the slight acidification of the media did not change the overall results seen in the resuscitation with R broth (Figure 5.2). Positive control *H. pylori* in early log phase showed the most robust growth on the slant agar surface and also showed confluent growth after subculturing onto TSA with sheep blood. The R slant color changed from a yellow-orange (phenol red at pH5.7) to a deep red color during incubation with healthy *H. pylori* indicating an active urease enzyme that hydrolyzed urea into ammonia and bicarbonate, thereby increasing the pH of the medium and causing the color change.

Consistent with results seen in the R broth, oxygen stressed cells (both 24 and 72 hour stress treatments) showed growth on the surface of the R agar slant. Growth on the R slant inoculated with oxygen stressed cells was less robust than the positive control R slants. The characteristic color change associated with urease activity was slightly less

red than the positive control slants indicating decreased urease activity compared to healthy cells. Nutrient deprived *H. pylori* behaved similarly, with growth on the agar slant occurring only after shorter periods of stress (24 hours). After 72 hours of nutrient deprivation, no visible growth was observed on the slant or after subculturing and there was only a slight change in color of the slant compared to the negative control, indicating lower levels of urease activity. The low level of urease activity is likely attributed to the dormant and nonculturable cells that were not able to be resuscitated using this method.

#### **4. Discussion**

Because growth of environmentally stressed *H. pylori* is essential to proving the viability of the organism, considerable time and effort have been expended to improve recovery of these cells. This has led to many formulations of nutrient rich media that are optimized to produce robust growth of *H. pylori* (Coudron and Stratton, 1995, Degnan et al., 2003, Testerman et al., 2001). Since the bacterium is sensitive to oxygen (Donelli et al., 1998) it must be grown in a microaerophilic environment with limited exposure to atmospheric conditions. It is well known that exposure to oxygen and starvation conditions lead to a nonculturable state *in vitro* (Kusters et al., 1997). This study has attempted to describe the conditions under which nonculturable *H. pylori* may be recovered. The stress conditions were carefully chosen; it was important to make stepwise changes to the stress treatments so that differences in results could be attributed to specific changes. Accordingly, we did not change the temperature for either stress conditions studied in this research.

Initially, we attempted to resuscitate cells grown confluent on a TSA plate with 5% sheep blood, given the stress treatments and then incubated in R broth. We increased the concentrations of FeSO<sub>4</sub>, MgCl<sub>2</sub>, and ZnSO<sub>4</sub> from the levels reported by Kurowawa and co-workers (1999) to achieve greater yields of healthy *H. pylori* in broth culture. Combined with the addition of fresh human serum and human erythrocyte lysate, this broth allowed robust and reproducible growth of *H. pylori*. However, we discovered that although calf serum may be frozen for long periods of time without a reduction in growth, human blood products stored at -20°C for over a month no longer allowed the recovery of stressed *H. pylori* cells. Therefore, it is important that fresh human blood be used. As a further development, and after optimization, the biphasic slant was used for all subsequent experiments due to the highly reproducible and robust growth observed with this culture method. *H. pylori* were grown in biphasic culture for 24 hours in a microaerophilic atmosphere, and then were either used directly or given the two stress treatments. All treatments (positive control and stress treatments) were plated on TSA with sheep blood prior to resuscitation to assess culturability. After being stressed for 24, 72, and 168 hours, the cells were heat shocked, placed in R broth, incubated for 48 hours, and finally subcultured into TSA with sheep blood.

The incubation of early log phase *H. pylori* (initially grown microaerophilically, in a TSA/TSB biphasic slant) in R broth did not increase the total number of cells but did increase the number of culturable cells (CFU/ml). This showed that even in a growing *in vitro* culture, a portion of the cells can be nonculturable. Because the total number of cells did not significantly change and the colony forming units nearly account for every

cell enumerated microscopically, we conclude that the nonculturable cells were resuscitated rather than stimulated to divide and regrow. This is consistent with the findings by Andersen and coworkers who saw the morphological conversion from a coccoid back to a rod when nonculturable *H. pylori* regained culturability (Andersen et al., 1997).

Oxygen stressed and nutrient deprived *H. pylori* exhibited differential growth in response to R broth. *H. pylori* that had access to ample nutrients but were stressed by atmospheric levels of oxygen had greater recovery of culturable colonies than cells that were suspended in sterile water and subjected to nutrient deprivation. The incubation of oxygen stressed cells in R broth followed by subculture onto TSA blood agar allowed the recovery of previously nonculturable cells after 24 and 72 hours of stress. However, after 7 days of incubation in atmospheric levels of oxygen, no colonies were recovered after resuscitation and subsequent subculture onto TSA with sheep blood. This is in contrast to nutrient deprived cells that recovered colonies only after 24 hours of exposure to stress. These results generally agree with other reports that found that *H. pylori* could be resuscitated using the proper nutrients and human blood products such as erythrocyte lysate and/ or serum (Andersen et al., 1997, Kurokawa et al., 1999). Our study has elaborated on these findings, showing that the recovery of stressed *H. pylori* depends on the type of stress and the length of time the organisms are exposed to that stress. It is important to note that it is not possible to conclude whether true resuscitation has occurred in either of the stress treatments we investigated. Because not every cell was recovered into a colony forming unit and our cultures were relatively dense, it is

impossible to discern whether a small subpopulation has retained the ability to divide and regrow or if the cells that formed colonies were actually resuscitated.

Because of our interest in eventually culturing this organism directly from the environment, the R broth was adapted into a selective and differential biphasic slant. This slant utilized antimicrobials known to select against normal flora of drinking water (Degnan et al., 2003, Stevenson et al., 2000). The addition of urea and phenol red, combined with slight acidification of the medium (pH 5.7) allowed the presumptive indication of *H. pylori* when it was inoculated into the slant. This strategy has been successfully used in research attempting to isolate *H. pylori* from complex biological samples (Degnan et al., 2003). By adding R broth to the selective and differential biphasic slant based on the same medium it was possible to recover *H. pylori*. This slant also gave an indication of the urease activity by a qualitative assessment of the color change. Although this method has potential for the regrowth of environmentally stressed *H. pylori*, there are limitations to the length of time the organism can be stressed and then recovered with this growth medium. The length of time *H. pylori* is exposed to an unfavorable condition has been shown to drastically affect culturability and has been well characterized (Donelli et al., 1998, Kusters et al., 1997, Shahamat et al., 1993). Typically, cells under nutrient deprivation at cold temperatures retain viability longer while starvation at warmer temperatures is known to cause a stringent response (Shahamat et al., 1993). It has also been observed that changing the oxygen concentration above or below the optimal level required by *H. pylori* results in a concomitant decrease in culturable cells (Donelli et al., 1998). More investigation will be

required to completely define the conditions under which *H. pylori* can be recovered from the nonculturable state.

Although it has been well documented, it is unknown why starved cells behave differently than cells subjected to oxygen stress alone (Azevedo et al., 2006, Donelli et al., 1998, Shahamat et al., 1993). Certainly the presence of nutrients is a major difference that dictates different survival strategies in *H. pylori*. In general, resuscitation of culturable cells occurs under nutritionally restricted conditions and can be enhanced by heat shock (Wai et al., 1996). Bloomfield and co-workers have proposed that the rapid change in nutrients utilized in most resuscitation experiments results in nonculturability due to “explosive transport” (Bloomfield et al., 1998). This is the rapid transport of nutrients into a cell resulting from the cells attempt to utilize the organic nutrients that are present, also known as nutrient shock. This idea is supported by our finding which showed that starved cells had decreased recovery on compared to cells under oxygen stress with available nutrients. The cells in the oxygen stress treatment may not have undergone explosive transport because they were never nutrient limited. The idea of explosive transport is supported by the findings of Azevedo and co-workers (Azevedo et al., 2004) who found that nutrient shock was a major factor in recovery of *H. pylori* from water and by Nilsson and coworkers ( Nilsson et al., 2002) who found that ATP production was increased dramatically with the addition of human erythrocytes.

It is known that *H. pylori* convert to a coccoid morphology as part of its normal stationary phase of growth and this process is likely adaptive (Azevedo et al., 2007). Both spiral and coccoid morphologies were present in varying fractions depending on the

stress with oxygen stressed cells converting to coccoid more quickly than the nutrient deprivation treatment. Consistent with other reports, we found that cell morphology did not dictate whether the cell was nonculturable *in vitro* (Adams et al., 2003). Depending on the length of time *H. pylori* is outside the mammalian host, the age of the individual cell, and the particular stress that is present, it is likely that either spiral or coccoid forms may be present and that resuscitation will vary depending on those factors.

In summary, we have provided an optimized resuscitation medium that shows promise for use on environmental samples. The longer term goal for this research is to develop a method that potentially allows growth of environmentally stressed *H. pylori*, with the added benefit of presumptive indication. Our data suggests that stressed *H. pylori* from pure cultures will regrow using this approach, and brings us closer to the elusive goal of a monitoring tool for this environmental pathogen. Additionally, we have shown that not all stress conditions are equal and that the potential for resuscitation is strongly affected by the type of stress and the length of exposure. These results are pertinent to researchers studying *H. pylori* physiology as well as individuals concerned with regrowing *H. pylori* from the environment.

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## REFERENCES

Adams, B.L., Bates, T.C., Oliver, J.D., 2003. Survival of *Helicobacter pylori* in a natural freshwater environment. *Appl Environ Microbiol.* 69, 7462-7466.

Andersen, A.P., Elliott, D.A., Lawson, M., Barland, P., Hatcher, V.B., Puszkin, E.G., 1997. Growth and morphological transformations of *Helicobacter pylori* in broth media. *J Clin Microbiol.* 35, 2918-2922.

Anonymous. 2005. Product information sheet for ATCC<sup>®</sup> 700392. American Type Culture Collection, Manassas, VA. pp. 1-2.

Asaka, M., Sepulveda, A.R., Sugiyama, T., Graham, D.Y., 2001. Gastric Cancer. In: H. L. T. Mobley, G. L. Mendz, S. L. Hazell (Eds.), *Helicobacter pylori: physiology and genetics*, American Society for Microbiology, Washington, D.C., pp. 481-498.

Azevedo, N.F., Almeida, C., Cerqueira, L., Dias, S., Keevil, C.W., Vieira, M.J., 2007. Coccoid form of *Helicobacter pylori* as a morphological manifestation of cell adaptation to the environment. *Appl Environ Microbiol.* 73, 3423-3427.

Azevedo, N.F., Pacheco, A.P., Keevil, C.W., Vieira, M.J., 2004. Nutrient shock and incubation atmosphere influence recovery of culturable *Helicobacter pylori* from water. *Appl Environ Microbiol.* 70, 490-493.

Azevedo, N.F., Pacheco, A.P., Keevil, C.W., Vieira, M.J., 2006. Adhesion of water stressed *Helicobacter pylori* to abiotic surfaces. *J Appl Microbiol.* 101, 718-724.

Bellack, N.R., Koehoorn, M.W., MacNab, Y.C., Morshed, M.G., 2006. A conceptual model of water's role as a reservoir in *Helicobacter pylori* transmission: a review of the evidence. *Epidemiol Infect.* 134, 439-449.

- Bloomfield, S.F., Stewart, G.S.A.B., Dodd, C.E.R., Booth, I.R., Power, E.G.M., 1998. The viable but not culturable phenomenon explained? *Microbiol Comm.* 144, 1-3.
- Bode, G., Mauch, F., Malfertheiner, P., 1993. The coccoid forms of *Helicobacter pylori*. Criteria for their viability. *Epidemiol Infect.* 111, 483-490.
- Bunn, J.E.G., MacKay, W.G., Thomas, J.E., Reid, D.C., Weaver, L.T., 2002. Detection of *Helicobacter pylori* DNA in drinking water biofilms: implications for transmission in early life. *Lett Appl Microbiol.* 34, 450-454.
- Byrd, J., 2000. Morphological changes leading to the nonculturable state. In: R. R. Colwell, J. D. Grimes (Eds.), *Nonculturable microorganisms in the environment*, American Society for Microbiology, Washington, D. C., pp. 7-18.
- Cellini, L., Robuffo, I., Di Campli, E., Di Bartolomeo, S., Taraborelli, T., Dainelli, B., 1998. Recovery of *Helicobacter pylori* ATCC 43504 from a viable but not culturable state: regrowth or resuscitation? *APMIS.* 106, 571-579.
- Coudron, P.E., Stratton, C.W., 1995. Factors affecting growth and susceptibility testing of *Helicobacter pylori* in liquid media. *J Clin Microbiol.* 33, 1028-1030.
- Degnan, A.J., Sonzogni, W.C., Standridge, J.H., 2003. Development of a plating medium for selection of *Helicobacter pylori* from water samples. *Appl Environ Microbiol.* 69, 2914-2918.
- Donelli, G., Matarrese, P., Fiorentini, C., Dainelli, B., Taraborelli, T., Di Campli, E., Di Bartolomeo, S., Cellini, L., 1998. The effect of oxygen on the growth and cell morphology of *Helicobacter pylori*. *Fems Microbiol Lett.* 168, 9-15.
- Janzon, A., Sjoling, A., Lothigius, A., Ahmed, D., Qadri, F., Svennerholm, A.M., 2009. Failure to detect *Helicobacter pylori* DNA in drinking and environmental water in Dhaka, Bangladesh, using highly sensitive real-time PCR assays. *Appl Environ Microbiol.* 75, 3039-3044.
- Kivi, M., Tindberg, Y., 2006. *Helicobacter pylori* occurrence and transmission: A family affair? *Scan J Infect Dis.* 38, 407-417.
- Knight, I.T., 2000. Molecular genetic methods for detection and identification of viable but nonculturable microorganisms. In: R. R. Colwell, D. J. Grimes (Eds.), *Nonculturable microorganisms in the environment*, American Society for Microbiology, Washington D. C., pp. 77-85.
- Kurokawa, M., Nukina, M., Nakanishi, H., 1999. Resuscitation from the viable but non culturable state of *Helicobacter pylori*. *Kansenshogaku Zasshi.* 73, 15-19.

- Kusters, J.G., Gerrits, M.M., Van Strijp, J.A.G., Vandenbroucke Grauls, C., 1997. Coccoid forms of *Helicobacter pylori* are the morphologic manifestation of cell death. *Infect Immun.* 65, 3672-3679.
- Kutner, M., C. Nachtsheim, J. Neter, and W. Li. 2004. *Applied linear statistical models*, Fifth ed. McGraw-Hill/Irwin, New York.
- Marshall, B.J., 1995. *Helicobacter pylori* - the etiologic agent for peptic ulcer. *J Amer Med Assoc.* 274, 1064-1066.
- Moss, S.F., Malfertheiner, P., 2007. *Helicobacter* and gastric malignancies. *Helicobacter.* 12, 23-30.
- Nilsson, H.O., Blom, J., Abu Al-Soud, W., Ljungh, A., Andersen, L.P., Wadstrom, T., 2002. Effect of cold starvation, acid stress, and nutrients on metabolic activity of *Helicobacter pylori*. *Appl Environ Microbiol.* 68, 11-19.
- Oliver, J.D., 2000. The public health significance of viable but not culturable bacteria. In: R. R. Colwell, D. J. Grimes (Eds.), *Nonculturable microorganisms in the environment*, American Society for Microbiology, Washington, D. C., pp. 277-300.
- Oliver, J.D., 2010. Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol Rev.* 34, 415-425.
- Saito, N., Konishi, K., Sato, F., Kato, M., Takeda, H., Sugiyama, T., Asaka, M., 2003. Plural transformation-processes from spiral to coccoid *Helicobacter pylori* and its viability. *J Infect.* 46, 49-55.
- Sasaki, K., Tajiri, Y., Sata, M., Fujii, Y., Matsubara, F., Zhao, M.G., Shimizu, S., Toyonaga, A., Tanikawa, K., 1999. *Helicobacter pylori* in the natural environment. *Scan J Infect Dis.* 31, 275-280.
- Shahamat, M., Mai, U., PaszkoKolva, C., Kessel, M., Colwell, R.R., 1993. Use of autoradiography to assess viability of *Helicobacter pylori* in water. *Appl Environ Microbiol.* 59, 1231-1235.
- Stevenson, T.H., Lucia, L.M., Acuff, G.R., 2000. Development of a selective medium for isolation of *Helicobacter pylori* from cattle and beef samples. *Appl Environ Microbiol.* 66, 723-727.
- Testerman, T.L., Conn, P.B., Mobley, H.L.T., McGee, D.J., 2006. Nutritional requirements and antibiotic resistance patterns of *Helicobacter* species in chemically defined media. *J Clin Microbiol.* 44, 1650-1658.

Testerman, T.L., McGee, D.J., Mobley, H.L.T., 2001. *Helicobacter pylori* growth and urease detection in the chemically defined medium Ham's F-12 nutrient mixture. *J Clin Microbiol.* 39, 3842-3850.

United States Environmental Protection Agency. 1998. Guidance for data quality assessment-Practical methods for data analysis. Office of Research and Development, Washington, D. C.

Veldhuyzen van Zanten, S.J., Sherman, P.M., 1994. *Helicobacter pylori* infection as a cause of gastritis, duodenal ulcer, gastric cancer, and nonulcer dyspepsia - a systematic overview. *Can Med Assoc J.* 150, 177-185.

Wai, S.N., Moriya, T., Kondo, K., Misumi, H., Amako, K., 1996. Resuscitation of *Vibrio cholerae* O1 strain TSI-4 from a viable but nonculturable state by heat shock. *FEMS Microbiol Lett.* 136, 187-191.

Table 5.1. Comparison of the differences in mean total cell (cells/ml) and mean culturable cell (CFU/ml) counts between the oxygen stress and nutrient deprivation treatments before and after resuscitation.

Total Cells (cells/ml)						
Time (h)	Oxygen before R broth	Nutrient before R broth	<i>P</i> value	Oxygen after R broth	Nutrient after R broth	<i>P</i> value
24	4.38 x 10 <sup>6</sup>	8.43 x 10 <sup>7</sup>	0.004	2.77 x 10 <sup>7</sup>	3.06 x 10 <sup>7</sup>	0.173
72	2.69 x 10 <sup>6</sup>	1.38 x 10 <sup>7</sup>	0.221	4.63 x 10 <sup>6</sup>	8.72 x 10 <sup>6</sup>	0.097
168	8.91 x 10 <sup>6</sup>	1.69 x 10 <sup>7</sup>	0.030	2.74 x 10 <sup>7</sup>	9.32 x 10 <sup>6</sup>	0.151

Viable Cells (CFU/ml)						
Time (h)	Oxygen before R broth	Nutrient before R broth	<i>P</i> value	Oxygen after R broth	Nutrient after R broth	<i>P</i> value
24	nd	nd	*	1.09 x 10 <sup>4</sup>	2.63 x 10 <sup>3</sup>	0.205
72	nd	nd	*	1.31 x 10 <sup>4</sup>	nd	0.000
168	nd	nd	*	nd	nd	*

\*No difference between oxygen stress and nutrient deprivation treatments  
(nd) not detected

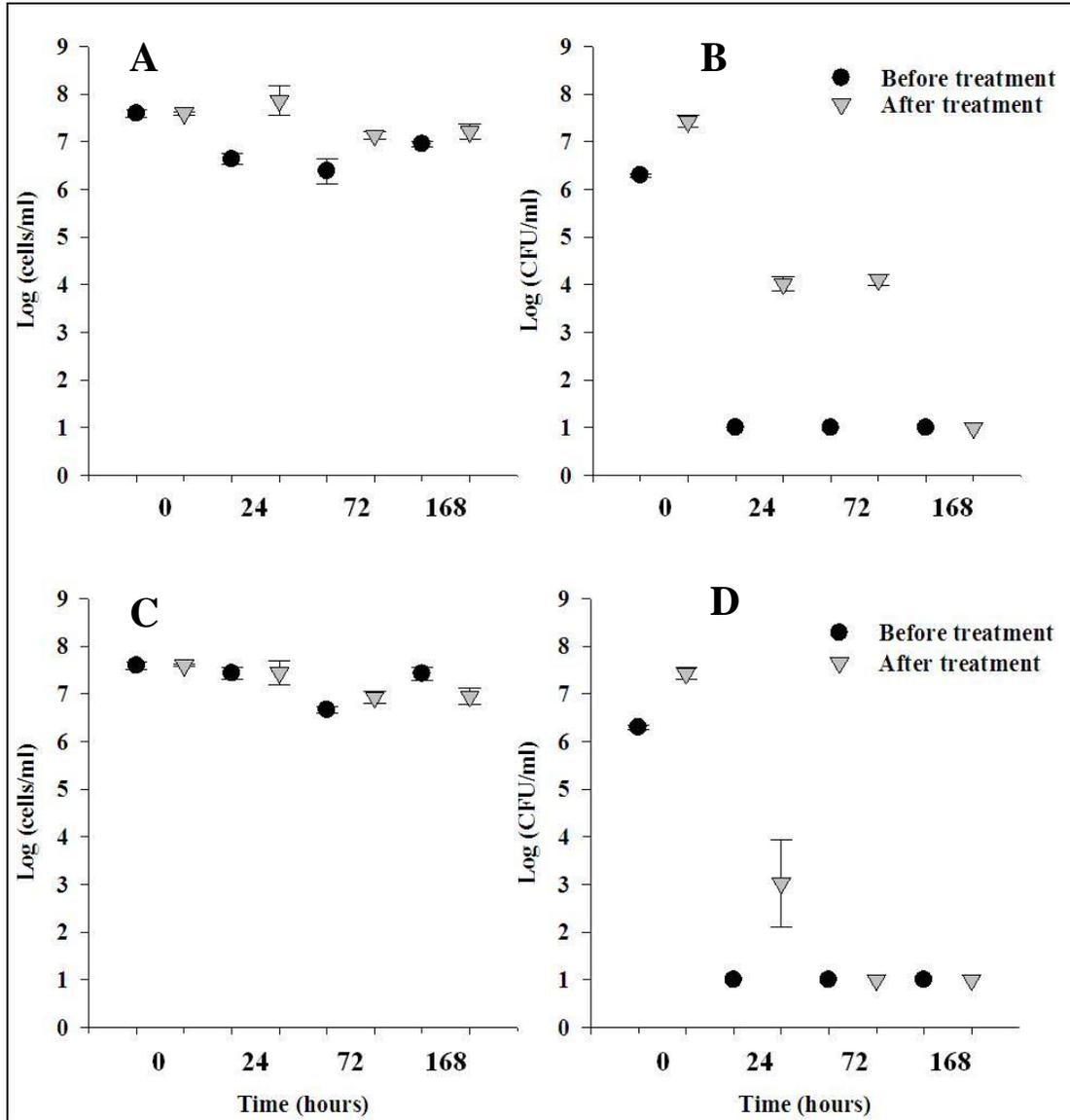


Figure 5.1. Depicts the total cell and culturable colony counts from *H. pylori* before and after resuscitation. Symbols indicate mean counts of three independent biological replicates (cells/ml or CFU/ml), error bar indicate 1 standard deviation. For comparison, time zero in all graphs is positive control healthy cells grown in biphasic slant before and after resuscitation 1A) Oxygen stressed total cell counts sampled at 24, 72 and 168 h before and after resuscitation. 1B) Oxygen stressed colony counts sampled at 24, 72, and 168h before and after resuscitation. 1C) Nutrient deprived total cell counts sampled at 24, 72 and 168 h before and after resuscitation. 1D) Nutrient deprived colony counts sampled at 24, 72 and 168 h before and after resuscitation. One log (CFU/ml) was the detection limit for culturable cells.



Figure 5.2. Depicts the selective and differential R slant. Slant on left is inoculated with early log phase *H. pylori* cells. The slant on the right is the negative control inoculated with sterile PBS.

## CHAPTER 6

## THESIS SYNTHESIS

The goal of the present work was to examine environmental factors that may lead to *H. pylori* survival and transmission. Transmission of a bacterial pathogen from host to host is a complex process that may involve survival of the pathogen outside the host for considerable lengths of time. The bacterium *H. pylori* causes severe gastritis and gastric ulcers, and infection can increase the risk of stomach cancer (11). The main mode of transmission is believed to be the oral-oral route, however other routes of transmission such as drinking water have been implicated (2, 4, 7, 8, 13). Environmental routes of transmission are difficult to prove because the organism enters a viable but not culturable state shortly after exiting the host due to a requirement for a microaerophilic atmosphere (1). Additionally, severe health outcomes restrict the use of human subjects in studying the transmission of this organism. In this dissertation, the environmental route of transmission of *Helicobacter pylori* was investigated using several approaches, and evidence for an environmental route of transmission was found.

A primary objective of this study was to determine if *H. pylori* could be detected in an environmental reservoir readily consumed by humans, such as drinking water, described in Chapter 3. *Helicobacter pylori* was detected by PCR but not culture in drinking water and biofilms that were obtained from groundwater and a municipal system. *Helicobacter pylori* contamination of drinking water and associated biofilms was sporadic and not associated with any of the environmental factors recorded, such as pH or

temperature. The presence of nonculturable *H. pylori* in drinking water systems suggests a potential role in disease transmission. These findings spurred interest in the nonculturable form and led to the characterization and resuscitation of *H. pylori* in the viable but nonculturable state.

The characterization of two conditions that cause the VBNC state in *H. pylori* was presented in Chapter 4. This study compared aging, oxygen stress and nutrient deprivation to examine differences in viability and cell morphology. During growth curve analysis, *H. pylori* cells exhibited a characteristic switch in cell morphology from a spiral to coccoid as they aged or were exposed to stressful culture conditions. However, results showed that cell morphology was not necessarily indicative of culturability, with spiral forms dominant in early nonculturable samples. Microarray analysis of the transition to a nonculturable state under the two defined conditions showed that cells under oxygen stress alone quickly modified their transcriptional activity and up-regulated genes associated with cell envelope processes, LPS and glycan biosynthesis, and translational activities. The cells exposed to nutrient deprivation under atmospheric levels of oxygen showed nearly undetected changes in transcriptional activities compared to the untreated control at early time intervals. Nutrient deprivation eventually led to significant changes in transcription with up-regulated genes related to the classic starvation response such as amino acid, carbohydrate, and co-factor/ vitamin metabolism. Finally, it was shown that the addition of nutrients to the two different stress treatments caused differential transcriptional responses. The oxygen stressed cells increased the transcription of virulence factors when incubated in a resuscitation medium containing

lysed human erythrocytes and serum. The addition of the resuscitation medium to nutrient deprived *H.pylori* cells caused a decrease in transcription of the same factors. This observation led to the conclusion that oxygen stressed and nutrient deprived cells are metabolically active but react differently to *in vitro* culture condition with starved cells likely undergoing nutrient shock.

The evaluation and optimization of an *H. pylori* specific growth medium was presented in Chapter 5. Preliminary work with *H. pylori* in various broth media showed that the organism required a complex media with many supplements to obtain robust growth. After careful characterization of the stress conditions (Chapter 4), resuscitation was attempted utilizing an optimized resuscitation medium that included human erythrocyte serum and lysate. Incubation in atmospheric conditions with nutrients (oxygen stress) at 37 °C allowed greater recovery of previously nonculturable *H. pylori* than incubation without nutrients but also under oxygen stress (nutrient deprivation in atmospheric conditions) in the presence of atmospheric conditions at 37 °C , and that exposure time to stress significantly affected resuscitation.

Collectively these data suggest that *H. pylori* can survive and persist under stressful conditions posed by the environmental mode of transmission. Under stressful conditions at least a portion of cells retain the ability to be recovered using modified *in vitro* culture techniques. Therefore, it is likely that *H. pylori* from the environment can pose a risk of infection to humans. This is important because it indicates that water could play a role in initial infections that are then spread by person to person contact.

This research is important because it provides insight into how *H. pylori* may survive the transmission from host to host. A complete understanding of the survival of *H. pylori* outside the human host will allow the identification of potential sources of infection. Additionally, characterizing the different morphological forms seen under environmental stress provides information that may be useful for the development of vaccines and other therapeutic approaches

#### Future Directions

More research is required to develop a better understanding of how *H. pylori* adapts to its changing environment. An organism that survives the harsh environment of the mammalian stomach has many ways of coping with stress. In future studies it would be helpful to consider some of the synergistic effects that accompany a transition through the environment such as oxygen stress plus temperature. Comparisons of multiple stressors would be useful and more realistic for modeling environmental modes of transmission.

Microarray technologies are very useful for gaining an understanding of how an organism responds to changing conditions (3). This technology has been applied to *H. pylori* with much success (5, 6, 9, 10, 12). However, one of the confounding problems encountered are the lack of characterized genes within the *H. pylori* genome. Future work should focus on characterizing genes and subsequently proteins and enzymes that have been identified in transcriptome studies and that play a role in surviving harsh conditions.

Another understudied aspect of *H. pylori* is the maintenance of a spiral shape under stringent conditions. The majority of research on *H. pylori* in the VBNC state focuses on the transition to a coccoid morphology. However, it is obvious from this work that there are two morphological populations in a nonculturable batch of cells of *H. pylori*, the spiral and the coccoid. More research is required to fully understand the ecological roles that these forms play and how they affect survival and transmission to the host.

Finally, it is obvious that human blood products are useful for resuscitating stressed *H. pylori*. However, under some conditions such as long stress exposures, this may not be the cue that allows *H. pylori* to resume active growth. More research extensively investigating the potential cues that may be involved in *H. pylori* resuscitation *in vivo* should be undertaken.

References

1. Adams, B. L., T. C. Bates, and J. D. Oliver. 2003. Survival of *Helicobacter pylori* in a natural freshwater environment. *Appl. Environ. Microbiol.* 69:7462-7466.
2. Bellack, N. R., M. W. Koehoorn, Y. C. MacNab, and M. G. Morshed. 2006. A conceptual model of water's role as a reservoir in *Helicobacter pylori* transmission: a review of the evidence. *Epidemiol. Infect.* 134:439-449.
3. Conway, T., and G. K. Schoolnik. 2003. Microarray expression profiling: capturing a genome-wide portrait of the transcriptome. *Mol. Microbiol.* 47:879-889.
4. Goodman, K. J., and P. Correa. 1995. The transmission of *Helicobacter pylori* - a critical review of the evidence. *Int. J. Epidemiol.* 24:875-887.
5. Han, Y. H., W. Z. Liu, Y. Z. Shi, L. Q. Lu, S. D. Xiao, and Q. H. Zhang. 2009. Gene expression profile of *Helicobacter pylori* in response to growth temperature variation. *J. Microbiol.* 47:455-465.
6. Israel, D. A., N. Salama, C. N. Arnold, S. F. Moss, T. Ando, H. P. Wirth, K. T. Tham, M. Camorlinga, M. J. Blaser, S. Falkow, and R. M. Peek. 2001. *Helicobacter pylori* strain-specific differences in genetic content, identified by microarray, influence host inflammatory responses. *J. Clin. Invest.* 107:611-620.
7. Ma, J. L., W. C. You, M. H. Gail, L. Zhang, W. J. Blot, Y. S. Chang, J. Jiang, W. D. Liu, Y. R. Hu, L. M. Brown, G. W. Xu, and J. F. Fraumeni. 1998. *Helicobacter pylori* infection and mode of transmission in a population at high risk of stomach cancer. *Int. J. Epidemiol.* 27:570-573.
8. Mackay, W. G., L. T. Gribbon, M. R. Barer, and D. C. Reid. 1998. Biofilms in drinking water systems - A possible reservoir for *Helicobacter pylori*. *Water Sci. Technol.* 38:181-185.
9. Merrell, D. S., M. L. Goodrich, G. Otto, L. S. Tompkins, and S. Falkow. 2003. pH-regulated gene expression of the gastric pathogen *Helicobacter pylori*. *Infect. Immun.* 71:3529-3539.
10. Merrell, D. S., L. J. Thompson, C. C. Kim, H. Mitchell, L. S. Tompkins, A. Lee, and S. Falkow. 2003. Growth phase-dependent response of *Helicobacter pylori* to iron starvation. *Infect. Immun.* 71:6510-6525.

11. Perez-Perez, G. I., D. Rothenbacher, and H. Brenner. 2004. Epidemiology of *Helicobacter pylori* infection. *Helicobacter* 9:1-6.
12. Thompson, L. J., D. S. Merrell, B. A. Neilan, H. Mitchell, A. Lee, and S. Falkow. 2003. Gene expression profiling of *Helicobacter pylori* reveals a growth-phase-dependent switch in virulence gene expression. *Infect. Immun.* 71:2643-2655.
13. Vaira, D., J. Holton, M. Menegatti, L. Gatta, C. Ricci, A. Ali, F. Landi, C. Moretti, and M. Miglioli. 1998. Routes of transmission of *Helicobacter pylori* infection. *Ital. J. Gastroenterol. Hepatol.* 30:S279-S285.

APPENDIX A:

COMMUNITY-BASED PARTICIPATORY RESEARCH IN INDIAN COUNTRY:  
IMPROVING HEALTH THROUGH WATER QUALITY RESEARCH AND  
AWARENESS

**Community-Based Participatory Research in Indian Country:  
Improving Health through Water Quality Research and Awareness**

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**Key Words**

Community-based participatory research, Native American, water quality, health  
disparities

**Abstract**

Water has always been held in high respect by the Apsaálooke (Crow) people of Montana. Tribal members questioned the health of the rivers and well water due to visible water quality deterioration and potential connections to illnesses in the community. Community members initiated collaboration among local organizations, the Tribe and academic partners, resulting in genuine community based participatory research. The article shares what we have learned as tribal members and researchers about working together to examine surface and groundwater contaminants, assess routes of exposure and use our data to bring about improved health of our people and our waters.

**Introduction**

Environmental health concerns of Native American and Canadian First Nations people are increasingly being addressed through community based participatory research (CBPR) partnerships among tribal communities, university researchers and others.<sup>1-6</sup> A frequently cited review of community-based research in public health defines it as “[A] collaborative approach to research that equitably involves, for example, community members, organizational representatives and researchers in all aspects of the research process.”<sup>7</sup> A number of health researcher teams conducting CBPR have shared their experiences in working with Native communities,<sup>8-14</sup> and in some cases the lead authors have been Native researchers.<sup>15-18</sup> However, rarely have Native American community members written about their perception of the value of the CBPR process to their

community, why they would participate in such research and how research should be conducted in their home community.<sup>15,19</sup> A better understanding of how to work with communities has been identified as a critical need in risk assessment research in particular.<sup>20</sup>

As members of the Apsaálooke (Crow) tribe in south central Montana, we identified deteriorating water quality as a critical environmental health issue in our community and recruited academic partners to help us conduct a local risk assessment of exposure to contaminants via water sources.<sup>21-29</sup> Data gathered in our collaborative efforts are quantifying our water quality problems, providing useful information to tribal members and are being successfully used to raise funds to improve water and wastewater treatment infrastructure in our community. We hope to also improve risk assessment modeling and methodology for Native American communities, in general. We meet monthly as a Steering Committee to work with our staff and academic partners to guide our collaborative work. Tribal members who are science majors at our local tribal college conduct most of the field work and community surveys; these students have been motivated to continue on in pursuing bachelor's degrees in environmental health and related fields and several are now in graduate school or are in the process of applying.

In this article, we describe how our project began, how our partnership with academic researchers developed and what we have learned and gained in the process. We hope that sharing our experiences will be helpful to other Reservation communities and possibly other minority communities who are faced with environmental health

challenges, as well as to University researchers partnering with communities to conduct community based research in environmental health.

### **Statement of the Problem: Battling for Healthy Water in Crow Indian Country**

When First Maker created the people that eventually became the Apsaálooke or Crow, he asked a duck to dive down in the water and bring up some earth. From this wet earth he sculpted the first Crow man and woman and breathed life into them (Harry Bull Shows, deceased). This creation story instills in the Crows a respect for the animals and for the earth; the earth is one of the three mothers of the Crows.

Like respect for the animals and mother earth, a fundamental tenet of the Crow is to respect water. Water comes in many forms (snow, ice, sleet, hail, rain and mist) and is powerful. Water has sustained the Crow people since their creation. Past and contemporary Crows grew up along the rivers, where they learned to swim, fish, and hunt – it was a way of life for us. Rivers provide sustenance to us and were our major source of home drinking water until the 1960s.

From the very beginning of the creation of the Crows to present, Crows have faced many adversities. One incident involved an attempt by enemy tribes to join forces and wipe out the Crows. A pitched battle took place along Arrow Creek. The Crows were losing and facing certain annihilation as they were vastly outnumbered until some of the Crow warriors sought spiritual guidance (Bear Don't Walk). Obviously, the Crows survived, but whom or what came to help them is reserved for later in this article.

Currently, the Crows, about 13,000 enrolled members, are faced with another insidious and ominous enemy: contamination and degradation of the Little Big Horn and other rivers which flow through the tribe's 2.2 million acre reservation located in south central Montana. As early as the 1950s, tribal members started filtering and boiling river water before drinking it. The river used to clear up after spring runoff, but by the mid 1960s it remained impaired and was aesthetically unpleasant (turbid, odiferous) throughout the summer. Although we no longer collect our drinking water directly from the rivers, the rivers continue to be essential to the maintenance of the Crow culture. Water from rivers and springs is used for spiritual ceremonies that are vital to the Crow people. Tribal members continue the traditional practice of feeding the river for protection and to show appreciation. For instance, after a successful hunt, a small portion of the raw meat is thrown into the river as an offering. The rivers are the municipal water source for the two largest communities in the area. Ground water sources are also impacted by contamination. Many rural residents obtain their water from wells that were drilled only to the first aquifer or "first water" to reduce costs. These wells are subject to over-flow from agricultural practices and are likely to be influenced by water drawn in from the nearby river. Another issue is the potential presence of radionuclides in water and air, since one reservation watershed has numerous abandoned uranium mines.

*Health Disparities:* Tribal members are concerned that the occurrence of disease seems greater on the Reservation than in other communities. A lack of Indian Health Service (IHS) funding for intervention and prevention has left the community with a sense of hopelessness. Health disparity data specific to the Crow Tribe are not readily

available; however, individuals noticed clusters of cancers and other ailments. Although most tribal members receive their healthcare at the local IHS hospital, some receive benefits through Medicaid or Medicare and are able to seek healthcare at off reservation clinics. Others have private health insurance; thus there is not a single source for tribal health statistics. This compounds the difficulty in collecting accurate health disparity data. Data from the Billings IHS Area Office includes eight Indian Reservations (seven in Montana and one in Wyoming) and is aggregated into a “Northern Plains Indian” group rather than by tribe or reservation. For example, a recent report by the Montana Central Tumor Registry (MCTR) indicates that American Indian residents in Montana have incidence rates of lung/bronchial cancer 1.6-fold higher and of stomach cancer 2.3-fold higher than white residents<sup>30</sup>. While smoking is believed to cause about 90% of lung cancer cases, radon exposure is responsible for about 10% and is the leading cause of lung cancer for non-smokers. The Reservation is in a high risk zone for radon but little is known about home air radon levels. Stomach cancer is also a concern: among Native Americans in Montana, *H. pylori* was associated with half of the cases of a specific stomach cancer, while this was one third for white Montanans<sup>30</sup>. As with home radon levels, little is known about exposure to *H. pylori*. Consequently, health disparities that are the focus of our efforts are lung and stomach cancers and gastro-intestinal illness.

The community began to suspect that increases in these diseases were associated with several environmental factors but primarily water. Additional changes in our rivers were noted in the early 1970s. The sewage lagoon at Crow Agency was leaking onto surrounding lands and into the river. Two community members went directly to the

Bureau of Indian Affairs (BIA) Facilities Manager to report their observations, but their concerns were summarily dismissed. One recalls the response he received from the Facilities Manager was that “the Navy” (probably meaning the Army Corps of Engineers) “approved the discharge of the raw sewage and if you don’t want to drink the water, you shouldn’t have been born.” Another red flag was raised when we began catching fish with lesions, clearly unfit for consumption. Frog, crawfish and clam populations were declining. Children contracted shigellosis in the summer, apparently from swimming in the rivers, which resulted in bloody diarrhea, fever and stomach cramps. The community members’ concerns were ignored by those who controlled the municipal water and wastewater systems.

As time passed, it became apparent no one else was going to take steps to clean up the rivers, thus two community members were compelled to organize and move forward with these issues. One had become a county commissioner and the other a respected construction manager; both had gained skills in voicing concerns. They began a campaign to improve the health of the rivers and the municipal water system. In 2000, these two men and others were appointed to the newly established Apsaálooke Water and Wastewater Authority (the “Authority”). This group was successful in acquiring BIA funding for a preliminary engineering report on the municipal water and waste water systems serving Crow Agency. Public recognition that the problems existed was finally confirmed.

The tribal health educator began sharing her concerns about health disparities related to the water with a local tribal college (Little Big Horn College, LBHC) science

faculty member while in the sweat lodge. The above-mentioned construction manager also started talking about local water quality issues with this same faculty member. She in turn sought assistance from the Indian Country Environmental Health Assessment Program to provide training for the community. This initial five-day training involved participants from a broad range of community agencies and included a reservation wide environmental health assessment. The assessment documented that water quality was the highest ranking environmental health issue. Some of the participants joined forces with the Authority and formed our working group, now known as the Crow Environmental Health Steering Committee (CEHSC). The Crow Water Project was on its way.

*Need for Data:* The Authority needed water quality data to submit grant applications for water and wastewater infrastructure improvements. The Tribe had neither the necessary data nor the capacity to generate it, but LBHC was already involved in local water quality monitoring. Data on the Reservation's rivers, collected by LBHC science majors with supervision from LBHC and Montana State University (MSU) faculty and staff and guidance from the CEHSC, has recently been used successfully by the Authority to attain grants. The data documents community concerns about water quality and its potential impact on our health. We now recognize that the pollution problems are worse than suspected.

Crow tribal members, like members of other Tribes, are sensitive about participating in research as we have been researched repeatedly with little or no benefit to the Tribe. This experience was different because Tribal members initiated the work, the data are useful to us and we are solving the problems we have identified. The fact that the

impetus for this research originated with the community continues to be the single most important factor in the overall success of our Crow Water Project.

Research has expanded beyond the initial efforts. Currently Tribal and MSU researchers are addressing community concerns about perceived cancer clusters by conducting a community based risk assessment of exposure to contaminants via water sources and select subsistence foods. This includes conducting community member surveys and comprehensive chemical and pathogen testing of drinking water sources. We focus on home well water, but we are also examining river water sources used for traditional activities and for municipal water systems.

### **Implementation of CBPR in a Native Community**

The critical element in making community based participatory research (CBPR) work is clear, unbiased and empowering communication between the University researchers and Tribal community members. It is important for key players to have a full comprehension of CBPR and how it impacts the community. Outreach to a broad spectrum of Tribal members is very important in developing the CBPR concept. Cultural sensitivity training for the University research team members - in the day to day life of Native communities - is hands on and in real life.

Our CBPR design is a creative, collaborative process that respects and takes into consideration: (a) the community's extensive knowledge of the local environment, environmental degradation and potentially related human health issues; (b) the community's traditional respect for and relationship to the land and rivers; (c) the

community's need for data to answer local questions; (d) Western scientific knowledge of environmental health and of risk assessment methodology; (e) legal considerations and (f) what research is valuable to other rural or minority communities and therefore worthy of outside funding. Community knowledge about cancer cases and historical and current sources of contamination has been invaluable in designing the research. Steering Committee members understand the scientific method and are able to connect it with tribal consciousness and knowledge.

Political support is critical to our ability to work together successfully. Since 2006, key officials of the Crow Tribe, LBHC and Montana State University (MSU) have all consistently supported our efforts and together signed a Memorandum of Understanding detailing conditions of the collaboration. The diversity of Tribal community members (age, gender, cultural and professional expertise, agencies represented) on our Steering Committee helps tremendously.

The CBPR concept pervades the organization of the research. We have a "flat hierarchy" for the CEHSC. Rather than elect a President, meeting facilitation is rotated through members of the Steering Committee. We share the responsibility of presenting our work, whether at local, state or national levels. Three Steering Committee members have traveled with LBHC project staff to co-present posters at national conferences. For the past three years our group has given a panel presentation annually at the state-wide Idea Network for Biomedical Research Excellence (INBRE) conference. This structure extends to our University partners and project principal investigators.

Much of the progress of the Crow Water Project has been accomplished on sheer people power. However, there was also a need for funding. After acquiring the initial data and working collaboratively, we have developed solid CBPR proposals. Community involvement was essential in every aspect from the initial planning to the writing of the proposals; collaboration was essential to express the community's initiative, commitment to and partnership in the research effort. Our partnership has grown from small projects to a much broader collaboration with INBRE funding from the National Center for Research Resources at the National Institutes of Health. The funding enhanced science education at the tribal college, including science faculty development and research capacity building. Funding has also come from the National Center for Minority Health and Health Disparities.

### **The Project as a Model for a CBPR Research Method**

From the researcher's perspective, the process developed to move the water related work forward serves as a model for how to develop a positive, functioning partnership between a community with health and environmental based research needs and university researchers. The learning experience is particularly helpful to those working with other Reservations, but is also applicable to other minority communities who are faced with environmental health challenges. The processes and insights are being viewed by the US EPA as a contribution to their initiative to incorporate community knowledge and insight into risk assessment. In particular, there is a growing need to manage risk in a community specific, culturally appropriate way that takes into account social, cultural and other non-chemical factors.

Our journey has also uncovered many of the challenges that are part of a community based effort. It is clear that the community has more trust if the research is driven by people in that community and when the main point of contact is a community member. Our project has been successful because the person who is conducting surveys and collecting samples on the Reservation is fluent in Crow and can communicate with those who value and routinely use their native language. Having students and staff from LBHC involved also demonstrates that the community is engaged in the work, rather than having it done by someone who is not local. It has been critical to know that cultural issues such as strong family ties will influence the pace of the work. Because there are many critical needs in the community, the work must continue to be relevant so that it retains its importance and focus. To serve this purpose, it is essential that the community knows that the work is important and yielding valuable information for them and not just the researcher. These are just a few of the lessons that we have learned in the process of developing, funding, and accomplishing the work that we collectively believe will improve the health of the environment and the people.

### **The Importance of Crow College Student Involvement**

Our projects are giving Crow science majors at LBHC and MSU the opportunity to participate in research in their home community on issues that are relevant and meaningful to them and to our Tribe as a whole. LBHC, like the majority of the 30+ tribal colleges around the country, offers Associates degrees and therefore student research experience is a relatively new addition to our educational programs. The CBPR

work has provided a unique opportunity to develop research capacity at the LBHC while also providing a meaningful research opportunity for the students. For the current research projects, field, lab and community-based survey and community education work is being conducted on the Reservation and at LBHC, while genetic analyses of organisms in water samples are being carried out at MSU. This provides Crow students with an opportunity to participate in research as freshmen and sophomores, without having to leave home. When they transfer to MSU, they can continue to stay involved as juniors and seniors and mentor the LBHC student interns. At least fifteen Crow students have participated in our Water Quality Project, either at LBHC or MSU or both; most have completed their bachelor's degree or are still in school in various science disciplines. One will finish her Master's degree this fall in science education, another will begin a Master's degree in community health this fall and others are now considering graduate school. For those of us who teach and mentor these students, it has been rewarding to see how much they are learning and how committed they are to the research.

The students contribute substantially to research in the community and in the university research laboratories. One intern commented that she sees her role, and that of other students as helping to connect the community and the academic researchers. In the Crow community, the term "researcher" has long had the connotation of "intruder." The survey and well water sampling work done by the research team simply could not be done by non-community members working alone. The interns and our project Coordinator are tribal members; they can translate the scientific language of water contamination into terms that make sense to other tribal members, thus increasing

community knowledge of water quality and health related issues. Educating the community to protect our surface and ground water can only be done effectively in person, for instance, by showing them potential problems with their well head. Distributing printed materials alone isn't very effective.

Our current community Project Coordinator began her involvement as a student intern and went on to complete her bachelor's degree in Environmental Science. She initially heard about the water quality project from other LBHC interns and applied to participate. Once she began her internship, she realized how polluted the rivers were, raising her awareness of the degradation of this resource. For example, as a child her family drank the river water untreated and swam in the river in the summer months without becoming ill. This is no longer the case, and as stated earlier, the water is sufficiently polluted that now it cannot safely be consumed without treatment. At LBHC she gained experience in baseline water quality monitoring. When she transferred to MSU for her bachelor's degree, she was accepted to work in our project's microbiology lab. There she was able to see what happened to the water samples when they got to the lab, and gained experience in molecular biology (polymerase chain reaction) techniques for identifying the isolated bacteria. This experience helped her in some of her other classes. Upon completing her bachelor's degree she was hired at LBHC as our Environmental Health Project Coordinator, where she is able to train, supervise and mentor the water quality interns. She finds that the students are eager to learn the protocols of water collecting and want to do the best job; they gain an appreciation for the importance of accuracy and precision in data collecting. The interns realize that their role

in the research project is very important, both to the community and to the university researchers.

Another former project intern went on to complete a bachelor's degree in hydrogeology and has just been hired for a one year math/science teaching position at LBHC. From the MSU side, a non-Native microbiology doctoral student who has worked with the project since being an undergraduate has also found the experience invaluable. She has gained a greater understanding of how to conduct research and outreach in a Native American community. On a Reservation where "research" has a toxic legacy, the increased success of our students in pursuing careers in environmental science and health and especially their interest in graduate school, is simply transformative.

## **Conclusion**

Earlier in this article, a survival story of the Crow was shared with a promise to tell what saved the Crows from certain annihilation. A warrior appeared seemingly from nowhere on the battlefield. He was invisible to the Crows, but the enemy said he was riding a beautiful pinto horse and wore a war bonnet with trailing extensions that nearly touched the ground. The Crows saw the enemy retreating, but they only learned of the appearance of this warrior upon parleying with the enemy years later. The enemy asked who the warrior was and the Crows did not immediately say, but after considerable discussion and thought, concluded that this must have been Isáahkawattee, a key character in Crow oral history who not only plays tricks on them, but is willing to come to their rescue in times of great need

(Bear Don't Walk). This begs the question: Is Isáahkawattee in some way involved in this community-based effort regarding the sacred waters and lands that Crow ancestors had bargained for in treaties and had set aside for future generations for as long as the grass shall grow and the rivers shall flow?

After years of inaction, indifference and/or the lack of resources to address our water quality, water infrastructure and related health disparity concerns, we have found that we can successfully take on these problems, identify the necessary researchers and effectively collaborate with them on mutually acceptable terms. We want to restore the health of our rivers and of our community. We realize it will take a broad based, grassroots effort to make this change. Passion, tenacity, persistence, mutual support and not letting one another quit are making this process work. We continue to learn more about health issues and how to facilitate change so that we can improve the well being of our Native community. We now feel empowered to take on other environmental health issues that affect our community.

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**Bibliography**

1. Roa Garcia CE, Brown S. Assessing water use and quality through youth participatory research in a rural Andean watershed. *J Environ Manage.* 2009;90:3040-3047.
2. Schell LM, Ravenscroft J, Cole M, Jacobs A, Newman J. Akwesasne task force on the environment. Health disparities and toxicant exposure of Akwesasne Mohawk young adults: A partnership approach to research. *Environ Health Perspect.* 2005;113(12):1826-1832.
3. Severtson DJ, Baumann LC, Will JA. A participatory assessment of environmental health concerns in an Ojibwa community. *Public Health Nurs.* 2002;19(1):47-58.

4. Wongittilin J. Traditional knowledge and contaminants project. *Int J Circumpolar Health*. 2001;60(4):454-460.
5. Garza D. Alaska Natives assessing the health of their environment. *Int J Circumpolar Health*. 2001;60(4):479-486.
6. Lawrence R. Community profiles/Indian housing in Yukon Territory. *Arctic Medical Research*. 1988;47(Suppl 1):149-154.
7. Israel BA, Schulz AJ, Parker EA, Becker AB. Review of community-based research: Assessing partnership approaches to improve public health. *Annu Rev Public Health*. 1998;19:173-202.
8. Holkup PA, Rodehorst TK, Wilhelm SL et. al. Negotiating three worlds: Academia, nursing science and Tribal communities. *Journal of Transcultural Nursing*. 2009;20(2):164-175.
9. Cashman SB, Adeky S, Allen AJ et. al. The power and the promise: Working with communities to analyze data, interpret findings and get outcomes. *Am J Public Health*. 2008;98(8):1407-1417.
10. Christopher S, Watts V, McCormick AKHG, Young S. Building and maintaining trust in a community-based participatory research partnership. *Am J Public Health*. 2008;98(8):1398-1406.
11. Schell LM, Ravenscroft J, Gallo M, Denham M. Advancing biocultural models by working with communities: A partnership approach. *A J Hum Biol*. 2007;19:511-524.

12. Wallerstein NB, Duran B. Using community-based participatory research to address health disparities. *Health Promot Pract.* 2006;7(3):1-12.
13. Quigley D. Perspective: A review of improved ethical practices in environmental and public health research: Case examples from Native communities. *Health Educ Behav.* 2006;33(2):130-147.
14. Holkup PA, Tripp-Reimer T, Salois EM, Weinert C. Community-based participatory research: An approach to intervention research with a Native American community. *Advances in Nursing Science.* 2004;27(3):162-175.
15. Manson SM, Buchwald DS. Enhancing American Indian and Alaska Native health research: A multi-faceted challenge. *J Interprof Care.* 2007;21(Suppl 2):31-39.
16. Salois EM, Holkup PA, Tripp-Reimer T, Weinert C. Research as spiritual covenant. *West J Nurs Res.* 2006;28(5):505-524.
17. Burhansstipanov L, Christopher S, Schumacher A. Lessons learned from community-based participatory research in Indian country. *Cancer Control.* 2005;(CC&L Suppl):70-76.
18. Cochran PAL, Marshall CA, Garcia-Downing C, et. al. Indigenous Ways of Knowing: Implications for Participatory Research and Community. *Am J Public Health.* 2008;98(1):22-27.
19. Arquette M, Cole M, Cook K et. al. Holistic risk-based environmental decision making: A Native perspective. *Environ Health Perspect.* 2002;110(Suppl 2):259-264.

20. Sanchez YA, Deener K, Hubal EC, Knowlton C, Reif D, Segal D. Research needs for community-based risk assessment: findings from a multi-disciplinary workshop. *J Expo Sci Environ Epidemiol.* 2009;1-10.
21. Cummins C, Eggers M, Hamner S, Camper A, Ford TE. Mercury levels detected in fish from rivers of the Crow Reservation, Montana. Poster presented at: 2009 SCREES National Water Conference; February 8-11, 2009; St. Louis, MO.
22. Eggers MJ, Cummins C, Crow Environmental Health Steering Committee, et. al. Community based risk assessment on the Crow Reservation. Poster presented at: NIH Summit: The Science of Eliminating Health Disparities; December 16-18, 2008; National Harbor, MD. (Abstract published.)
23. Sylvester AW, Tuthill D, Williams KE, Cummins C, Eggers MJ. Water quality assessment: Developing PCR-based methods to enhance environmental research at tribal colleges in the Rocky Mountain EPSCoR states. Poster presented at: NSF EPSCoR Water Workshop; November 9-12, 2008; Burlington, VT.
24. Eggers MJ, Cummins C, Plaggemeyer S, et. al. Community based risk assessment on the Crow Reservation. Poster presented at: National Institute for Health's IDeA Conference; August 6-8, 2008; Washington, D.C.
25. Richards CL, Eggers MJ, Broadaway S, Pyle B, Ford T. Occurrence of opportunistic pathogens in drinking water and associated biofilms in rural Montana. Poster presented at: National Institute for Health's IDeA Conference; August 6-8, 2008; Washington, D.C.

26. Eggers MJ, Morsette J, Whiteman GA, et. al. Community based risk assessment on the Crow Reservation. Poster presented at: USDA CSREES National Water Conference; Feb 3-6, 2008; Sparks, NV.
27. Eggers MJ, Jackson D, Sylvester A. Tribal college outreach: Academic partnerships and educational exchanges. Poster presented at: 49<sup>th</sup> Maize Genetics Conference; March 22-25, 2007; St. Charles, IL. (Abstract published.)
28. Eggers MJ. Global trends In human and ecosystem health: Links between land use, trade and climate change. Presented at: Environmental Protection Agency STAR/GRO Conference; September 24-26, 2006; Washington, D.C. (Abstract published.)
29. Eggers MJ. University/Tribal College partnerships: Hope now and for generations to come. Presented at: NSF Plant Genome Research Program Conference; Sept. 7-11, 2006; Washington, D.C. (Abstract published.)
30. Lemons D, Ballew C. Cancer in Montana - 2002-2006 - An annual report of the Montana Central Tumor Registry. 2008. Montana Department of Public Health and Human Services, Public Health and Safety Division. Helena, MT.

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APPENDIX C:

SUPPLEMENTARY TABLE OF COMPLETE LIST OF SIGNIFICANTLY  
REGULATED GENES

Supplementary Table. Total set of up and down regulated *H. pylori* genes stimulated by oxygen stress and nutrient deprivation treatments.

Gene ID	Gene function and classification	Oxygen stress (24h)	Nutrient stress (24h)	Nutrient stress (72h)	Nutrient stress (168h)
Up regulated genes					
Amino acid biosynthesis					
HP0652	phosphoserine phosphatase, <i>serB</i>	3.4	-0.4	-0.7	-0.9
jhp1150	aspartate kinase, monofunctional class	1.8	0.2	0.0	1.3
HP1210	serine acetyltransferase, <i>cysE</i>	1.5	0.6	0.7	1.7
HP1050	homoserine kinase, <i>thrB</i>	1.4	0.2	0.5	0.7
HP0330	ketol-acid reductoisomerase, <i>ilvC</i>	1.1	0.4	0.4	0.8
HP1468	branched-chain-amino-acid aminotransferase, <i>ilvE</i>	1.1	0.0	0.0	1.3
HP0672	solute-binding signature and mitochondrial signature protein, <i>aspB</i>	1.0	-0.2	-0.3	-0.3
HP0290	diaminopimelate decarboxylase (dap decarboxylase), <i>lysA</i>	0.7	0.4	0.6	1.4
jhp1294	Prephenate dehydrogenase	0.5	0.4	-0.1	2.0
Biosynthesis of cofactors, prosthetic groups, and carriers					
HP1221	undecaprenyl diphosphate synthase	2.8	0.0	0.1	-1.0
HP0239	glutamyl-tRNA reductase, <i>hemA</i>	1.3	0.5	-1.1	-0.2
HP0798	molybdenum cofactor biosynthesis protein C, <i>moaC</i>	1.3	0.0	0.8	1.0
HP0843	thiamin phosphate pyrophosphorylase/hydroxyethylthiazole kinase, <i>thiB</i>	1.0	0.3	0.0	2.1
HP0804	GTP cyclohydrolase II/3,4-dihydroxy-2-butanone 4-phosphate synthase, <i>ribAB</i>	1.0	-0.1	0.5	1.4
HP1140	biotin operon repressor/biotin acetyl coenzyme A carboxylase synthetase, <i>birA</i>	0.9	0.1	0.8	1.5
Cell envelope					
HP0725	outer membrane protein, <i>omp17</i>	3.8	0.2	0.0	0.6
jhp0424	outer membrane protein	3.4	0.6	0.2	0.3
jhp1002	fucosyltransferase	3.1	0.7	0.6	2.9

**Table continued**

HP0009	outer membrane protein, <i>omp1</i>	2.7	0.9	0.2	0.7
HP1052	UDP-3-0-acyl N-acetylglucosamine deacetylase, <i>envA</i>	2.7	0.3	-0.1	-0.4
HP0472	outer membrane protein, <i>omp11</i>	2.6	0.2	-0.4	0.6
HP0655	protective surface antigen D15	2.2	0.4	-0.2	-0.4
HP0122	lipoprotein, putative	2.1	0.3	0.1	-0.6
HP0805	lipooligosaccharide 5G8 epitope biosynthesis-associated protein, <i>lex2B</i>	1.9	-0.2	-0.2	-0.8
HP0638	outer membrane protein, <i>omp13</i>	1.8	0.2	0.1	0.4
HP0087	lipoprotein, putative	1.6	-0.1	0.0	-0.3
jhp0593	UDP-N-acetylglucosamine 1-carboxyvinyltransferase, <i>murA</i>	1.3	0.2	0.9	2.6
HP0018	lipoprotein, putative	1.3	0.8	0.1	1.1
HP0289	toxin-like outer membrane protein	1.2	0.3	0.2	1.0
HP1501	outer membrane protein, <i>omp32</i>	1.0	0.2	0.6	0.4
HP1373	rod shape-determining protein, <i>mreB</i>	0.7	0.3	0.5	1.1
jhp0007	outer membrane protein	0.5	-0.2	0.7	2.1
HP1157	outer membrane protein, <i>omp26</i>	-0.2	0.8	0.4	2.2
HP0648	UDP-N-acetylglucosamine enolpyruvyl transferase, <i>murZ</i>	-0.3	0.3	1.2	0.9
HP0859	ADP-L-glycero-D-mannoheptose-6-epimerase, <i>rfaD</i>	-0.6	0.3	1.4	0.0
HP0057	lipoprotein, putative	-2.0	0.2	1.3	0.0
Cellular processes-adaptation to atypical environments					
HP1228	invasion protein, <i>invA</i>	3.3	0.4	0.3	0.7
Cellular processes-cell division					
HP1556	cell division protein, <i>ftsI</i>	1.4	0.2	-0.5	-0.7
jhp1161	maf protein, <i>maf</i>	0.2	-0.3	-0.1	1.1
Cellular processes-chemotaxis and motility					
HP0584	flagellar switch protein, <i>fliN</i>	2.2	0.3	1.0	0.3
HP1558	flagellar basal-body rod protein (proximal rod protein), <i>flgC</i>	2.0	0.3	0.0	-0.2
HP0246	flagellar basal-body P-ring protein, <i>flgI</i>	1.9	0.5	-0.2	0.3

**Table continued**

HP0325	flagellar basal-body L-ring protein, <i>flgH</i>	1.3	0.0	-2.1	-0.5
Cellular processes-detoxification					
jhp0252	chlorohydrolase	-0.6	0.0	0.4	2.9
HP1563	alkyl hydroperoxide reductase, <i>tsaA</i>	-1.9	0.2	1.0	0.4
Cellular processes-pathogenicity					
HP0535	cag pathogenicity island protein, <i>cagI4</i>	1.9	0.0	-0.5	-1.3
Cellular processes-toxin production and resistance					
HP1431	16S rRNA (adenosine-N6,N6-)- dimethyltransferase, <i>ksgA</i>	1.6	0.3	0.0	0.1
Central intermediary metabolism					
jhp0976	probable aminotransferase	1.2	-0.8	-1.2	0.0
DNA metabolism					
HP1208	ulcer associated adenine specific DNA methyltransferase	3.8	-0.5	0.3	1.8
HP0092	type II restriction enzyme M protein, <i>hsdM</i>	3.4	0.2	0.2	1.5
HP0012	DNA primase, <i>dnaG</i>	2.0	0.4	0.0	0.7
HP0091	type II restriction enzyme R protein, <i>hsdR</i>	1.6	0.2	0.2	0.9
HP1209	ulcer-associated gene restriction endonuclease, <i>iceA</i>	1.3	-0.2	0.1	1.6
jhp0435	cytosine-specific DNA methyltransferase	1.2	0.0	-0.1	0.8
HP0883	Holliday junction DNA helicase, <i>ruvA</i>	1.0	0.1	0.8	1.3
HP1529	chromosomal replication initiator protein, <i>dnaA</i>	0.1	1.0	0.1	0.8
Energy metabolism					
HP1261	NADH-ubiquinone oxidoreductase, NQO6 subunit	1.8	0.4	0.3	0.5
HP1212	ATP synthase F0, subunit c, <i>atpE</i>	1.5	0.4	0.6	1.0
HP1227	cytochrome c553	1.4	0.2	0.7	-0.4

**Table continued**

HP1386	D-ribulose-5-phosphate 3 epimerase, <i>rpe</i>	1.4	0.3	0.1	0.1
jhp0661	l-asparaginase	1.2	0.3	0.6	1.6
jhp0908	phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent, <i>gpmA</i>	0.2	0.3	0.6	2.0
jhp1281	Carbohydrate kinase family	0.1	0.8	0.9	4.6
HP0277	ferredoxin	-0.4	0.0	1.1	-0.2
jhp0126	iron-sulfur cluster binding protein	-0.6	-0.4	0.0	1.6
HP0378	cytochrome c biogenesis protein, <i>ycf5</i>	-1.7	0.4	1.5	0.5
Fatty acid and phospholipid metabolism					
HP0808	holo-acp synthase, <i>acpS</i>	1.7	-0.7	-0.6	-0.6
jhp0968	cyclopropane-fatty-acyl-phospholipid synthase	-0.1	-0.2	0.0	1.4
Hypothetical proteins					
HP1440	hypothetical protein	3.8	-0.6	-0.6	-0.7
HP0708	hypothetical protein	3.4	0.2	0.2	0.5
HP1207	hypothetical protein	3.2	0.1	0.3	0.4
HP1515	hypothetical protein	3.1	0.2	0.0	0.1
HP1230	hypothetical protein	3.1	0.2	0.6	0.6
HP0015	hypothetical protein	3.0	0.0	-0.7	0.2
HP1589	conserved hypothetical protein	2.9	0.1	-0.1	0.5
HP1449	conserved hypothetical protein	2.9	0.2	0.4	0.5
HP1502	hypothetical protein	2.8	-0.6	-0.1	-0.8
HP0682	hypothetical protein	2.7	-0.8	-0.1	3.0
HP1001	hypothetical protein	2.7	0.2	-1.4	-0.7
HP0016	hypothetical protein	2.7	-1.0	-1.4	-1.9
HP1504	conserved hypothetical protein	2.6	-0.1	0.0	0.8
HP0412	hypothetical protein	2.5	0.7	1.6	1.6
HP0707	conserved hypothetical protein	2.5	0.0	0.2	0.3
HP0342	hypothetical protein	2.4	-0.4	0.0	-0.2
HP1334	hypothetical protein	2.4	-0.1	0.4	1.1
HP0341	hypothetical protein	2.3	0.2	0.2	1.2
HP1331	conserved hypothetical integral membrane protein	2.3	0.0	-0.1	0.3
HP0533	hypothetical protein	2.3	0.5	-0.2	-0.1

**Table continued**

HP0036	hypothetical protein	2.2	0.5	0.2	1.1
HP1056	hypothetical protein	2.2	0.3	0.3	1.0
jhp1208	conserved hypothetical protein	2.2	-0.1	-0.5	0.4
HP0186	hypothetical protein	2.1	-0.1	0.0	0.0
jhp0623	conserved hypothetical protein	2.1	0.3	0.6	2.6
HP1145	hypothetical protein	2.0	0.0	-0.1	0.2
HP1590	hypothetical protein	2.0	-0.1	-0.5	-0.6
HP1049	conserved hypothetical protein	2.0	0.1	0.0	0.3
HP0337	hypothetical protein	2.0	-0.3	-0.1	0.0
HP0339	hypothetical protein	2.0	0.1	-0.1	0.5
HP0199	hypothetical protein	1.9	-0.8	0.4	1.7
HP0408	hypothetical protein	1.9	-0.3	-0.4	-0.7
HP1055	hypothetical protein	1.9	-0.4	-0.4	-0.1
HP1330	conserved hypothetical integral membrane protein	1.8	0.5	-0.7	0.4
HP1054	hypothetical protein	1.8	0.4	0.0	0.1
HP1146	hypothetical protein	1.7	-0.4	-0.7	-0.6
HP0129	hypothetical protein	1.7	0.2	0.1	-0.5
HP0809	hypothetical protein	1.6	-0.2	-0.3	-0.9
HP0563	hypothetical protein	1.5	0.3	0.4	0.5
HP0398	hypothetical protein	1.5	-0.3	-0.7	-0.6
HP0656	conserved hypothetical protein	1.5	-0.1	0.6	0.3
HP0270	hypothetical protein	1.5	0.2	0.6	-0.1
HP0030	hypothetical protein	1.5	0.2	-0.2	0.1
HP0811	hypothetical protein	1.5	0.3	0.3	0.2
HP0131	hypothetical protein	1.4	0.1	0.3	0.7
HP0014	hypothetical protein	1.4	0.4	-0.8	-0.4
HP1288	hypothetical protein	1.4	-0.6	-0.8	-0.1
HP0487	hypothetical protein	1.4	-0.2	0.2	-0.7
HP0188	hypothetical protein	1.3	-0.2	-0.5	-0.4
HP0128	hypothetical protein	1.3	-0.5	-0.5	-0.6
HP0486	hypothetical protein	1.2	0.1	0.1	0.4
HP0758	conserved hypothetical integral membrane protein	1.2	0.2	0.8	0.8
jhp0173	conserved hypothetical protein	1.2	-0.2	0.0	-0.4
HP0035	conserved hypothetical protein	1.2	0.1	-0.1	0.5
HP0161	hypothetical protein	1.2	0.2	-0.5	-1.0
HP1053	hypothetical protein	1.2	0.0	0.0	0.8

**Table continued**

HP0013	hypothetical protein	1.2	0.2	-0.1	-0.2
HP0038	hypothetical protein	1.1	0.0	-0.5	0.1
HP0465	conserved hypothetical protein	1.1	-0.1	0.0	1.5
HP1548	conserved hypothetical integral membrane protein	1.0	0.1	0.0	-0.7
jhp0153	conserved hypothetical protein	1.0	0.4	2.4	2.6
HP0628	hypothetical protein	1.0	0.2	0.0	2.0
jhp0457	conserved hypothetical protein TIGR000	0.9	0.4	0.6	2.0
HP0556	hypothetical protein	0.8	-0.2	0.1	3.4
HP0489	hypothetical protein	0.8	0.6	0.7	2.0
HP0206	hypothetical protein	0.7	0.3	1.2	2.5
HP1240	conserved hypothetical protein	0.7	0.4	0.0	1.1
HP0582	hypothetical protein	0.7	-0.2	-0.2	1.8
HP0233	conserved hypothetical protein	0.6	0.1	1.1	0.2
HP0046	hypothetical protein	0.5	-0.3	1.3	1.4
HP0114	hypothetical protein	0.5	0.0	0.1	1.5
HP1570	conserved hypothetical protein	0.4	1.5	1.2	1.6
HP0226	conserved hypothetical integral membrane protein	0.2	0.3	-0.7	1.1
HP1029	hypothetical protein	0.1	0.7	1.2	2.9
HP1525	hypothetical protein	0.0	0.6	1.3	0.8
HP1414	conserved hypothetical protein	-0.1	0.5	1.1	-0.5
jhp0527	conserved hypothetical protein	-0.2	1.4	0.0	0.0
HP0507	conserved hypothetical protein	-0.3	0.3	0.9	1.5
HP0812	hypothetical protein	-0.3	-0.2	-0.2	1.1
HP0944	conserved hypothetical protein	-0.5	0.0	-0.1	2.2
Protein fate					
HP1549	protein-export membrane protein, <i>secF</i>	2.1	0.1	0.4	0.1
HP0033	ATP-dependent C1p protease, <i>clpA</i>	1.9	0.2	-0.5	-0.7
HP1332	co-chaperone and heat shock protein, <i>dnaJ</i>	1.8	-0.2	0.4	0.1
HP1550	protein-export membrane protein, <i>secD</i>	1.3	0.4	0.4	0.4
HP0657	processing protease, <i>ymxG</i>	1.2	0.2	0.2	0.2
Protein synthesis					

**Table continued**

HP0123	threonyl-tRNA synthetase, <i>thrS</i>	2.6	0.3	0.3	0.2
HP1201	ribosomal protein L1, <i>rp11</i>	1.8	0.2	0.2	-0.1
jhp1236	ribosomal protein L2, <i>rplB</i>	1.7	-0.3	0.3	-0.2
HP1319	ribosomal protein L3, <i>rpl3</i>	1.7	0.2	0.4	0.6
HP1318	ribosomal protein L4, <i>rpl4</i>	1.6	-0.4	-0.8	-0.3
HP1048	translation initiation factor IF-2, <i>infB</i>	1.6	0.5	0.6	1.5
HP0084	ribosomal protein L13, <i>rpl13</i>	1.3	-0.1	-0.1	-0.4
HP1315	ribosomal protein S19, <i>rps19</i>	1.3	-0.2	-0.2	-0.7
HP1295	ribosomal protein S11, <i>rps11</i>	1.2	0.1	0.2	0.0
HP0124	translation initiation factor IF-3, <i>infC</i>	1.2	0.2	-1.0	-2.7
HP1310	ribosomal protein S17, <i>rps17</i>	1.2	-0.3	-0.5	-1.2
HP0083	ribosomal protein S9, <i>rps9</i>	1.2	0.1	0.6	0.1
HP1316	ribosomal protein L2, <i>rpl2</i>	1.1	-0.1	0.3	0.1
HP1311	ribosomal protein L29, <i>rpl29</i>	1.1	-0.1	-0.1	-0.6
HP1147	ribosomal protein L19, <i>rpl19</i>	1.0	-0.1	0.4	-0.9
HP1294	ribosomal protein S4, <i>rps4</i>	1.0	0.2	0.5	0.2
HP1497	peptidyl-tRNA hydrolase, <i>pth</i>	0.7	-0.5	0.1	1.7
HP0281	tRNA-guanine transglycosylase, <i>tgt</i>	0.6	0.3	0.1	4.1
HP0402	phenylalanyl-tRNA synthetase, beta subunit, <i>pheT</i>	0.2	0.4	0.4	1.3
jhp0170	lysyl-tRNA synthetase, <i>lysS</i>	-0.1	0.7	0.8	1.4
Mobile and extrachromosomal element functions					
HP1000	PARA protein	2.1	0.3	0.2	-0.5
Purines, pyrimidines, nucleosides, and nucleotides					
HP1218	glycinamide ribonucleotide synthetase, <i>purD</i>	1.9	0.4	0.9	0.6
HP0409	GMP synthase, <i>guaA</i>	1.8	-0.3	0.2	-0.6
HP0919	carbamoyl-phosphate synthase (glutamine-hydrolysing), <i>pyrAb</i>	0.0	-0.3	1.2	-0.3
HP0198	nucleoside diphosphate kinase, <i>ndk</i>	0.0	0.6	1.1	1.1
Regulatory functions					
HP0088	RNA polymerase sigma-70 factor, <i>rpoD</i>	1.2	0.0	-0.3	-0.4

**Table continued**

jhp1180	transcriptional regulator, Sir2 family	0.4	0.3	1.1	1.5
jhp1282	probable histidine kinase sensor protein	0.4	0.1	0.5	2.4
Transcription					
HP1514	transcription termination factor, <i>nusA</i>	2.0	0.6	0.6	0.7
HP1448	ribonuclease P, protein component, <i>rnpA</i>	1.7	-0.1	-0.5	-0.4
Transport and binding proteins					
HP1220	ABC transporter, ATP-binding protein, <i>yhcG</i>	3.4	0.2	0.2	-0.3
HP0471	glutathione-regulated potassium-efflux system protein, <i>kefB</i>	3.0	-0.4	-0.5	-0.1
HP1491	phosphate permease	2.9	0.5	-0.4	0.5
HP1503	cation-transporting ATPase, P-type, <i>copA</i>	2.3	0.1	0.2	0.4
HP0251	oligopeptide ABC transporter, permease protein, <i>oppC</i>	1.9	0.2	0.2	0.2
HP1180	pyrimidine nucleoside transport protein, <i>nupC</i>	1.5	0.3	0.3	0.3
HP0300	dipeptide ABC transporter, permease protein, <i>dppC</i>	1.4	0.5	0.3	0.0
HP0301	dipeptide ABC transporter, ATP-binding protein, <i>dppD</i>	1.4	0.2	-0.2	-0.2
HP0299	dipeptide ABC transporter, permease protein, <i>dppB</i>	1.2	0.5	-0.5	-0.6
HP1577	ABC transporter, permease protein, <i>yae</i>	0.7	0.3	0.4	1.3
Unknown function					
HP0405	nifS-like protein	2.3	0.5	-0.6	0.0
jhp0376	Protein of unknown function (DUF448) superfamily	1.9	0.0	0.5	1.1
HP0431	protein phosphatase 2C homolog, <i>ptc1</i>	1.8	0.3	-0.4	0.4
jhp1123	50s ribosomal protein L10	1.8	-0.4	-0.5	-1.2
jhp0432	GTP-binding protein, <i>typA</i>	0.6	0.1	0.3	2.4
jhp0395	Domain of unknown function (DUF386) superfamily	0.4	0.1	0.7	1.9

**Table continued**

HP0207	ATP-binding protein, <i>mpr</i>	0.1	0.3	0.3	1.1
Down regulated genes					
Amino acid biosynthesis					
	hydantoin utilization protein A,				
HP0695	<i>hyuA</i>	-2.6	-0.4	-0.8	-0.8
HP0098	threonine synthase, <i>thrC</i>	-1.7	0.5	-0.6	-0.7
	serine hydroxymethyltransferase,				
HP0183	<i>glyA</i>	-1.6	-0.1	0.0	-1.1
HP1038	3-dehydroquinase type II, <i>aroQ</i>	-1.4	0.1	0.2	-0.4
	tryptophan synthase, alpha subunit,				
HP1277	<i>trpA</i>	-1.1	-0.6	0.4	0.0
HP0510	dihydrodipicolinate reductase, <i>dapB</i>	-1.0	0.3	0.6	0.0
HP0649	aspartate ammonia-lyase, <i>aspA</i>	-1.0	-0.3	-0.3	-0.2
Biosynthesis of cofactors, prosthetic groups, and carriers					
	glutamate-1-semialdehyde 2,1-				
HP0306	aminomutase, <i>hemL</i>	-1.6	0.1	0.6	-0.5
	nicotinate-nucleotide				
HP1355	pyrophosphorylase, <i>nadC</i>	-1.4	0.6	0.8	0.4
HP1118	gamma-glutamyltranspeptidase, <i>ggt</i>	-1.2	0.3	0.3	0.3
	octaprenyl-diphosphate synthase,				
HP0240	<i>ispB</i>	-0.2	0.1	-0.3	-1.9
Cell envelope					
HP0913	outer membrane protein, <i>omp21</i>	-2.3	-0.3	-0.8	-0.8
HP1039	membrane protein, putative	-1.9	0.0	0.1	-0.6
HP0229	outer membrane protein, <i>omp6</i>	-1.7	-0.1	-0.4	-0.4
HP1395	outer membrane protein, <i>omp30</i>	-1.6	0.0	-0.1	-0.3
	soluble lytic murein				
HP0645	transglycosylase, <i>slt</i>	-1.6	0.3	-0.1	-1.2
HP1564	outer membrane protein	-1.4	0.0	-0.2	-0.6
HP1177	outer membrane protein, <i>omp27</i>	-1.1	0.2	0.2	0.9
HP0317	outer membrane protein, <i>omp9</i>	-1.0	0.1	-0.4	-0.2
HP0743	rod shape-determining protein	-1.0	-0.3	0.5	-0.3
Cellular processes-chemotaxis and motility					
	purine-binding chemotaxis protein,				
HP0391	<i>cheW</i>	-3.5	0.0	0.0	-1.1

**Table continued**

	secreted protein involved in				
HP1192	flagellar motility	-2.2	0.2	0.6	0.0
HP0392	histidine kinase, <i>cheA</i>	-1.9	-0.2	-0.3	-0.6
jhp1355	putative motility protein	-1.1	-0.2	-1.0	-2.4
HP0099	methyl-accepting chemotaxis protein, <i>tlpA</i>	-1.0	-0.1	-0.6	-0.8
Cellular processes-detoxification					
HP0874	KapA protein, <i>kapA</i>	-3.0	-0.6	-1.4	-1.8
HP0875	catalase	-2.1	-0.6	-0.6	-0.7
HP0389	superoxide dismutase, <i>sodB</i>	-2.0	-0.2	-0.2	-1.0
HP0243	neutrophil activating protein, <i>napA</i>	-1.8	-0.3	-0.1	0.0
Cellular processes-other					
HP0517	GTP-binding protein	-1.1	0.0	0.0	-0.7
Cellular processes-pathogenesis					
HP0543	<i>cag</i> pathogenicity island protein, <i>cag22</i>	-2.6	-0.5	-1.1	-1.8
HP0545	<i>cag</i> pathogenicity island protein, <i>cag24</i>	-2.1	-0.4	-0.9	-1.1
HP0547	<i>cag</i> pathogenicity island protein, <i>cag26</i>	-1.4	-0.1	0.1	0.1
HP0546	<i>cag</i> pathogenicity island protein, <i>cag25</i>	-1.2	0.2	0.3	0.2
Cellular processes-toxin production and resistance					
HP0630	modulator of drug activity, <i>mda66</i>	-2.7	-0.2	0.1	-0.7
HP0887	vacuolating cytotoxin, <i>vacA</i>	-1.7	-0.2	-0.5	-0.4
Central intermediary metabolism					
HP0071	urease accessory protein, <i>ureI</i>	-3.7	0.2	0.3	-0.3
HP0068	urease accessory protein, <i>ureG</i>	-3.2	0.2	0.5	0.0
HP0073	urease, alpha subunit, <i>ureA</i>	-2.1	0.0	-0.3	-0.6
HP1186	carbonic anhydrase	-2.0	0.0	-0.6	-0.3
HP0070	urease accessory protein, <i>ureE</i>	-1.8	-0.5	-1.1	-1.0
HP0072	urease beta subunit (urea amidohydrolase), <i>ureB</i>	-1.6	0.2	0.1	0.2
HP0696	N-methylhydantoinase	-1.5	0.0	-0.1	-0.1

**Table continued**

HP0067	urease accessory protein, <i>ureH</i>	-1.5	0.3	0.6	0.3
	hydrogenase expression-formation				
HP0900	protein, <i>hypB</i>	-1.4	-0.2	-0.3	-0.3
DNA metabolism					
jhp0141	recombinase A, <i>recA</i>	-1.7	-0.2	-0.1	-0.9
HP0275	ATP-dependent nuclease, <i>addB</i>	-1.2	-0.2	-0.5	-0.3
HP1526	exodeoxyribonuclease	-1.1	0.0	-0.4	-0.5
HP0548	DNA helicase, putative	-0.1	0.2	0.7	-1.2
Energy metabolism					
	indolepyruvate ferredoxin				
jhp1035	oxidoreductase	-3.4	-1.1	-1.5	-2.8
	NADH-ubiquinone oxidoreductase,				
HP1267	NQO8 subunit	-2.7	0.1	0.2	0.0
HP0824	thioredoxin, <i>trxA</i>	-2.6	0.0	-0.2	-0.7
HP1161	flavodoxin, <i>fldA</i>	-2.6	0.3	0.1	-0.5
	NADH-ubiquinone oxidoreductase,				
HP1273	NQO14 subunit	-2.4	0.2	0.6	0.3
jhp0764	Thioredoxin reductase, <i>trxB</i>	-2.2	-0.3	-0.3	-0.8
	pyruvate ferredoxin oxidoreductase,				
HP1108	gamma subunit	-2.1	0.2	0.2	-0.4
	ferredoxin oxidoreductase beta				
jhp1038	subunit	-2.0	0.2	-0.3	-0.5
	NADH-ubiquinone oxidoreductase,				
HP1269	NQO10 subunit	-2.0	0.4	0.2	-0.3
HP0779	aconitase B, <i>acnB</i>	-1.9	0.0	-0.7	-0.2
	pyruvate ferredoxin oxidoreductase,				
HP1111	beta subunit	-1.8	0.0	-0.4	-0.1
HP0154	enolase, <i>eno</i>	-1.8	-0.7	-0.1	-0.7
	ubiquinol cytochrome c				
	oxidoreductase, cytochrome b				
HP1539	subunit, <i>fbcH</i>	-1.7	0.1	0.2	-0.1
	cytochrome c oxidase, monoheme				
HP0145	subunit, membrane-bound, <i>fixO</i>	-1.7	-0.1	0.7	0.2
	ferredoxin oxidoreductase, gamma				
HP0591	subunit	-1.6	-0.4	-0.6	-1.1
	NADH-ubiquinone oxidoreductase,				
HP1266	NQO3 subunit	-1.6	0.1	0.1	0.0
	NADH-ubiquinone oxidoreductase,				
HP1271	NQO12 subunit	-1.6	0.5	0.2	0.0

**Table continued**

HP1109	pyruvate ferredoxin oxidoreductase, delta subunit	-1.5	-0.1	-0.1	-0.4
HP1272	NADH-ubiquinone oxidoreductase, NQO13 subunit	-1.5	0.3	0.9	-0.6
HP1268	NADH-ubiquinone oxidoreductase, NQO9 subunit	-1.5	0.0	0.1	0.2
HP1134	ATP synthase F1, subunit alpha, <i>atpA</i>	-1.5	0.0	-0.2	0.2
HP1133	ATP synthase F1, subunit gamma, <i>atpG</i>	-1.4	-0.2	0.1	-0.3
HP0691	3-oxoadipate coA-transferase subunit A, <i>yxjD</i>	-1.4	-0.3	-1.0	-0.8
HP0176	fructose-bisphosphate aldolase, <i>tsr</i>	-1.4	-0.1	0.5	-0.2
HP0193	fumarate reductase, cytochrome b subunit, <i>frdC</i>	-1.4	0.3	-0.2	-0.8
HP0146	cbb3-type cytochrome c oxidase subunit Q, <i>CcoQ</i>	-1.4	-0.4	-0.1	-1.4
HP1495	transaldolase, <i>tal</i>	-1.4	0.1	0.4	-0.5
HP1345	phosphoglycerate kinase	-1.3	-0.3	-0.2	-0.2
HP0056	delta-1-pyrroline-5-carboxylate dehydrogenase	-1.3	0.5	0.3	0.1
HP0147	cytochrome c oxidase, diheme subunit, membrane-bound, <i>fixP</i>	-1.3	-0.1	0.4	-0.8
HP0294	aliphatic amidase, <i>amiE</i>	-1.2	0.1	0.1	0.1
HP0589	ferredoxin oxidoreductase, alpha subunit	-1.2	-0.3	-0.3	-0.5
HP0590	ferredoxin oxidoreductase, beta subunit	-1.1	-0.2	-0.3	-0.4
HP0633	quinone-reactive Ni-Fe hydrogenase, cytochrome b subunit, <i>hydC</i>	-1.1	0.0	0.2	-0.3
HP0954	oxygen-insensitive NAD(P)H nitroreductase	-1.1	0.0	0.3	0.0
HP1399	arginase, <i>rocF</i>	0.0	-0.8	-1.5	-1.9
Fatty acid and pholpholipid metabolism					
HP0700	diacylglycerol kinase	-2.7	-1.0	-0.9	-2.4
HP0371	biotin carboxyl carrier protein	-1.1	0.6	0.9	0.7
HP0557	acetyl-coenzyme A carboxylase	-0.2	-0.3	0.0	-1.0

## Hypothetical proteins

**Table continued**

	conserved hypothetical secreted				
HP1286	protein	-3.5	-0.1	0.4	-0.7
HP0318	conserved hypothetical protein	-1.9	-0.2	0.2	-0.5
HP1020	conserved hypothetical protein	-1.9	0.1	0.6	-0.4
	conserved hypothetical secreted				
HP0139	protein	-1.8	-0.2	-0.7	-0.8
HP1588	conserved hypothetical protein	-1.8	-0.1	-0.1	-0.1
HP1037	conserved hypothetical protein	-1.8	-0.1	-0.5	-0.4
HP0891	conserved hypothetical protein	-1.7	0.0	0.1	-0.2
	conserved hypothetical secreted				
HP1098	protein	-1.6	0.2	-0.3	0.1
	conserved hypothetical integral				
HP0693	membrane protein	-1.5	-0.1	-1.1	-1.4
	conserved hypothetical integral				
HP0284	membrane protein	-1.5	0.0	0.5	0.0
HP0388	conserved hypothetical protein	-1.4	-0.4	-0.1	-1.0
jhp1045	hypothetical protein	-1.3	-1.5	0.1	-0.3
HP0105	conserved hypothetical protein	-1.3	-0.1	0.2	-0.6
	conserved hypothetical integral				
HP0851	membrane protein	-1.2	0.1	0.5	-0.8
	conserved hypothetical secreted				
HP1117	protein	-1.1	-0.2	-0.4	-0.7
HP0710	conserved hypothetical protein	-1.1	-0.1	-0.5	-0.4
jhp1462	hypothetical protein	-1.0	-0.2	0.0	0.3
	conserved hypothetical secreted				
HP0190	protein	-0.8	-0.3	0.1	-1.9
HP0813	conserved hypothetical protein	-0.8	0.0	-0.6	-1.8
	conserved hypothetical secreted				
HP0518	protein	-0.8	0.6	0.5	-1.1
jhp1160	hypothetical protein	-0.7	-1.1	-3.8	-3.0
HP0162	conserved hypothetical protein	-0.2	-0.2	0.4	-1.2
jhp0926	hypothetical protein	0.2	0.1	-1.5	0.0
HP1173	hypothetical protein	-4.2	-0.4	-1.0	-3.1
HP0721	hypothetical protein	-3.0	0.2	0.4	-0.7
HP0697	hypothetical protein	-2.8	0.0	0.0	-0.7
HP0719	hypothetical protein	-2.4	0.1	0.3	-0.7
HP0720	hypothetical protein	-2.4	-0.1	-0.2	-1.3
HP1033	hypothetical protein	-2.3	-0.5	-1.3	-2.2
HP0788	hypothetical protein	-2.0	0.1	-0.1	-1.1
HP0156	hypothetical protein	-1.6	-0.2	0.3	0.2

**Table continued**

HP1349	hypothetical protein	-1.6	-0.3	0.2	-1.1
HP0852	hypothetical protein	-1.6	-0.5	-0.6	-0.8
HP0307	hypothetical protein	-1.5	-0.1	0.1	0.0
HP0155	hypothetical protein	-1.5	-0.4	-0.4	-1.1
HP0184	hypothetical protein	-1.5	-0.2	0.0	-0.6
HP1524	hypothetical protein	-1.5	-0.3	-0.4	-1.0
HP0185	hypothetical protein	-1.4	-0.2	-0.3	-0.3
HP1018	hypothetical protein	-1.3	-0.3	0.4	-0.9
HP1527	hypothetical protein	-1.3	0.1	-0.7	0.0
HP0776	hypothetical protein	-1.3	-0.2	0.0	-0.5
HP0650	hypothetical protein	-1.3	0.0	-0.3	-0.4
HP1128	hypothetical protein	-1.3	-0.3	-0.3	-0.6
HP0993	hypothetical protein	-1.3	0.2	-1.2	-1.2
HP1074	hypothetical protein	-1.3	-0.4	-0.1	-1.0
HP0953	hypothetical protein	-1.3	-0.2	-0.3	-0.4
HP0873	hypothetical protein	-1.2	0.1	-0.1	-1.0
HP0137	hypothetical protein	-1.2	-0.3	-0.1	-0.6
HP0932	hypothetical protein	-1.0	0.2	0.5	-0.9
HP0241	hypothetical protein	-1.0	-0.8	-1.1	-2.7
HP0933	hypothetical protein	-0.9	0.3	0.6	-1.0
HP1455	hypothetical protein	-0.6	-0.4	-0.6	-1.2
HP0659	hypothetical protein	-0.5	0.1	0.0	-1.5
HP0636	hypothetical protein	-0.5	0.6	-1.7	-1.6
HP0560	hypothetical protein	-0.4	-1.2	-1.1	-1.9
HP1154	hypothetical protein	-0.4	-0.3	-0.7	-1.6
HP0336	hypothetical protein	-0.2	-1.0	-2.5	-3.2
HP0991	hypothetical protein	-0.1	-0.6	-0.9	-1.3
HP1236	hypothetical protein	0.0	-1.9	-2.6	-3.1
HP0291	hypothetical protein	0.6	-0.1	-0.4	-1.1
Protein fate					
HP1019	serine protease, <i>htrA</i>	-2.5	-0.2	-0.1	-0.2
HP1012	protease, <i>pqqE</i>	-1.6	0.0	-0.2	-0.6
HP0210	chaperone and heat shock protein, <i>htpG</i>	-1.4	-0.2	-0.5	-0.5
HP0382	zinc-metallo protease	-1.2	0.0	-0.1	-0.5
HP0570	aminopeptidase a-I, <i>pepA</i>	-1.2	0.0	0.1	0.2
HP0011	co-chaperone, <i>groES</i>	-1.1	0.2	-0.1	-0.5

**Table continued**

HP1300	preprotein translocase subunit, <i>secY</i>	-1.1	0.1	0.1	-0.9
Protein synthesis					
HP1040	ribosomal protein S15, <i>rps15</i>	-2.4	0.3	0.2	-0.9
jhp1117	putative motility protein	-2.2	-0.2	-0.5	-1.5
HP0643	glutamyl-tRNA synthetase, <i>gltX</i>	-2.1	0.0	0.1	-0.7
HP1298	translation initiation factor EF-1, <i>infA</i>	-1.8	-0.6	-0.7	-2.1
jhp0186	50S ribosomal protein L32, <i>rpmF</i>	-1.8	-0.6	-0.2	-1.6
HP0177	translation elongation factor EF-P, <i>efp</i>	-1.7	-0.4	-0.2	-1.1
HP0200	ribosomal protein L32, <i>rpl32</i>	-1.7	-0.4	0.0	-1.1
HP1555	translation elongation factor EF-Ts, <i>tsf</i>	-1.5	-0.3	-0.6	-0.4
HP1196	ribosomal protein S7, <i>rps7</i>	-1.5	0.1	0.1	0.0
HP1195	translation elongation factor EF-G, <i>fusA</i>	-1.4	0.4	0.7	0.4
HP1303	ribosomal protein L18, <i>rpl18</i>	-0.7	-0.2	-0.6	-1.2
HP0126	ribosomal protein L20, <i>rpl20</i>	-0.5	-0.2	0.0	-1.3
HP1301	ribosomal protein L15, <i>rpl15</i>	-0.4	-0.5	-0.8	-1.8
HP1307	ribosomal protein L5, <i>rpl5</i>	-0.1	-0.1	-0.7	-1.3
HP0417	methionyl-tRNA synthetase, <i>metS</i>	-0.1	-0.9	-1.2	-1.2
HP1253	tryptophanyl-tRNA synthetase, <i>trpS</i>	0.0	0.0	-0.3	-0.9
HP0972	glycyl-tRNA synthetase, beta subunit, <i>glyS</i>	0.5	-0.7	-1.1	0.5
Purines, pyrimidines, nucleosides, and nucleotides					
HP0825	thioredoxin reductase, <i>trxB</i>	-2.7	0.3	-0.2	0.0
HP0045	nodulation protein, <i>nolK</i>	-1.5	0.2	-0.3	-0.5
HP1112	adenylosuccinate lyase	-1.3	-0.1	0.5	-0.6
Regulatory functions					
HP0224	peptide methionine sulfoxide reductase, <i>msrA</i>	-2.1	0.1	-0.2	0.0
HP0400	penicillin tolerance protein, <i>lytB</i>	-1.3	0.4	0.0	-1.2
HP0166	response regulator, <i>ompR</i>	-1.2	0.0	0.1	-0.5
Transcription					
HP0550	transcription termination factor Rho	-1.5	0.2	0.4	-0.3

**Table continued**

## Transport and binding proteins

HP1512	iron-regulated outer membrane protein	-2.4	0.4	0.0	-0.2
HP1172	glutamine ABC transporter, periplasmic glutamine-binding protein, <i>glnH</i>	-2.0	-0.1	0.2	-0.5
HP0055	proline permease, <i>putP</i>	-1.7	0.4	0.6	-0.2
HP1073	copper ion binding protein, <i>copP</i>	-1.6	0.1	0.2	-0.6
HP1432	histidine and glutamine-rich protein	-1.4	0.3	0.5	0.6
HP1427	histidine-rich, metal binding polypeptide, <i>hpn</i>	-1.2	0.4	0.4	0.2
HP0916	iron-regulated outer membrane protein	-1.2	0.0	0.3	-0.2
HP0607	acriflavine resistance protein, <i>acrB</i>	-1.2	0.1	-0.3	-0.8
HP1130	biopolymer transport protein, <i>exbB</i>	-1.0	-0.2	-0.2	0.2
HP0687	iron(II) transport protein, <i>feoB</i>	-1.0	-0.4	-0.6	0.3

## Unknown/unclassified

jhp1188	NADH dehydrogenase subunit H, <i>chainH</i>	-3.0	0.1	-0.4	0.0
HP0377	thiol:disulfide interchange protein, <i>dsbC</i>	-2.6	-0.2	-0.7	-0.9
HP0390	adhesin-thiol peroxidase, <i>tagD</i>	-2.4	0.0	-0.1	-0.9
HP0136	bacterioferritin comigratory protein, <i>bcp</i>	-1.8	-0.2	0.3	-0.3
HP1104	cinnamyl-alcohol dehydrogenase ELI3-2, <i>cad</i>	-1.6	-0.3	-0.4	-0.2
HP0569	GTP-binding protein, <i>gtp1</i>	-1.4	-0.4	-0.3	-0.5
HP0485	catalase-like protein	-1.1	-0.3	-0.6	-0.9

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