

2013

## Survival, injury, and antibiotic resistance patterns of *Cronobacter sakazakii* and *Escherichia coli* resulting from exposure to low-temperature ground water

Alisha L. Dailey

Follow this and additional works at: <https://researchrepository.wvu.edu/etd>

---

### Recommended Citation

Dailey, Alisha L., "Survival, injury, and antibiotic resistance patterns of *Cronobacter sakazakii* and *Escherichia coli* resulting from exposure to low-temperature ground water" (2013). *Graduate Theses, Dissertations, and Problem Reports*. 7308.

<https://researchrepository.wvu.edu/etd/7308>

This Thesis is protected by copyright and/or related rights. It has been brought to you by the The Research Repository @ WVU with permission from the rights-holder(s). You are free to use this Thesis in any way that is permitted by the copyright and related rights legislation that applies to your use. For other uses you must obtain permission from the rights-holder(s) directly, unless additional rights are indicated by a Creative Commons license in the record and/ or on the work itself. This Thesis has been accepted for inclusion in WVU Graduate Theses, Dissertations, and Problem Reports collection by an authorized administrator of The Research Repository @ WVU. For more information, please contact [researchrepository@mail.wvu.edu](mailto:researchrepository@mail.wvu.edu).

# Survival, injury, and antibiotic resistance patterns of *Cronobacter sakazakii* and *Escherichia coli* resulting from exposure to low-temperature ground water

Alisha L. Dailey

Thesis submitted to the Davis College of Agriculture, Forestry and Consumer

Sciences at West Virginia University

in partial fulfillment of the requirements for

the degree of

Master of Science

In

Applied and Environmental Microbiology

Gary Bissonnette, Ph.D., Chair

Alan Sexstone, Ph.D.

Jacek Jaczynski, Ph.D.

Department of Plant and Soil Sciences

Morgantown, West Virginia

2013

Keywords: Antibiotic resistance patterns, *Cronobacter sakazakii*, injury, survival

Copyright 2013 Alisha Dailey

UMI Number: 1549740

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



UMI 1549740

Published by ProQuest LLC (2013). Copyright in the Dissertation held by the Author.

Microform Edition © ProQuest LLC.

All rights reserved. This work is protected against unauthorized copying under Title 17, United States Code



ProQuest LLC.  
789 East Eisenhower Parkway  
P.O. Box 1346  
Ann Arbor, MI 48106 - 1346

## ABSTRACT

### **Survival, injury, and antibiotic resistance patterns of *Cronobacter sakazakii* and *Escherichia coli* resulting from exposure to low-temperature ground water**

**Alisha L. Dailey**

Bacteria are constantly being exposed to changing conditions in the environment. Exposure to adverse conditions such as pH, temperature, and desiccation may cause stress to the bacteria, potentially resulting in loss of viability and growth. *Cronobacter sakazakii* and *Escherichia coli* were exposed to cold temperature (4°C) and room temperature (24°C) nutrient-limiting groundwater. As a function of time (0 to 15 days), samples were removed from the stress and analyzed. Response of stressed cells was evaluated by three approaches: (i) traditional viable cell counts (nonselective and selective media), (ii) substrate utilization patterns (BIOLOG GN microplates), and (iii) antibiotic resistance patterns (modified Kirby-Bauer disk diffusion assay). The cold, nutrient-limiting environment resulted in physical changes as reflected by survival and sublethal injury, changes in metabolic activity as reflected by differences in utilization of carbon substrates, and changes in antibiotic resistance patterns as reflected by increased resistance or susceptibility to six antibiotics. Three major findings were observed. First, analyses of the survival/injury data indicated *C. sakazakii* was more tolerant to extended ground water exposure than *E. coli*, regardless of water temperature. Second, changes in substrate utilization patterns (BIOLOG profiles) for both organisms as a result of stress present an option in which recovery media could be altered or supplemented in order to enhance detection of stressed cells from low-temperature aquatic environments. Finally, changes in antibiotic resistance patterns due to cold temperature stress may also have implications regarding detection of stressed cells, as well as the efficacy of clinical use of certain antibiotics to treat infectious disease. Findings from this research should provide a better understanding of the physiological response of *C. sakazakii* and *E. coli* to low temperature aquatic environments.

## **Acknowledgements**

I would like to thank my graduate committee for their cooperation and assistance with this project. I am especially grateful for the guidance and patience of my advisor, Dr. Gary K. Bissonnette, without his guidance this project would never have been completed. In addition I would like to thank Dr. Alan J. Sexstone for all his help with the statistics for this project and analysis of BIOLOG microplates, and Dr. Jacek Jacyński for his help with D-value calculations.

I greatly appreciate all the help from Chelse Ward, and all other faculty and graduate students in the Plant and Soil Science Department. They were always there to add their suggestions and let me bounce ideas off of them.

I would also like to thank my husband, mom, and brother for their love and encouragement throughout my graduate journey.

Finally, this project was supported by the WVU Agricultural Experiment Station.

## Table of Contents

ABSTRACT.....	ii
Acknowledgements.....	iii
Table of Contents.....	iv
List of Figures.....	vi
List of Tables.....	vii
LITERATURE REVIEW.....	1
1. Bacterial Stress Response.....	1
2. Cold Shock Response.....	3
3. Antibiotic Resistance.....	5
4. Growth Conditions.....	9
5. Identifying Physiological Changes.....	10
6. BIOLOG Microplate System.....	11
7. <i>Cronobacter sakazakii</i> .....	13
RESEARCH OBJECTIVES & RATIONAL for the CURRENT STUDY.....	17
Chapter 1.....	19
Survival and injury of <i>Cronobacter sakazakii</i> and <i>Escherichia coli</i> following exposure to cold temperature groundwater.....	19
1. Introduction.....	20
2. Material and Methods.....	21
Preparation and growth of test species.....	21
Cold stress.....	22
Observation of cell survival and sublethal injury.....	22
3. Statistical analysis.....	23
4. Results and discussion.....	23
Survival.....	23
Sublethal injury.....	25
5. Summary.....	26
Chapter 2.....	33
Physiological response of <i>Cronobacter sakazakii</i> and <i>Escherichia coli</i> following exposure to cold temperature groundwater.....	33
1. Introduction.....	34
2. Material and Methods.....	35
Preparation and growth of test species.....	35

Cold stress.....	35
Carbon source utilization patterns.....	35
3. Statistical analysis.....	36
4. Results and discussion.....	36
5. Summary.....	39
Chapter 3.....	43
Changes in antibiotic resistance patterns of <i>Cronobacter sakazakii</i> and <i>Escherichia coli</i> as the result of exposure to cold stressed groundwater.....	43
1. Introduction.....	44
2. Material and Methods.....	46
Preparation and growth of test species.....	46
Cold stress.....	46
Optimum temperature and nutrient-rich growth conditions.....	47
Antibiotic disks.....	47
3. Statistical analysis.....	47
4. Results and discussion.....	48
Susceptibility of <i>C.sakazakii</i> to antibiotics.....	48
Susceptibility of <i>E.coli</i> to antibiotics.....	49
5. Summary.....	51
6. Appendix.....	55
Literature Cited.....	57
VITA.....	66

## List of Figures

### Chapter 1

Fig. 1. Survival and injury of *C. sakazakii* following 9 day exposure to 4 °C, filter-sterilized ground water compared to 24°C.....27

Fig. 2. Survival and injury of *E.coli* following 9 day exposure to 4 °C, filter-sterilized ground water compared to 24°C.....28

Fig. 3. D-value determination of cold-stressed *C. sakazakii* and *E.coli*.....31

Fig. 4. D-value determination of room temperature *C.sakazakii* and *E.coli*.....32

### Chapter 3

Fig. 1. Responses of *C.sakazakii* to six antibiotics during 15 days of exposure to filter-sterilized groundwater held at 4°C as compared to optimal growth conditions in TSB at 35°C.....52

Fig. 2. Responses of *E.coli* to six antibiotics during 15 days of exposure to filter-sterilized groundwater held at 4°C as compared to optimal growth conditions in TSB at 35°C.....53



## List of Tables

### Chapter 1

Table 1. Survival and Injury of *C.sakazakii* following exposure to 4°C and 24°C filter-sterilized ground water.....29

Table 2. . Survival and Injury of *E.coli* following exposure to 4°C and 24°C filter-sterilized ground water.....30

### Chapter 2

Table 1. Average well color development (AWCD) for *C.sakazakii* and *E.coli* following 6 day exposure to nutrient-deplete groundwater at 4°C or 24 °C.....40

Table 2. Utilization of specific BIOLOG substrates by cold-stressed (4°C) *C.sakazakii* and *E.coli* comparing day 0 to day 6 exposure in nutrient-deplete groundwater. Expressed as percent difference.....41

Table 3. Utilization of specific BIOLOG substrates by room temperature (24°C) *C.sakazakii* and *E.coli* comparing day 0 to day 6 exposure in nutrient-deplete groundwater. Expressed as percent difference.....42

### Chapter 3

Table 1. Resistance (R) and susceptibility (S) of *C.sakazakii* and *E.coli* following a 15 day exposure to non-growth conditions in nutrient-deplete groundwater at 4°C or optimal growth conditions in TSB at 35°C. Resistance (R) and susceptibility (S) determined with a modified Kirby-Bauer disk diffusion assay by measuring zones of growth inhibition.....54

Table 2. Regression equations and R<sup>2</sup> values for *C.sakazakii* and *E.coli* following a 15 day exposure to non-growth conditions in nutrient-deplete groundwater at 4°C or optimal growth conditions in TSB at 35°C.....56

# LITERATURE REVIEW

## 1. Bacterial Stress Response

Bacteria are constantly being exposed to ever changing conditions in the environment. These changes (acid, heat, cold, etc.) subject the bacteria to stress, potentially resulting in loss of viability and growth. As a result of environmental stresses, many bacteria have evolved mechanisms that allow for their continued survival. Such changes to the bacterial genome allow for the preservation of vital life processes and continued cellular homeostasis. These genome changes allow bacteria to colonize specific niches present in extreme environments (Aertsen and Michiels, 2004).

When bacteria enter or experience a stressful environment, they sometimes/ or often enter the stationary phase (Hengge-Aronis, 1993). Prolonged stationary phase triggers a response stage called stringent response. Stringent response causes an increased gene expression of  $\sigma^S$  and  $\sigma^{70}$ . The regulation of these genes is directly related to the quality and quantity of nutrients in the environment as they direct the role of sigma factor competition for RNA polymerase. In nutrient-depleted environments the  $\sigma^S$  and  $\sigma^{70}$  genes signal the bacterium to allocate resources normally used for growth to now use them for protection and repair of vital cellular components (Aertsen and Michiels, 2004). The ability of bacteria to enter stationary phase when nutrient concentrations are insufficient to sustain steady growth allows the cells to remain viable under stressed conditions. Stationary phase is defined as the ability to withstand long periods of time without access to energy-yielding substrates (Llorens et al., 2010). Gram-negative and gram-positive cells can react to stresses in different ways. In the example of starvation, some gram-

positive cells have the ability to produce dormant spores that allow the cell to significantly slow or halt metabolic processes under starvation conditions. However, gram-negative cells lack the ability to enter into a dormant spore stage so they must overcome low-nutrient conditions by developing resistant cells without dormancy. Both dormant spores and resistant cells sustain the bacteria until more nutrients become available and “normal” metabolic functions can resume until all nutrients are depleted once again and the cells enter back into stationary phase (Kolter et al., 1993).

The regulatory gene *rpoS* signals the cell to enter stationary phase and increase stress resistance. Hengge-Aronis (2002) indicated that *rpoS* regulation extends to transcription, translation, and post-translational controls, all of which are tightly coordinated. Stressful conditions that can trigger an up regulation of *rpoS* gene expression are low levels of carbon, nitrogen or phosphorus, or amino acid starvation. In the stationary phase with *rpoS* regulation, morphological changes occur such as cells become smaller and spherical in shape with a more rigid cellular envelope. Also, metabolic changes may occur such as decreased protein synthesis and increased peptidases/proteases synthesis. These changes allow the cell to withstand various stress conditions (oxidative stress, temperature changes, pH changes, etc.). *RpoS* genes are so important for *Escherichia coli* that they account for ten percent of the genome (Llorens et al., 2010).

Under adverse environmental conditions for extended periods of time, bacterial cells enter what is known as the death phase. The death phase occurs when nutrient-depleted cells in the stationary phase accumulate waste products from metabolism and 90-99% of the population dies as a result. Finkel (2006) showed that *E. coli* grown in Luria-Bertani (LB) medium start dying after three days of incubation. However, this amount of cell death is not always negative,

as these “dead” cells release their nutrients into the environment where surviving cells can take up the nutrients and maintain viability (Finkel, 2006).

As a last resort to ensure survival, bacteria can enter into a viable but nonculturable (VBNC) state. VBNC cells are described as those that fail to grow under standard laboratory conditions, but still retain metabolic activity (Llorens et al., 2010). To date, little is known about the molecular mechanisms that allow bacteria to become dormant and then under richer conditions recover almost instantly, permitting their normal ability to proliferate and demonstrate pathogenesis (Tufariello et al., 2006). A better understanding of physiological changes to bacteria that occur under adverse conditions may lead to development of more reliable methods of determining bacterial presence, when normal culturing methods seem to indicate non-presence.

## **2. Cold Shock Response**

One such extreme environment is cold temperature. Cold temperatures lead to a reduction in membrane fluidity, protein biosynthesis, and stability of secondary structures associated with deoxyribonucleic acid and ribonucleic acid. All of these processes are essential for bacterial cell survival. To overcome these changes, bacteria synthesize cold-shock proteins (CSP's), molecular chaperones compatible solutes, and structural modifications to maintain membrane fluidity (Prisco et al., 2012). Since the bacterial membrane is in constant contact with the outer environment, it appears to serve as the primary signaling mechanism. Marr and Ingraham (1962) demonstrated that *E. coli* when exposed to low temperatures increased the amount of oleic acid (C<sub>18:1</sub>) (a monounsaturated acid) and reduced palmitic acid (C<sub>16:0</sub>) (a saturated fatty acid) in its

membrane. The change from saturated to unsaturated fatty acids allows for the bacterium to better maintain membrane fluidity under low temperature stress (Shivaji et al., 2010).

Molecular chaperone compatible solutes such as trehalose, glycine betaine, and carnitine help prevent denaturation and aggregation of proteins within the cell. Kandror et al. (2002) showed that when *E. coli* was shifted from a 37 to 16 °C environment there was an increase in trehalose from the upregulation of otsAB operon (generates trehalose from glucose). In the same study, the growth and viability of otsA null mutants under low temperatures were also examined. At 16°C, mutants were able to grow without issue; however, at 4°C viability was reduced. Thus, the increased production of trehalose may serve as an anticipatory defense mechanism to overcome temperatures below 16°C.

CSP's are thought to bind messenger RNA and regulate ribosomal translation, rate of mRNA degradation, and termination of transcription (Ermolenko and Makhatadze, 2002). Studies have shown that CSP's are highest in the cell during the acclimation phase, and decline to new steady expression level after acclimation has occurred. This new CSP expression level is lower than protein expression before cold-shock was induced (Ermolenko and Makhatadze, 2002). Bacterial cells counteract this effect by altering lipid composition of cell membranes and remodeling transcription and translational machinery (Cao-Hoang et al., 2008). At temperatures below 0°C, bacterial metabolic activity and cell growth are halted. In contrast to when the temperature is above 0°C, bacteria mount an adaptive response by synthesizing specific proteins. However, a temperature of 0°C can be lethal to a bacterial cell (Cao-Hoang et al., 2008). Cao-Hoang et al. (2008) observed membrane fluidity of *E. coli* decreased as temperature decreased and was less during the exponential growth phase than during the stationary phase. An increased expression of flagella genes has been reported for *E. coli* that has been exposed to cold shock,

thus indicating a possible correlation between flagella genes and environmental fluctuations (Friedman et al., 2005). Eight CSP's have been discovered in *E.coli*; however, cold shock protein A (CspA) and proteins for its family have been found to play a major role in cold-shock adaptation and also are important under normal growing conditions (Ermolenko and Makhatadze, 2002). Although research into the role and effects of CSP has increased in the past decade, much about them is still not understood.

### **3. Antibiotic Resistance**

There are hundreds of different antibiotics in today's world and the majority fall into seven classes: penicillins, cephalosporins, macrolides, fluoroquinolones, sulfonamids, tetracyclines, and aminoglycosides (Tintinalli et al., 1995). The use of antibiotics plays an important role in treatment of diseases. About one-half of all life threatening nosocomical diseases are caused by gram-negative bacteria. Gram-negative bacteria have a higher rate of enhanced resistance to antibiotics than gram positive bacteria (Poole, 2001). The most widely used antibiotics are those that contain a  $\beta$ -lactam ring in their molecular structures.  $\beta$ -lactam antibiotics, such as penicillins and cephalosporins, are prescribed because of their broad spectrum target activity, safety profile, and proven clinical efficacy. The wide use of antibiotics has contributed to the rapid emergence and spread of resistance. Bacteria acquire resistance predominantly in three ways: antibiotic inactivation, target site modification, and altered uptake by restricted entry and/or enhanced efflux (Poole, 2001). In gram-negative bacteria, the bacteriostatic or bactericidal action of the antibiotic requires passage of the antibiotic across the outer membrane (Poole, 2001). Changes in the outer bacterial membrane might trigger changes in antibiotic resistance in the organism. Membrane changes can stop antibiotics from gaining access to the penicillin binding proteins (PBPs), thus causing the bacteria to become resistant.

Changes to the outer membrane are due to a gain or loss of porins resulting in inefficient transport of the antibiotic through porin channels (Maiti et al., 2006).

Changes in the outer membrane alone are not usually enough to increase resistance on its own. Antibiotic resistance becomes much more significant when there are additional resistance mechanisms such as efflux and  $\beta$ -lactamases that work synergistically with membrane changes to enhance resistance (Nikaido, 1994). Antibiotics containing a  $\beta$ -lactam ring function by inactivating the cell wall synthesizing enzymes. Bacterial cells that are exposed over an extended period of time to  $\beta$ -lactam can produce the enzyme  $\beta$ -lactamase. This enzyme catalyzes the opening of the  $\beta$ -lactam ring, inactivating the antibiotic before it reaches the PBPs to act. Each strain of bacteria has the ability to produce different  $\beta$ -lactamases resulting in penicillin resistance. Since the target sites for  $\beta$ -lactams are PBPs, the bacteria can modify these sites resulting in decreased affinity for the antibiotics. These modifications could occur through the substitutional mutation of a single amino acid in the protein chain (Maiti et al., 2006).

Fluoroquinolones (FQ), such as ciprofloxacin, are valued and prescribed for their broad spectrum activity, excellent tissue penetration, and ability to be administered both orally or intravenously (Laurence et al., 2005). FQ resistance is attributed to mutations affecting the FQ targets, DNA gyrase and topoisomerase (Hooper, 2000). The majority of FQ antibiotics target DNA gyrase or topoisomerase IV, which allows for just one single mutation to start providing resistance (Hooper, 2000). Both DNA gyrase and topoisomerase IV are enzymes present when DNA is being replicated in cells. DNA gyrase relieves the strain on the double-stranded DNA which is being unwound by the enzyme helicase. Topoisomerase IV has two functions, to relieve the strain of the double-stranded DNA, and to assist in unlinking DNA strands so the

chromosomes and plasmids can segregate to opposite sides of the cell for division (Gore et al., 2006).

Tetracyclines are broad-spectrum antibiotics commonly used to treat urinary tract and intestinal infections. They are especially useful when a patient is allergic to  $\beta$ -lactams and macrolide antibiotics (Finberg and Guharoy, 2012). Tetracyclines are protein synthesis inhibitors that bind the 16S part of the 30S ribosomal subunit, thus inhibiting translation (Connell et al., 2003). Tetracyclines are prescribed for moderate to severe acne/rosacea and administered to animals, especially farm animals to prevent illness and promote growth (Finberg and Guharoy, 2012). Over use of tetracyclines is contributing to increased resistance. There are at least three mechanisms involved: enzymatic inactivation, efflux, and ribosomal protection. Inactivation is the rarest way to gain resistance (Roberts, 1996). Resistance from efflux is encoded by *tet* determinants, which are usually plasmid or transposon-encoded; thus, resistance is acquired from resistance genes from outside sources (Roberts, 1996). The resistance of ribosomal protection depends on what gene is transferred and where it is inserted (Levy and Nelson, 1998).

Aminoglycosides are used for their highly potent, broad-spectrum targeting abilities when treating life-threatening infections (Mingeot-Leclercq et al., 1999). They cause a disruption in protein synthesis by binding to prokaryotic ribosomes (Mingeot-Leclercq et al., 1999). Two antibiotics from this class of drugs are streptomycin and kanamycin. Resistance is caused by changes to ribosomal binding, decrease in drug uptake/accumulation, or aminoglycoside-modifying enzymes (Mingeot-Leclercq et al., 1999). A chemical modification of the aminoglycoside, which alters how the antibiotic binds to its ribosomal target, only affects streptomycin resistance (Poole, 2001). A reduction of drug uptake is primarily seen in *Pseudomonas* spp. and other non-fermenting gram-negative bacilli, due do to membrane



impermeabilization (Chambers and Sande, 1995). This is of particular significance in a clinical setting as it can affect all aminoglycosides, causing moderate resistance in bacteria (Mingeot-Leclercq et al., 1999). Changes in the bacterial membrane as a result of an accumulation of an antibiotic in the body can lead to an increase in resistance (Karlowsky et al., 1997).

Aminoglycoside-modifying enzymes cause a catalyzation of specific amino or hydroxyl functions of the antibiotic, resulting in a chemical modification that now binds poorly to the ribosomal target (Mingeot-Leclercq et al., 1999). This modification results in very high levels of resistance. As other classes of antibiotics have been evaluated in recent years, the use of aminoglycosides has decreased because of the negative side effects. Nevertheless, as more and more gram-negative bacteria are emerging with advanced patterns of microbial resistance, physicians and scientists are starting to reevaluate the use of aminoglycosides since they are still effective against many gram-negative bacteria.

Chloramphenicol is one such antibiotic that does not fall into the seven major categories of antibiotics. It is closely related to the macrolide class of antibiotics as both are broad-spectrum, acting against gram-negative, gram-positive, and most anaerobic organisms that act upon the 50S ribosomal subunit to stop bacterial growth. The difference is chloramphenicol directly interferes with substrate binding, but macrolides sterically block the progression of the growing peptide (Neu and Gootz, 1996). Chloramphenicol is used because of its wide bacteriostatic property, cheapness, and ease in manufacture. In the developing world, chloramphenicol is the most prescribed antibiotic (Falagas et al., 2008). In the developed world, chloramphenicol is not routinely administered to humans because of the negative side-effects that are known to occur without warning. However, before 2003, it was widely administered to cattle to prevent diseases and increase growth (Ribeiro et al., 2012). Resistance is attributed to

three mechanisms: efflux, mutation of the 50S ribosomal subunit, and changes by acetyltransferases (Poole, 2001). Efflux resistance can very easily be selected for *in vitro* and confers low-level resistance (Mine et al., 1998; Arcangioli et al., 1999). High-levels of resistance occur when the *cat*-gene mutates. This gene codes for chloramphenicol acetyltransferase, which causes a covalent linking of one or two acetyl groups to the hydroxyl groups on the chloramphenicol molecule (Murray and Shaw, 1997). Mutations to the 50S ribosomal subunit are very rare.

#### **4. Growth Conditions**

In laboratory experiments, growth conditions for the microorganisms are often ideal. These microbes receive ample nutrients, pH, and temperature to permit optimal growth. However, in nature these optimal growing conditions almost never occur, thus, leading to microbial adaptations to ensure survival, rather than growth. Thunberg et al. (2001) demonstrated that nutrient depleted environments can alter the effectiveness of disinfectants and growth of microbes, thus, indicating that nutrient availability due to environmental changes would have an effect on microbial changes due to cold shock.

One limiting factor to adequate growth conditions is nutrient availability. One example of this is *E.coli*, an enteric bacterium, ideally suited to a life cycle taking place in a mammalian host gut, which is rich in nutrients and constant temperature of 37°C. However, *E.coli* can survive in soil and water environments where nutrient availability is often low. Under starvation conditions, bacterial cells after exhausting all present nutrients will progressively metabolize their cellular carbohydrates, followed by proteins and RNA, while initially protecting the DNA (Van Elsas et al., 2010). Ihssen et al. (2007) proposed that *E.coli* can survive low-nutrient environments by

increased activity of ABC- transporters, which readily absorb any amino acids and sugars the cell may encounter. Vital et al. (2008) showed that *E.coli* O157:H7 cannot only grow in low-nutrient aquatic environments, but has a growth rate of  $\sim 0.19\text{h}^{-1}$  in sterile freshwater. Stewart and Olson (1992) observed that bacterial cells subjected to low temperatures during growth are more resistant to disinfectants than cells grown at higher temperatures.

## 5. Identifying Physiological Changes

Various methods have been used to identify physiological changes in microorganisms following exposure to environmental stress. The traditional way to detect physiological changes is performed by examining total plate counts on nonselective, nutrient rich and selective media to determine survival and sublethal injury. The method of spread plating allows for direct counting of culturable bacteria on a rich non-selective medium such as trypticase soy agar (TSA). Sublethal injury to bacterial cells is the outcome of physiological/structural damage that is the product of exposure to an environmental stress resulting in an inability to grow on selective media which allows the growth of normal cells (Brenner et al., 1996). Estimation that 99% of all bacteria are sublethally injured could lead to a serious underestimation of the total concentration of bacteria that are present in food and water.

Since many bacteria are difficult to culture outside of their natural environmental niche, better enumeration methods for detecting injured cells need to be developed. Direct viable counts can be used to determine bacterial density and viability. Direct viable counts use membrane and nucleic acid stains to determine if cells are living or dead. In recent years, flow cytometry has been used to distinguish between the population of living and dead cells after being exposed to an environmental stressor. The dyes used during flow cytometry distinguish membrane

permeability and membrane potential of dead and live cells. To date, there is no one stain that allows for the complete analysis of a bacterium's physiology. Virta et al. (1998) observed that staining a living bacterial cell that has a damaged membrane from stress is difficult. Also, there is the factor of investigator bias, as the likely differences in estimation of cells between observers depends on what that particular observer determines is a countable bacterial cell (Kepner and Pratt, 1994). The biggest problem associated with the direct viable count method is the lack of agreement on standard procedures, since most studies do not indicate the magnification, preservation, dispersion techniques, filter types, and counting strategies (Kepner and Pratt, 1994). This lack of a standard direct viable count method could result in inaccurate numbers of physiologically injured bacterial cells for different environmental stressors.

## **6. BIOLOG microplate system**

The BIOLOG microplate system was introduced in the early 1990's and was used to identify microorganisms based on the exchange of electrons that are produced during bacterial respiration. The oxidation rate of the 95 different carbon sources causes the tetrazolium oxidation/reduction indicator to change to violet (Miller and Rhoden, 1991). Tetrazolium is an indicator dye used to show a positive result for wells in which bacterial samples are utilizing that carbon source. BIOLOG was designed to assist in identification of human isolates and environmental contaminants in a clinical setting; however, in recent years it has been used to generate community level physiological profiles of heterotrophic communities to evaluate environmental diversity (O'Connell et al., 2000). Garland and Mills (1991) used BIOLOG to examine whole environmental soil samples for microbial community analysis from different environmental sample areas. This testing method has permitted distinction between microbial communities found in soils, water, and contamination sites. Graves et al. (2009) used BIOLOG

to determine if different species of enterococci were present in dry or fresh manure and if there was a difference in microbial communities among cattle, horse, and sheep feces. They were able to determine that the two main species of enterococci, *Enterococcus casseliflavus* and *Enterococcus mundtii*, found in all three animal species in both fresh and dry manure were very similar. However, grain vs. pasture grass fed animals resulted in slightly altered microbial composition, as reflected by higher detection of *E. casseliflavus* in grain feed animals and with higher *E. mundtii* populations in pasture grass feed animals. Another study performed by Ihssen and Egli (2005) examined the catabolic functions of *E. coli* when exposed to different growth conditions, carbon-limiting medium vs. medium containing excess carbon. The BIOLOG system indicated that *E. coli* grown on glucose-limiting medium was able to oxidize 43 of the 95 different substrates, whereas *E. coli* was only able to oxidize seven of the 95 substrates when grown in the glucose rich medium.

McCarroll (2008) utilized the BIOLOG microplate system to examine changes in the utilization of substrates by *E. coli*, *Salmonella typhimurium*, and *C. sakazakii* after being heat stressed at 52°C for 90 minutes. He observed a decreased utilization of  $\alpha$ -D-glucose, L-rhamnose, D-glucose-6-phosphate, D-trehalose, and L-alanine for *E. coli*. *S. typhimurium* illustrated an increased utilization in succinic acid mono methyl, L-ornithine, and bromosuccinic acid. For *C. sakazakii*, there was increased utilization of xylitol, adonitol and glycogen. Such observations (Graves et al., 2009; Ihssen and Egli, 2005; McCarroll, 2008) suggest that the BIOLOG microplate system could be beneficial in evaluating specific changes in carbon utilization of bacteria under stress conditions. These results may provide increased knowledge as to what substrates are important to bacteria found in food and water under various stress conditions.

## 7. *Cronobacter sakazakii*

*Cronobacter sakazakii* is a gram-negative, yellow-pigmented bacterium belonging to the *Enterobacteriaceae* family. It was reclassified in 1980, when testing performed on yellow-pigmenting *Enterobacter cloacae* revealed it as a new species (Farber and Forsythe, 2008). *C.sakazakii* was known as *Enterobacter sakazakii* before 2007 when the genus name was changed as a result of full-length 16S rRNA gene sequencing, DNA-DNA hybridization, multilocus sequence typing (MLST), f-AFLP, and automated ribotyping (Iversen et al., 2008 and 2007, Joseph et al., 2011) . It has been linked to several neonatal meningitis outbreaks over the years. Both the World Health Organization and the Food and Drug Administration list *C. sakazakii* as an emerging food and waterborne pathogen.

The first case of an infant death as a result of *C.sakazakii* was reported in 1958 (Farber and Forsythe, 2008). In 2011, two cases of infants with *C.sakazakii* infection were reported to the Missouri Department of Health, with one of the infants dying from the infection (FDA and CDC, 2011). The source of the bacterium was thought to be Enfamil commercial powdered infant formula. This bacterium causes life-threatening neonatal sepsis and meningitis with a mortality rate of 40-80%, and those infants that survive often suffer from severe mental impairments (Stock and Wiedemann, 2001). *C.sakazakii* infections are primarily associated with the use of powdered infant formula.

To date, scientists are unsure of the exact environmental reservoirs for this bacterium, infectious dose, as well as its mode of transmission. Most infections with *C.sakazakii* have been blamed on contaminated powdered infant (milk) formulas (PIF). PIF is commercially pasteurized, and research shows that the *C. sakazakii* bacterium is not present at that time (Farber

and Forsythe, 2008; Kandhai et al., 2004). It has been hypothesized that contamination occurs after pasteurization when nutrient additives are blended into the formula, or when it is rehydrated in kitchens, hospitals, etc. (Gurtler et al., 2005). *C. sakazakii* is present in about 2.4%-14.2% of all infant formulas tested (Iverson and Forsythe, 2004; Leuscher et al., 2004; Muytjens et al., 1988). In its dry state, infant formula has an  $a_w$  of 0.2, which largely impacts the survival of bacteria present. With a low  $a_w$ , *C. sakazakii* has been found to survive at least two years and upon rehydration be pathogenic (Beuchat et al., 2009). *C. sakazakii* has a competitive advantage in infant formula due to its ability to withstand osmotic and dry stress environments. Breeuwer et al. (2003) reported that accumulation of trehalose in the cell allows for bacterial cells to survive in low osmotic and dry conditions. Trehalose replaces water in the cell, thus allowing the cell to survive prolonged exposure to dry environments and subsequently allow normal cellular activity when rehydration occurs without lethal damage.

*C. sakazakii* has been isolated from a wide spectrum of environments including water, soil, household dust, food production-lines, and drinking water system biofilms (Farmer et al. 1985; Drudy et al., 2006). Kandhai et al. (2004) found when examining milk powder, chocolate, cereal, potato flour, pasta, and spice factories and household dust using spread plating methods, eight out of the nine factories' samples and five of the sixteen household dust samples were positive for *C. sakazakii*. Lee and Kim (2003) identified, through the use of spread plating, *C. sakazakii* as an indigenous bacterium in their analysis of biofilm formation in the water distribution system of Seoul, Korea. Schindler (1994) reported the presence of *C. sakazakii* in bottled mineral water. When examining the bacterial flora of dishwasher rinsed beer mugs, Schindler and Metz (1990) found *C. sakazakii* to be a common isolate using the API 20E system. Beauchamp et al. (2006) isolated *C. sakazakii* from paper sludge by isolation on MacConkey medium with subsequent

isolation of 16S rDNA sequences. *C.sakazakii* has also been isolated from the intestines of rats and guts of flies (Gakuya et al., 2001; Kuzina et al., 2001).

In recent years, scientists have used quantitative polymerase chain reaction (qPCR), also referred to as real-time PCR, in conjunction with traditional assay methods such as spread plating to isolate this bacterium from different environmental sources. qPCR is used because of its ability to detect and quantify the bacterium in question from a sample in less time. Liu et al. (2006) used qPCR to detect 35 strains of *C.sakazakii* in infant formula. They were able to detect and positively identify 1.1 CFU/100g infant formula within two business days versus five days with conventional methods. Nair and Venkitanarayanan (2006) used 17 *C.sakazakii* isolates from clinical and infant formula to study the specificity of primers used in PCR. They found that amplification of ompA gene specific to *C.sakazakii* used with enrichment (incubation at 37°C for 4 -8 h) could detect fewer than 3 CFU/g of infant formula. Turcovský et al. (2011) isolated 71 *Cronobacter* strains from 602 food samples in their lab, with most strains being identified as *C. sakazakii*, and the highest contaminated foods were of plant origin (spices, teas, chocolate, nuts, vegetables).

Since this bacterium has been found in a wide range of environments, physiological changes due to temperature differentials are bound to occur. *C.sakazakii* has a growth range between 5 and 47°C with a doubling time of around 20 minutes on nutrient rich media at 37°C, depending on the strain (Farber and Forsythe, 2008). The ability of this bacterium to produce exopolysaccharides enhances the formation of biofilms, resulting in increased resistance to disinfectants (Beuchat et al., 2009). This increase in resistance to disinfectants at low temperatures may be similarly important in aquatic environments wherein *in situ* temperatures can be lower than 10°C. Such low aquatic temperatures could impact bacterial survival. The



increase in resistance to disinfectants by *C. sakzarii* and the wide range of environments that it has been isolated from illustrates the importance of gaining a better understanding of how the bacterium can be recovered from the environment where stressors are present.

# RESEARCH OBJECTIVES AND RATIONALE FOR THE CURRENT STUDY

The bacterial species used in the current research were chosen as a result of their significance in public health. A bacterium of particular interest that could enter into a VBNC state and thus elude detection is *Cronobacter sakazakii*. Over the past 49 years there have been at least 111 cases and 26 reported deaths from *C. sakazakii* (Mullane et al., 2007). Between October and December 2004, five infants in France were diagnosed with *C. sakazakii* infection, with two of the five dying (CDR Weekly, 2004). The majority of *C. sakazakii* infections are blamed on tainted powdered infant (milk) formulas (PIF). The current standard for PIF states that *C. sakazakii* should be absent from dried formula and dried dietary foods intended for infants below six months of age as they are at an increased risk of infection from this bacterium (Commissions Regulation (EC), 2005).

Most *E. coli* strains are normally harmless to their host and are present in the intestines of humans and other warm-blooded animals. However, certain strains of *E. coli* can cause severe gastroenteritis. The CDC estimates that there are 265,000 cases of new “Shiga toxin-producing” *E. coli* (STEC) infections each year. Of those infections about 36% are *E. coli* O157 H7, with the remainder caused by other strains of *E. coli* (CDC, 2012). *E. coli* has been found in both aquatic and terrestrial habitats and can survive in such environments until ingested by a mammalian host. Wang and Doyle (1998) showed that enterohemorrhagic *E. coli* O157:H7 was present in water after 91 days at 8, 15, 25° C and could be cultured on TSA agar for up to 12 weeks. The lower the water temperature the longer the bacterium persisted.

## **Objectives**

The overall goal of the proposed study was to evaluate the physiological response (survival, sublethal injury, nutritional demands, and antibiotic resistance profiles) of *C.sakazakii* and *E.coli* following exposure to groundwater held at low temperatures. The specific objectives were as follows:

### **Objective 1:**

To determine the overall survival and degree of sublethal injury incurred by *C.sakazakii* and *E.coli* as a result of prolonged exposure to cold temperature groundwater.

Hypothesis: Cold water stress will result in death as a function of time to *C.sakazakii* and *E.coli*; however, a significant proportion of the survivors will be sublethally injured.

### **Objective 2:**

To determine whether nutritional requirements of *C.sakazakii* and *E.coli* change as a result of exposure to cold temperature stress.

Hypothesis: Surviving *C.sakazakii* and *E.coli* cells will demonstrate an altered demand for specific nutrients during *in vitro* recovery.

### **Objective 3:**

To determine whether cold temperature stress impacts the response of *C.sakazakii* and *E.coli* to antibiotics.

Hypothesis: Cold temperature stress in groundwater will result in altered antibiotic resistance patterns for *C.sakazakii* and *E.coli*.

## **CHAPTER 1**

**Survival and injury of *Cronobacter sakazakii* and  
*Escherichia coli* following exposure to cold temperature  
ground water**

## 1. Introduction

Bacteria are constantly being exposed to changing conditions in the environment and over time have evolved mechanisms that assist in their ability to survive (Aertsen and Michiels, 2004). For example, physiological changes occurring under low-nutrient conditions include cell size shrinkage, modifications to metabolic functions, and increased resistance to environmental stresses (Van Elsas et al., 2010). In classic laboratory experiments examining the effects of different environmental stresses, bacteria are traditionally cultured and grown at optimum temperature and supplied ample nutrients for growth. Unfortunately, optimal growth conditions are rarely found in nature, and thus experimental results from optimal conditions may not accurately reflect behavior from *in situ* conditions. For many bacteria present in aquatic environments, temperatures of 35°C or higher rarely occur and if bacteria have adapted to an aquatic environment then temperatures over 20°C could prove to be lethal (Roszak and Calwell, 1987). Thunberg et al. (2001) found low-nutrient environments can alter effectiveness of certain disinfectants. Therefore, it is possible that changes in environmental conditions could affect how bacteria respond to cold, nutrient depleted environments. When bacteria encounter harsh environments, sublethal injury may occur. Understanding how different environmental conditions affect bacterial sublethal injury is of importance because under favorable conditions sublethally injured cells may have the ability to repair and grow, resulting in increased numbers of pathogens (Lu et al., 2011). This is important when considering the role sublethally injured bacterial cells could play in drinking water and during food production/preservation.

Selective and non-selective plating media are commonly used to assay levels of injury following environmental stress. The number of colonies that appear after incubation on the respective agar plates allows for the determination of how many bacterial cells are present in the

sample at different periods of time. Selective media are designed to permit the growth of the organism of interest while simultaneously inhibiting growth of non-target organisms by incorporating selective agents in the growth medium. However, target cells that have sustained physiological/structural damage as a result of exposure to harsh environmental conditions may lose their ability to grow on selective media.

One selective medium that has this ability is MacConkey agar. MacConkey agar is both a selective and differential medium. It is selective due to the presence of bile salts, which inhibits most gram-positive bacteria (MacConkey, 1908). The addition of neutral red dye acts as a pH indicator and accounts for the differential aspect of this medium. Bacteria that utilize lactose produce acid and lower the pH of this agar below 6.8, which results in the colonies appearing reddish/pink, and these bacteria are lactose positive. Bacteria can also be lactose negative by utilizing peptone instead of lactose, resulting in ammonia production which leads to an increase in pH, leading to colonies that appear white or colorless (MacConkey, 1905). Accordingly, comparison of growth (number of colonies) following simultaneous inoculation and incubation of non-selective and selective media permits evaluation of the degree of injury to surviving cells (Brenner et al., 1996).

In the current study, selective and non-selective media were used to determine the overall survival and degree of sublethal injury incurred by *C.sakazakii* and *E.coli* as a result of prolonged exposure to cold temperature, nutrient-limiting groundwater.

## **2. Methods and Materials**

*Preparation and growth of test bacteria.* *Esherichia coli* (ATCC 23559) and *Cronobactor sakazakii* (ATCC 51329) were grown in 10 ml trypticase soy broth (TSB) at 35°C for 24 h. An

inoculating loop was used to plate the bacterium on a trypticase soy agar (TSA) plate and incubated at 35°C for 24 h. A single colony was selected and grown in 50 ml of TSB at 35°C for 24 h. Ten ml of the 50 ml stationary phase culture was centrifuged at 3020 x g for 11 min. The supernatant was removed, and the pellet was resuspended in 0.1% peptone buffer and washed twice.

*Cold stress.* One ml each of washed cells was added to two flasks of filter-sterilized (0.22µm) well water (initial population ~ 10<sup>6-7</sup> CFU/ml). One flask was held at room temperature (24 °C). The second flask was used for the cold stress experiments (tempered to 4°C prior to inoculation and continuation of 4°C stress for the duration of experiments). Cells in the two different flasks were immediately plated onto TSA and the appropriate selective medium (0-time samples). Flasks were incubated at 24°C and 4°C and samples removed periodically over 9 days (3, 6, and 9 days). Temperature stressed cells (4 °C) and non- temperature stressed cells (24 °C) were plated on nonselective and selective media using a spread plate technique at each stress interval.

*Observation of cell survival and sublethal injury.* Tryptic soy agar (TSA) was used as the non-selective medium for *C. sakazakii* and *E. coli* permitting determination of overall survival (noninjured and injured cells). MacConkey agar was used as the selective medium for both species permitting determination of nonlethally injured survivors. Cold-stressed and non-temperature stressed cells were plated in triplicate and incubated at 35°C for 24 h. After incubation, colonies were counted. Sublethal injury was calculated by determining the difference in the number of colonies able to grow on nonselective (TSA) and selective media (MacConkey).

### 3. Statistical analysis

The colony counts from TSA were used to determine the overall survival of stressed cells by being expressed as the difference in surviving populations detected at the different exposure times vs. the initial (0-day) population. These data were used to determine overall cell density at time intervals (time zero, 3, 6, and 9 days). Sublethal injury was expressed as the percentage of cells which failed to be detected on selective MAC agar vs. cells detected on nonselective TSA. Decimal reduction time (D-value) was estimated using standard regression analysis based on log linear models for each bacterial species tested. A linear model of time versus log CFU of the plate counts on TSA were used to estimate the D-values.

### 4. Results and Discussion

*Survival.* *C.sakazakii* proved to be quite persistent upon 9 days of exposure to nutrient-deplete groundwater (Fig. 1 and Table 1). Nonselective TSA counts were only reduced by 1.1 log at 4°C as compared to a 0.9 log reduction at 24°C (Table 1). The only substantial difference in survival at the two temperatures occurred after 3 days as reflected by a 0.5 log reduction at 4°C versus no change in survivors from day-0 for the 24°C exposed cells (Table 1). Beuchat et al. (2009) studied *C.sakazakii* on stainless steel plates reporting an approximate 2 log reduction after 10 days at 4°C, whereas the same reduction required 25 days at 25°C. The difference in the time acquired to achieve similar reduction in survivors between the study of Beuchat et al. (2009) and the current experiment is likely the result of the difference in stress conditions (aqueous environment versus dry environment on steel plates).

Calculated D-values also reflect the relatively similar survival patterns at the two exposure temperature as demonstrated by a slightly higher  $D_{24^{\circ}\text{C}}=8.57$  as compared to  $D_{4^{\circ}\text{C}}=7.69$



(Fig. 3 and 4). These findings suggest that *C. sakazakii* can withstand the nutrient-deplete environments of groundwater nearly equally as well at 4°C compared to 24°C.

Exposure of *E.coli* to groundwater for 9 days also resulted in a reduction in survivors at both temperatures (Fig. 2 and Table 2). In particular, the warmer 24°C temperature was more detrimental to survival (2.0 log reduction) versus the colder 4°C temperature (1.5 log reduction). The enhanced survival at lower temperatures suggests that *E.coli* may possess physiological mechanisms to permit better persistence during cold stress. Studies performed by Sampson et al. (2006) and Bogosian et al. (1996) concur with the findings of this study. Sampson et al. (2006) studied the effects of temperature on *E.coli* survival in northern lake water, observing rapid reduction in survivors after one week at 10, 14 and 25°C, followed by a dramatic decrease in survivors after 10 days. However, *E.coli* held at 4°C only showed a slight decrease. Bogosian et al. (1996) determined that *E. coli* was able to survive for up to 6 d at 37°C, for 8 d at 20°C, and for 12 d at 4°C in non-sterile river water. This suggests the temperature of an aquatic environment is important in the survival of bacteria.

Calculated D-values also reflect the difference in survival patterns at the two exposure temperatures as reflected by the higher  $D_{4^{\circ}\text{C}}=5.36$  as compared to  $D_{24^{\circ}\text{C}}=4.41$  (Fig. 3 and 4). These finding suggest that *E.coli* can withstand the nutrient-deplete environment of groundwater slightly better at 4°C than at 24°C.

The findings from this study indicate *C.sakazakii* survives better in groundwater at both 4°C and 24°C than *E.coli*. At 4°C *C.skazakii* had slightly better survival in the cold, nutrient-depleted aquatic environments than *E.coli* as evident by a 1.1 log versus 1.5 log reduction by day

9. The difference in survival between *C.sakazakii* and *E.coli* at day 9 is evident at 24°C, with a 0.9 log reduction for *C.sakazakii* and a 2.0 log reduction for *E.coli*.

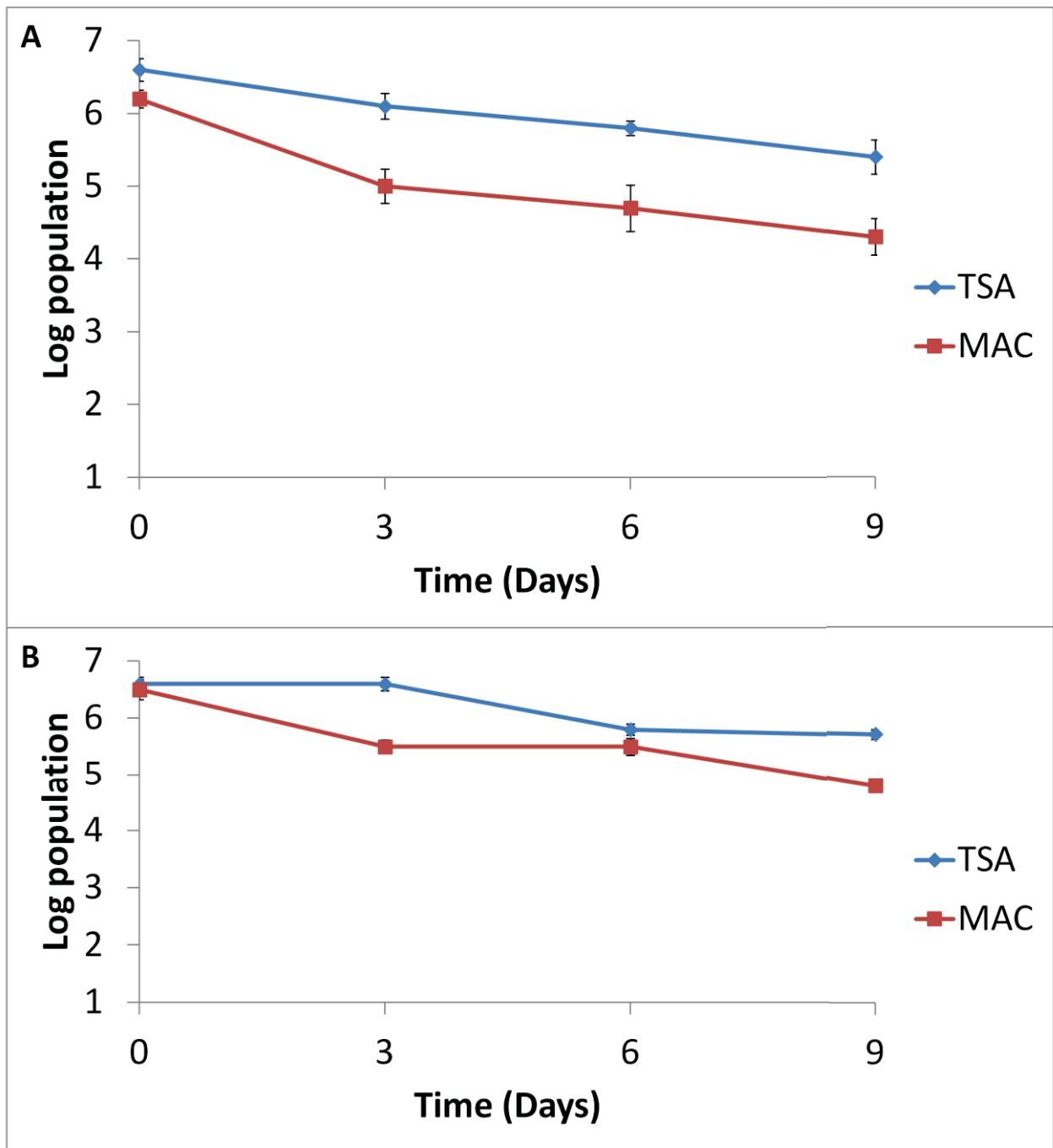
*Sublethal injury.* *C.sakazakii* placed in nutrient-deplete groundwater had less than 90% sublethal injury rate regardless of water temperature (Fig. 1 and Table 1). When *C.sakazakii* viable cell counts are compared on TSA to MacConkey agar after a 9 day incubation period, cold-stressed (4° C) cells sustained 82% versus non-temperature stressed (24°C) 86% sublethal injury (Table 1). The only substantial difference in sublethal injury at the two temperatures occurred at day 6 as reflected by a 77% sublethal injury at 4°C compared to 42% sublethal injury at 24°C (Table 1).

*E. coli* exposed to groundwater for 9 days demonstrated sublethal injury greater than 90% at both temperatures (Fig. 2 and Table 2). When comparing *E.coli* viable cell counts on TSA to MacConkey agar after a 9 day incubation period, 4° C cells sustained 99% injury versus 97% sublethal injury at 24°C (Table 2). The similarity between injury rates at the two temperatures suggests that under the conditions of this study *E.coli* cells are sublethally injured at about the same rate regardless of water temperature.

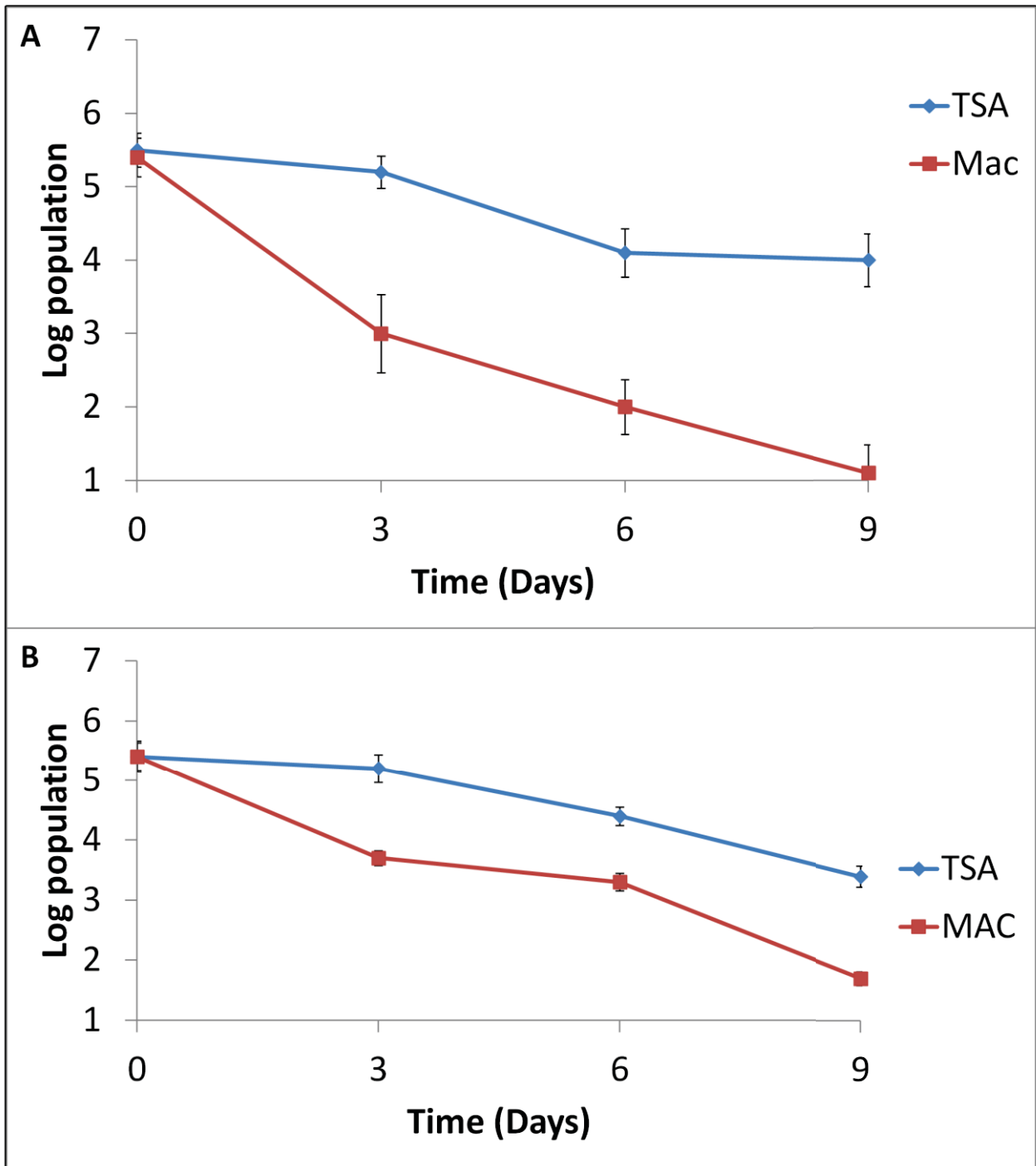
Comparison of injury rates between the two organisms after 9 days of exposure demonstrates that *E.coli* incurs higher levels of injury than *C.sakazakii*, regardless of temperature (Table 1 and 2). *C.sakazakii* may have become less sublethally injured than *E.coli* as a result of higher naturally occurring levels of trehalose in the cells. Kandror et al. (2002) hypothesized that under low temperatures bacterial cells use trehalose to prevent inactivation and aggregation of proteins, thus stabilizing the cell membrane.

## 5. Summary

In this study, physiological changes of *C.sakazakii* and *E.coli* exposed to nutrient-limiting groundwater, and two different temperatures (4°C and 24°C) were examined for survival and sublethal injury using the spread plating method. Cells exhibited decreased survival and sublethal injury over the 9 day experiment. *E.coli* exhibited greater sublethal injury and larger log reduction values when compared to *C.sakazakii* (Table 1 and 2). This observation has at least one potential implication. Since *E.coli* is often used as a universal indicator bacterium for pathogen presence in aquatic environments, it is desirable that *E.coli* survive at least as long as the pathogen (e.g., *C.sakazakii*). Based on the current findings, the use of *E.coli* as a predictor of *C.sakazakii* presence and persistence in an aquatic environment may result in an underestimation of *C.sakazakii* as this bacterium not only survived longer than *E.coli* but also incurred less injury under both temperatures. Future studies should include an investigation of the reliability of a recently developed isolation medium, designated *Enterobacter sakazakii* chromogenic plating medium (ESPM) (Restaino et al., 2006), specifically designed to detect *C.sakazakii* from environmental sources.



**Fig. 1.** Survival and injury of *C. sakazakii* following 9 day exposure to 4 °C, filter-sterilized ground water (panel A) compared to 24°C (panel B). Each point represents the mean of three separate experiments. Surviving populations detected on TSA and MAC agar were performed on triplicate spread plates. Bars represent standard error.



**Fig. 2.** Survival and injury of *E.coli* following 9 day exposure to 4 °C, filter-sterilized ground water (panel A) compared to 24°C (panel B). Each point represents the mean of three separate experiments. Surviving populations detected on TSA and MAC agar were performed on triplicate spread plates. Bars represent standard error.

Table 1. Survival and injury of *C. sakazakii* following exposure to 4°C and 24°C filter-sterilized ground water

Time(days)	4°C Exposure		24°C Exposure	
	Survival (log reduction) <sup>a</sup>	Injury (%) <sup>b</sup>	Survival (log reduction) <sup>a</sup>	Injury (%) <sup>b</sup>
3	0.5	79%	0	91%
6	0.8	77%	0.9	42%
9	1.1	82%	0.9	86%

<sup>a</sup> Expressed as the difference in surviving populations detected at the different exposure times vs. the initial (0-day) population on nonselective TSA agar.

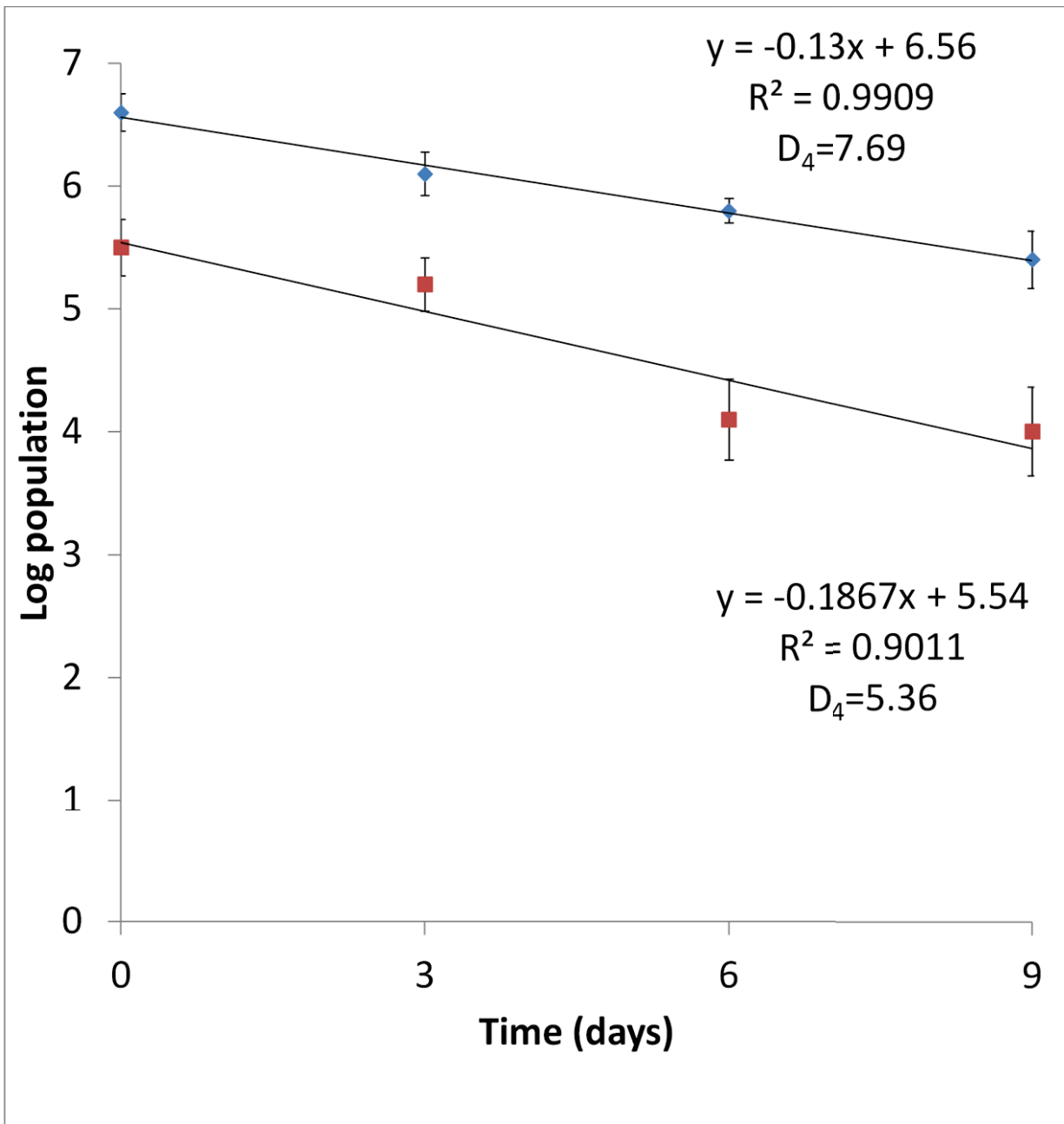
<sup>b</sup> Expressed as the percentage of cells which failed to be detected on selective MAC agar vs. cells detection on nonselective TSA agar at the respective time intervals.

Table 2. Survival and injury of *E. coli* following exposure to 4°C and 24°C filter-sterilized ground water

Time(days)	4°C Exposure		24°C Exposure	
	Survival (log reduction) <sup>a</sup>	Injury (%) <sup>b</sup>	Survival (log reduction) <sup>a</sup>	Injury (%) <sup>b</sup>
3	0.2	97%	0.2	95%
6	1.3	97%	1.0	89%
9	1.5	99%	2.0	97%

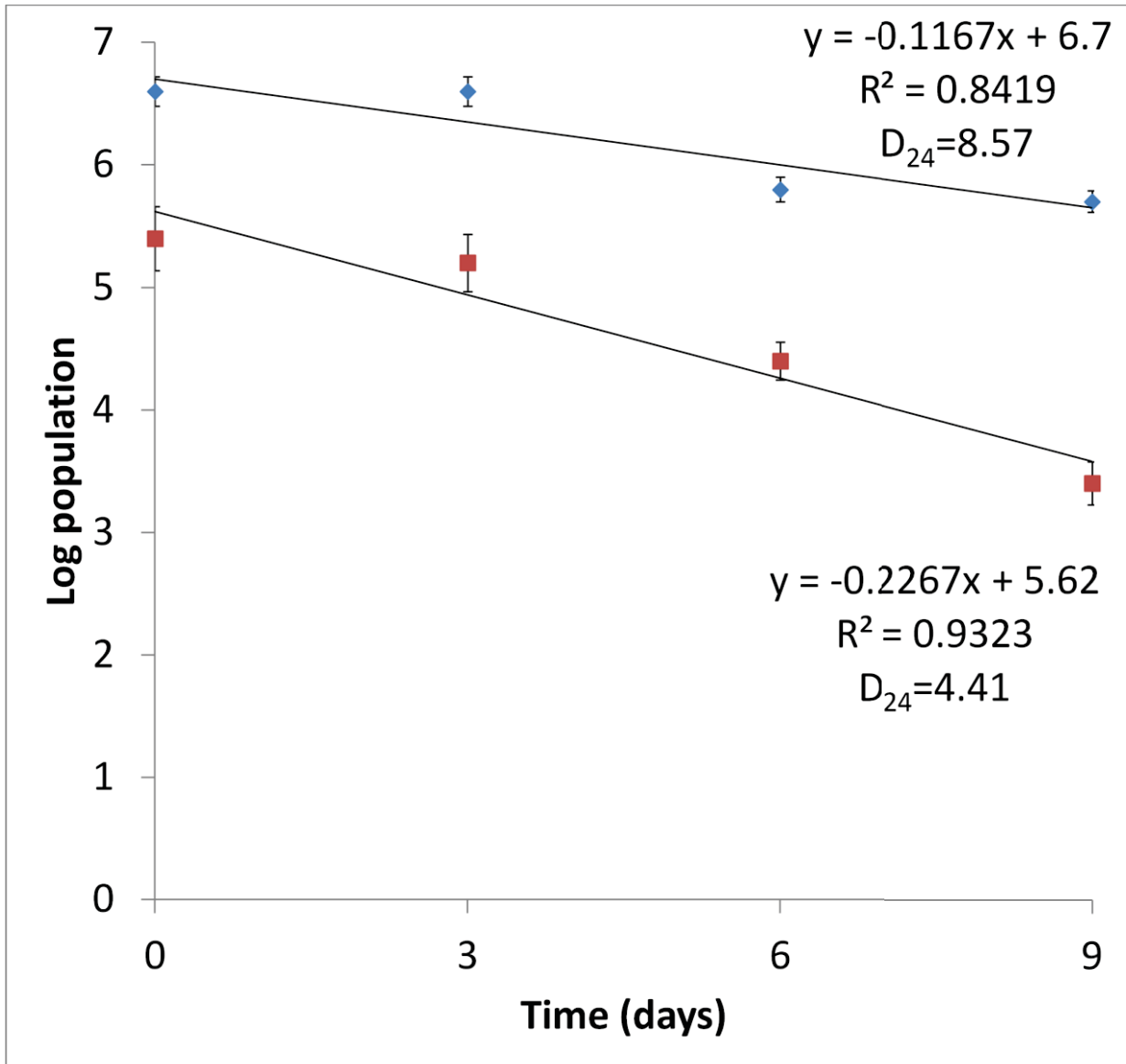
<sup>a</sup> Expressed as the difference in surviving populations detected at the different exposure times vs. the initial (0-day) population on nonselective TSA agar.

<sup>b</sup> Expressed as the percentage of cells which failed to be detected on selective MAC agar vs. cells detection on nonselective TSA agar at the respective time intervals.



**Fig.3.** D-value determination of cold-stressed *C. sakazakii* (◆) and *E.coli* (■). Points are cell counts graphed by linear regression of triplicate TSA counts to determine slope. Each point represent the average of three independent experiments for *C. sakazakii* and *E.coli*. Bars represent standard error.





**Fig.4.** D-value determination of room temperature *C. sakazakii* (◆) and *E. coli* (■). Points are cell counts graphed by linear regression of triplicate TSA counts to determine slope. Each point represent the average of three independent experiments for *C. sakazakii* and *E. coli*. Bars represent standard error.

## **CHAPTER 2**

**Physiological response of *Cronobacter sakazakii* and *Escherichia coli* following exposure to cold temperature ground water**

## 1. Introduction

Identification of physiological changes that occur when bacterial cells are under stressful conditions often involve microscopic methods, such as direct viable counts and flow cytometry. Direct viable count assays are performed to determine the amount of living vs. dead cells in a sample. This is achieved by focusing on increases in intra-cellular rRNA that when present is bound by fluorescence during in situ hybridization (FISH). Fluorescing cells are said to be metabolically alive as evidenced by increasing amounts of rRNA (Piqueres et al., 2006). Flow cytometry is performed by suspending cells in a stream of fluid and then passing them through a detection apparatus where they are sorted by specific criteria. These cells are then analyzed based on physical and chemical characteristics. Dyes are used to distinguish membrane permeability and potential, thus allowing distinction of living vs. dead cells (Loken, 1990). These methods of detection can be time consuming and there is a lack of agreement on standard procedures.

Miller and Rhoden (1991) evaluated the BIOLOG microplate system for its ability to correctly identify clinical and environmental bacterial isolates within 24 h. BIOLOG identifies microorganisms based on the exchange of electrons produced during bacterial respiration of carbon substrates causing the tetrazolium indicator dye to be reduced, changing to various shades of purple. The microplate is able to determine the rate and which of the 95 different carbon substrates an organism can utilize, thereby generating a “metabolic fingerprint” to enable identification of the unknown bacterial species (Miller and Rhoden, 1991).

BIOLOG was originally designed for bacterial identification in clinical settings; however, in recent years it has been utilized for ecological purposes. Garland and Mills (1991) used

BIOLOG to determine microbial community analysis in different environmental samples. BIOLOG permitted rapid visualization of community structure, in conjunction with the ability to compare microbial communities with different parameters. Since the BIOLOG system contains 95 different substrates, any changes in substrate utilization as a result of exposure to environmental stress can be examined. Using BIOLOG microplates, Ihssen and Egli (2005) found that *E.coli* oxidized more substrates when grown on a carbon-limiting medium than when grown on a medium containing excess carbon. McCarroll (2008) reported increased utilization of xylitol, adonitol, and glycogen for heat stressed *C.sakazakii* (52°C for 90 min) using the BIOLOG system. These observations suggest that BIOLOG microplates could provide beneficial data regarding changes in carbon utilization of a pure culture under stress conditions.

In the current study, the BIOLOG microplate system was used to determine differences in substrate utilization of *C.sakazakii* and *E.coli* as a result of exposure to low-nutrient groundwater incubated at two different temperatures (4 °C and 24°C). Determination of substrate utilization profiles could allow for more realistic determination of which specific carbon substrates these bacteria use for metabolic functions when under cold stress, perhaps leading to modification of the nutritional contents of selective media used to detect cold-stressed bacteria.

## **2. Materials & Methods**

*Preparation and growth of test bacteria.* Preparation of *C.sakazakii* and *E.coli* were the same as those described in Chapter 1.

*Cold stress.* Preparation of *C.sakazakii* and *E.coli* were the same as those described in Chapter 1.

*Carbon source utilization patterns.* Carbon source utilization patterns of cold stressed cells were determined with a respiration assay based on BIOLOG GN MicroPlates. BIOLOG microplates

were inoculated with  $10^{-2}$  dilutions of both room temperature (24°C) and cold stressed cells (4°C) of *C.sakazakii* and *E.coli* at day 0, 3, 6. Each bacterium's ability to use the 95 different substrates was determined by comparing tetrazolium dye color development in the wells to the control well. BIOLOG GN plates contain 95 substrates arranged into six different guilds: carbohydrates, carboxylic acids, polymers, amines/amides, amino acids, and miscellaneous (Zak et al., 1994). The rate of absorption of the tetrazolium dye was determined at 590 nm using a SpectraMax 340PC. This wavelength was chosen as it is the maximum absorbance rate for tetrazolium violet (Lindstrom et al., 1998). Positive readings were considered to be those wells with an absorbency greater than 0.40 (De Paolis and Lippi, 2008 and Verschuere et al., 1997) BIOLOG GN microplates were incubated at 35 °C and read at 48 h.

### **3. Statistical analysis**

Data from the BIOLOG microplates were taken from Soft MAX Pro (version) and transferred into an Excel spreadsheet. The net absorbance value for each of the 95 well was determined by subtracting the control well from each of the substrates. The net absorbance values of each substrate at 48h incubation for day zero at each temperature were compared to the same substrate values at day six for increased or decreased use of substrates. Any increase or decrease in substrate utilization over 60 percent was noted. Average well color development (AWCD) for day 0, 3, and 6 was calculated and significance was determined by a t-test ( $p < 0.05$ ).

### **4. Results and Discussion**

Average well color development (AWCD) of the BIOLOG GN microplates were calculated by averaging the absorbance reading for all 95 substrates for room temperature and cold stressed cells (Table 1). AWCD for *C.sakazakii* decreased over the six day exposure period

at both temperatures. It is of interest to note that AWCD was approximately 2-3 times greater ( $p < 0.05$ ) at all exposure times for cells stressed in the nutrient-deplete groundwater at 24°C as compared to 4°C. This observation suggests that the lower temperature stress (4°C) more severely impaired overall substrate utilization.

AWCD for *E.coli* at day 3 increased for both temperatures, before decreasing at day 6 (Table 1). Notably, the AWCD for cold stressed *E.coli* cells at day 6 was higher than at day 0 (Table 1), suggesting that cold stressed cells were able to utilize the substrates more efficiently. The only significant difference ( $p < 0.05$ ) for *E.coli* AWCD between the two stress temperature was at day 0 (Table 1). The difference in 0-day AWCD between the two different temperatures could be the result of initial cold shock to the bacteria during the introduction of the washed cells into the 4°C groundwater.

Differences in substrate utilization (greater or less than 60%) for *E.coli* and *C.sakazakii* between day 0 and day 6 after incubation for 48 h are expressed in Table 2 for cold-stressed 4°C and Table 3 for room temperature 24°C groundwater. *E.coli* exposed to cold stress for 6 days showed a decrease of over 60% for one substrate D-glucose-6-phosphate (Table 2). D-glucose-6-phosphate is used to produce pyruvate which is utilized in the citric acid cycle to produce NADH, FADH<sub>2</sub>, and ATP (Kessler et al., 2005). *C.sakazakii* cells exposed to 6 day nutrient-limiting cold stress showed a decrease in L-arabinose and  $\alpha$ -D-lactose (Table 2). L-arabinose is one of the substrates used in the pentose-phosphate pathway, which results in the synthesis of nucleotides and aromatic amino acids (Caspi et al., 2006). McCarroll (2008) found that 90 min of heat stress resulted in a significant decrease in the utilization of D-glucose-6-phosphate for *E.coli* and a decrease in L-arabinose utilization for *C.sakazakii* under the same conditions. These results suggest when *E.coli* and *C.sakazakii* encounter stress conditions catabolic pathways are

repressed. There was not increase in any substrate utilization for either bacterium under cold stress when comparing day 0 to day 6 cells. When examining the results from cold stress, one should note that the cells were first cold shocked (4°C), then placed in the BIOLOG system, and incubated at 35°C for 48 h, resulting in potential heat shock. This additional shock might mask or change true cold response to differences in substrate utilization.

*E. coli* cells held at room temperature resulted in decreased utilization of D-mannitol and succinic acid (Table 3). D-mannitol is an energy source and carbon storage molecule in nature and many microbes use it as an alternative to traditional synthesis (Song and Vieille, 2009). Also, under room temperature conditions, *E. coli* demonstrated an increase in  $\alpha$ -D-glucose, D-trehalose, turanose, D-glucuronic acid, Tween 80, m-inositol, and urocanic acid utilization (Table 3). Both  $\alpha$ -D-glucose and D-trehalose metabolism produces pyruvate which when entered into the citric acid cycle produces NADH, FADH<sub>2</sub>, and ATP (Kessler et al., 2005). Turanose is involved in signal transduction, and can be used as a carbon source by many bacteria (Sinha et al., 2002). Of interest is the increased utilization of urocanic acid, which goes from zero at day 0 to an absorbance value of greater than 0.40 at day 6. Urocanic acid under nutrient-deplete conditions can be used as a carbon source, also it has been found to trigger VjbR protein expression. VjbR expression allows cross-talk among neighboring cells about changes in the environment (Arocena et al., 2012). This suggests that *E. coli* could use urocanic acid as not only an additional carbon source but also to allow cells within an environment to communicate.

*C. sakazakii* cells exposed to room temperature showed decreased utilization of p-Hydroxy phenylacetic acid, D-serine, D-glucose-1-phosphate, and D-glucose-6-phosphate

(Table 3). D-glucose-1-phosphate and D-glucose-6-phosphate are both used for the production of energy (Kessler et al., 2005). This suggests that under nutrient-limiting conditions *C.sakazakii* regulates catabolic functions.

## **5. Summary**

In this study, the physiological changes of *E.coli* and *C.sakazakii* exposed to cold stress, nutrient-limiting conditions were examined with the BIOLOG microplating system. Differences in AWCD and utilization of different substrates over the six day experiment suggest that BIOLOG may be useful in identifying physiological changes in environmentally stressed bacterial cells. The use of BIOLOG in conjunction with traditional assay methods may lead to a better understanding of how metabolic changes, as a result of stress, effect bacterial survival in the environment. One possible application is to modify the selective media used to detect these bacteria from environmental sources with substrates that indicated increased utilization.



**Table 1.** Average well color development (AWCD) for *C.sakazakii* and *E.coli* following 6 day exposure to nutrient-deplete groundwater at 4°C or 24 °C.

Exposure time (days)	AWCD <sup>a</sup>			
	<i>C.sakazakii</i>		<i>E.coli</i>	
	4°C/ND <sup>b</sup>	24°C/ND <sup>c</sup>	4°C/ND <sup>b</sup>	24°C/ND <sup>c</sup>
Day 0	0.55	1.06 <sup>^</sup>	0.26	0.49 <sup>^</sup>
Day 3	0.53	1.00 <sup>^</sup>	0.50	0.70 <sup>*</sup>
Day 6	0.39	0.90 <sup>*^</sup>	0.45	0.49

<sup>a</sup> Average well color development after 48 hour incubation in BIOLOG GN microplates

<sup>b</sup> Exposed to 4°C nutrient-deplete filter sterilized groundwater

<sup>c</sup> Exposed to 24°C nutrient-deplete filter sterilized groundwater

<sup>\*</sup>t-test (p<0.05) shows significance when compared to day 0 of each temperature

<sup>^</sup> t-test (p<0.05) shows significance when comparing 4°C to 24°C at the same time interval

**Table 2.** Utilization of specific BIOLOG substrates by cold-stressed (4°C) *C.sakazakii* and *E.coli* comparing day 0 to day 6 exposure in nutrient-deplete groundwater (expressed as percent difference).

Organism	Substrates	
	Increased utilization	Decreased utilization
<i>E.coli</i>	<sup>a</sup> None	D-glucose-6-phosphate (71%)
<i>C.sakazakii</i>	<sup>a</sup> None	L-arabinose (62%) α-D-lactose (61%)

<sup>a</sup>None=value less than 60%.

**Table 3.** Utilization of specific BIOLOG substrates by room temperature stressed (24°C) *C.sakazakii* and *E.coli* comparing day 0 to day 6 exposure in nutrient-deplete groundwater (expressed as percent difference).

Organism	Substrates	
	Increased utilization	Decreased utilization
<i>E. coli</i>	α-D-glucose (194%) D-trehalose (90%) Turanose (136%) D-glucuronic acid (130%) Tween 80 (^) m-Inositol (^) Urocanic acid (^)	D-mannitol (*) Succinic acid (*)
<i>C.sakazakii</i>	None <sup>a</sup>	p-Hydroxy phenylacetic acid (*) D-serine (*) D-glucose-1-phosphate (*) D-glucose-6-phosphate (*)

\*Indicates substrate at day 0 was greater than 0.40 but by day 6 was zero.

^ Indicates substrate at day 0 was zero but by day 6 was greater than 0.40.

<sup>a</sup>None = value less than 60%.

## **CHAPTER 3**

**Changes in antibiotic resistance patterns of *C.sakazakii* and *E.coli* resulting from exposure to low-temperature ground water**

## 1. Introduction

*Enterobacteriaceae* is a large family of gram-negative bacteria that range from harmless saprophytes to pathogens of humans, animals, and plants. Two bacteria of importance are *E.coli* and *C.sakazakii*. *E.coli* is present in a variety of different environments from soils, to water, to intestinal tracts of humans and animals. In many cases, *E.coli* is not a pathogen and even beneficial to its host. However, as with all bacteria, pathogenic strains such as *E.coli* O157: H7 do exist. Approximately 0.1% of the intestinal gut flora consists of *E.coli* and other closely related bacteria (Eckburg et al., 2005). Since *E.coli* can survive in the environment for varying time periods and pathogenic strains are mainly transmitted by the fecal-oral route, it is used as an indicator organism of fecal contamination in environmental samples (Ribeiro et al., 2012; Feng et al., 2002; Ishii and Sadowsky, 2008).

*C.sakazakii* is listed as an opportunistic pathogen by the World Health Organization and the Food and Drug Administration because it primarily infects infants less than one year in age, immunocompromised individuals, and elderly patients (FAO/WHO, 2006). *C.sakazakii* infections have been linked to life-threatening neonatal meningitis, septicemia, and necrotizing enterocolitis (Dancer et al., 2009). *C.sakazakii* has been isolated from a wide range of foods, such as processed milk, cheeses, meats, herbs, spices, and rice (Al-Nabulsi et al., 2011). Also, this bacterium has been found in a wide spectrum of environments such as water, soil, and household dust (Friedemann, 2007; Schindler, 1994). One environment of particular concern for *C.sakazakii* is powdered infant formula (PIF) because it is an ideal medium for bacterial growth, given that it is not a sterile product and is rich in carbohydrates and fats (Farber and Forsythe, 2008; Dancer et al., 2009; Al-Nabulsi et al., 2011), with detection ranging from 2.4 to 14.2% of samples tested. When using PIF, many parents or caregivers reconstitute enough formula to last

for at least a day. The Centers for Disease Control and Prevention, Food and Agriculture Organization, and World Health Organization all recommend that powdered infant formula be held at refrigeration temperatures ( $< 5^{\circ}\text{C}$ ) for no more than 24 hours after being reconstituted (CDC, 2012; FAO/WHO, 2006). Detection and treatment of *C.sakazakii* infections are important since infant infection rates can result in 40-80% fatality within a short time period. In infants less than one year of age, a combination of ampicillin and gentamycin/chloramphenicol antibiotics are administered (CDC, 2012; Farber and Forsythe, 2008). Survivors often suffer severe mental impairment (Stock and Wiedemann, 2002). These statistics highlight the need for adequate detection and treatment.

*C.sakazakii* and *E.coli* may become antibiotic resistant like other bacteria through spontaneous induced mutation events or acquisition of resistance genes from other bacteria during horizontal gene transfer via conjugation, transduction, or transformation (Donadio et al., 2010). Antibiotics have naturally occurred in the environment for centuries because some bacteria naturally produce them as a bi-product of natural cell processes and defense against neighboring bacteria in the same ecosystem. As long as antibiotics have been produced, resistance genes have been present (D'Costa et al., 2011). For example, a sample of dried soil on plant roots preserved from 1689 in a British Museum was analyzed in 1962, and the presence of penicillinase was detected from dormant *Bacillus licheniformis* endospores (Umbreit, 1970).

The increasing prevalence of antibiotic resistance is directly linked to the large numbers of antibiotics that are administered for the treatment of human, animal, and plant diseases (D'Costa et al., 2011; Donadio et al., 2010; Hawkey and Jones, 2009). Humans have contributed to the antibiotic resistance problem through misuse and overuse. For animals, antibiotics are added to feed to increase their size and decrease death from pathogens, thus increasing the profit

margin in the food industry. As more and more antibiotics are polluting the environment from human and animal waste and the pharmaceutical industry, resistance in bacteria shortly follows. These bacteria rapidly replicate, with those containing resistance genes having a survival advantage over those that do not. Since horizontal gene transfer may occur among bacteria, these resistance genes can be transferred long after antibiotics are removed from the environment (Sapkota et al., 2007). Once transferred genes encoding for resistance incorporate into the bacterial cells, genome antibiotic resistance develops in usually one of four ways: drug inactivation or modification, alteration of target site, alteration of metabolic pathway, or reduced drug accumulation (Tenover, 2006; Ki and Nikadio, 2009).

In the present study, the differences in antibiotic resistance patterns of *C. sakazakii* and *E.coli* exposed to a cold temperature, nutrient-limiting aquatic environment vs. an optimal temperature, nutrient-rich environment were examined. Changes in antibiotic resistance for *C. sakazakii* and *E.coli* were determined using a modified version of the Kirby-Bauer disk diffusion assay.

## 2. Methods and Materials

*Preparation and growth of test bacteria.* Preparation of *C.sakazakii* and *E.coli* were the same as those described in Chapter 1.

*Cold stress.* One ml aliquots of *C.sakazakii* and *E.coli* washed cells were added to twelve flasks (six for each bacterium) containing 99 ml of filter-sterilized (0.22 $\mu$ m) well water (initial population  $\sim 10^{6-7}$  CFU/ml). These flasks were used for the cold stress experiments (tempered to 4°C prior to inoculation and continuation of 4°C stress for survival experiments). Cells of either *C.sakazakii* or *E.coli* were harvested from one flask at each time interval.

*Optimum temperature and nutrient-rich growth conditions.* One ml of *C.sakazakii* and *E.coli* washed cells were added to flasks containing 99 ml of autoclaved Trypticase Soy Broth (TSB) (initial population  $\sim 10^{6-7}$  CFU/ml). These flasks served as the temperature and nutrient controls and were held at 35 °C. Cells of either *C.sakazakii* or *E.coli* were harvested from one flask at each time interval.

*Antibiotic disks.* Determination of the degree of antibiotic resistance within surviving populations at different time intervals of stress was evaluated by a modification of the Kirby-Bauer disk diffusion assay (Bauer et al., 1959). A 10 mL sample of groundwater or TSB containing *C.sakazakii* or *E. coli* was removed from the (4 °C) flasks and (35° C) flasks at time zero and every 3 days for 15 days and subsequently filtered through a 0.22µm filter. The filter was aseptically placed in the center of a TSA plate, followed by aseptic placement of two different antibiotic disks on the filter. This process was done three times for each flask to evaluate the six different antibiotics. Antibiotic disks used were ampicillin (10µg), ciprofloxacin (5µg), kanamycin (30µg), streptomycin (10µg), chloramphenicol (30µg), and tetracycline (30µg). The total diameter of any zones of inhibition was measured (in millimeters) after incubation at 35°C for 24 h to determine changes in antibiotic resistance patterns.

### **3. Statistical analysis**

Determination of differences in antibiotic disk zones of inhibition were examined by linear regression for each antibiotic and significance was determined by a t-test ( $p < 0.05$ ).



#### 4. Results and Discussion

##### *Susceptibility of C.sakazakii to antibiotics.*

*C.sakazakii* illustrated a steady increase in susceptibility ( $p < 0.05$ ) to streptomycin and ciprofloxacin (Fig. 1 B) during the 15 days of exposure to 4°C groundwater. Also, there was a slight increase in susceptibility to tetracycline (Fig. 1 B), but this was found to not be statistically significant ( $p > 0.05$ ). On the contrary, a steady increase in resistance to chloramphenicol, ampicillin, kanamycin (Fig. 1 A) was found under these identical stress conditions. These observed decreases and increases in antibiotic susceptibility or resistance as a result of cold stress are supported by earlier findings of Al-Nabulsi (2011), who examined *C.sakazakii* cells exposed to 4°C in a sample of sterile potassium phosphate buffer for 24 hours.

Non-temperature stressed, nutrient-rich *C.sakazakii* cells grown in TSB and held at the optimal temperature of 35°C over the same 15 day period demonstrated increased susceptibility ( $p < 0.05$ ) to streptomycin, chloramphenicol, tetracycline, ciprofloxacin, ampicillin, and kanamycin (Fig. 1 C and D). However, the increased susceptibility to tetracycline (Fig. 1 D) and ampicillin (Fig. 1 C) were not statistically significant ( $p > 0.05$ ). Young-Duck (2012) found that the same strain of *C.sakazakii* used in this experiment under non-stressed conditions was susceptible to ampicillin, streptomycin, tetracycline, and chloramphenicol. Using five strains of *C.sakazakii*, Al-Nabulsi (2011) observed unstressed cells were sensitive to streptomycin, kanamycin, and ciprofloxacin, but were resistant to tetracycline. Susceptibility to ampicillin was strain dependent. The results from the current study suggest that cold-stressed *C.sakazakii* in aquatic environments may demonstrate different antibiotic resistance patterns compared to cells cultured under ideal growth conditions. However, it should be noted that by day 15 of this

experiment, cells held at 35°C in TSB were also stressed and were in stationary phase, possibly triggering the stringent response due to utilization of the nutrients contained in TSB within 24 h of bacterium addition.

Since low-temperature storage (refrigeration and freezing) is a common method to preserve the quality and integrity of foods and drinks, a better understanding of the physiological effects of cold stress is of major importance. In order to counter exposure to lower temperatures, bacterial cells may synthesize cold-shock proteins (CSP's) which aid in the process of changing saturated fatty acids to unsaturated fatty acids in the outer membrane, thereby allowing for better maintenance of membrane fluidity (Prisco et al., 2012; Shavaji et al., 2010). In the current study, efforts were focused on determining how environmental conditions (low temperature and nutrient starvation) may impact the antibiotic resistance profiles of *C.sakazakii*. The lack of a known reservoir for *C.sakazakii*, combined with its detection in a variety of locations (water, soil, house dust, and various foods), illustrates the importance of gaining a better understanding of the impact of environmental stress on this pathogen (Friedemann, 2007; Schindler, 1994).

#### *Susceptibility of E.coli to antibiotics.*

In this study, *E. coli* cells were exposed to cold stressed (4°C), nutrient-limiting aquatic environment of filter-sterilized groundwater. As the exposure time extended over the 15 days, survivors of the stress demonstrated an increase in resistance ( $p < 0.05$ ) to ampicillin and kanamycin (Fig. 2 A), with a corresponding increase in susceptibility ( $p < 0.05$ ) to chloramphenicol, tetracycline ( $p > 0.05$ ), and ciprofloxacin ( $p > 0.05$ ) (Fig. 2 B). Stressed *E.coli* cells were completely resistant to streptomycin ( $p > 0.05$ ) over the 15 days (Fig. 2 A), with the lawn of growth extending to the edge of the antibiotic disk. Results from an experiment

performed by Scherer et al. (2013) support the theory that when *E. coli* is exposed to streptomycin in soil, the population of resistant cells increases and these resistant cells can remain in soil for years. They found a 25.2% increase in resistance to streptomycin in the test population (sheep grazing on grass sprayed with streptomycin levels equivalent to those used in treatment of orchards). In contrast, only a 6.5% increase in streptomycin resistance occurred the control group (sheep grazing on grass not exposed to streptomycin sprayed grass) for *E. coli* isolated from sheep fecal samples over a three month period. They attributed the increased resistance in the control to manure added five years previously. These results illustrate that antibiotic resistant bacteria can exist in the environment long after the source of contamination is removed.

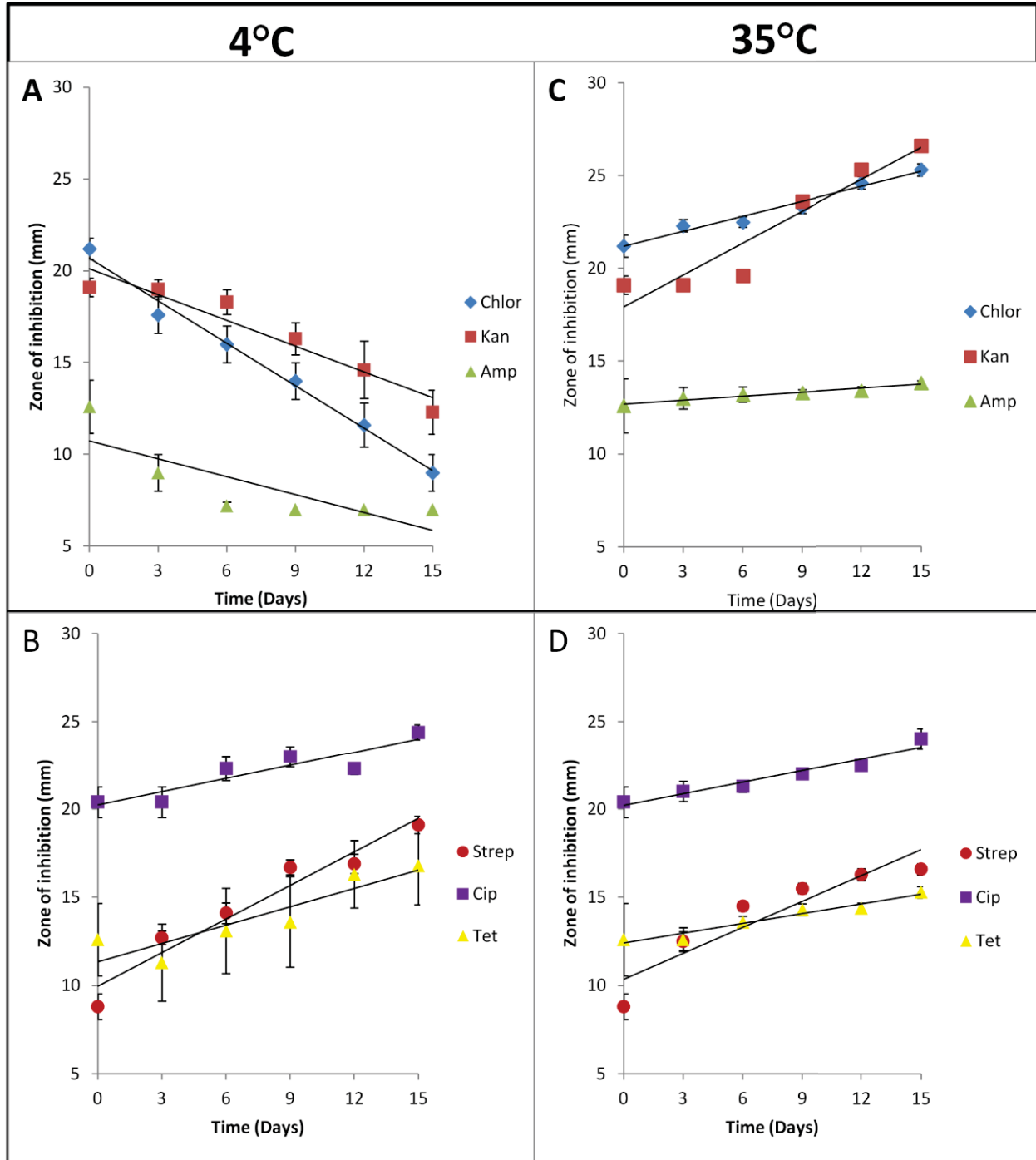
*E. coli* cells grown in nutrient-rich TSB and held at the optimal temperature of 35°C over the same 15 day period reflected an increase in susceptibility to tetracycline ( $p > 0.05$ ), ciprofloxacin (Fig. 2 D), and kanamycin ( $p < 0.05$ ) (Fig. 2 C), with a corresponding increase in resistance ( $p < 0.05$ ) to chloramphenicol (Fig. 2 D) and ampicillin (Fig. 2 C). Similar to observations with cold temperature stressed conditions, *E. coli* remained resistant to streptomycin ( $p > 0.05$ ) throughout the 15 days (growth to the edge of the disk) (Fig. 2 C). A six decade study examining antibiotic resistance of *E. coli* conducted by the Food and Drug Administration using human and animal *E. coli* isolates resulted in a steady increase in resistance to streptomycin, ampicillin, and tetracycline; intermediate resistance to chloramphenicol and kanamycin due to their past large use in cattle; and little to no resistance to ciprofloxacin (Tadesse et al., 2012). The results from the present study suggest that cold, nutrient limiting environments may have the ability to alter *E. coli*'s susceptibility or resistance *in vitro* to antibiotics and closer examination of the significance these changes requires further study. Similar to observations with *C. sakazakii*,

consideration must be given to the fact that cells held at 35°C in TSB also were stressed as the result of stationary phase conditions being created in the flask within 24 h of inoculum addition.

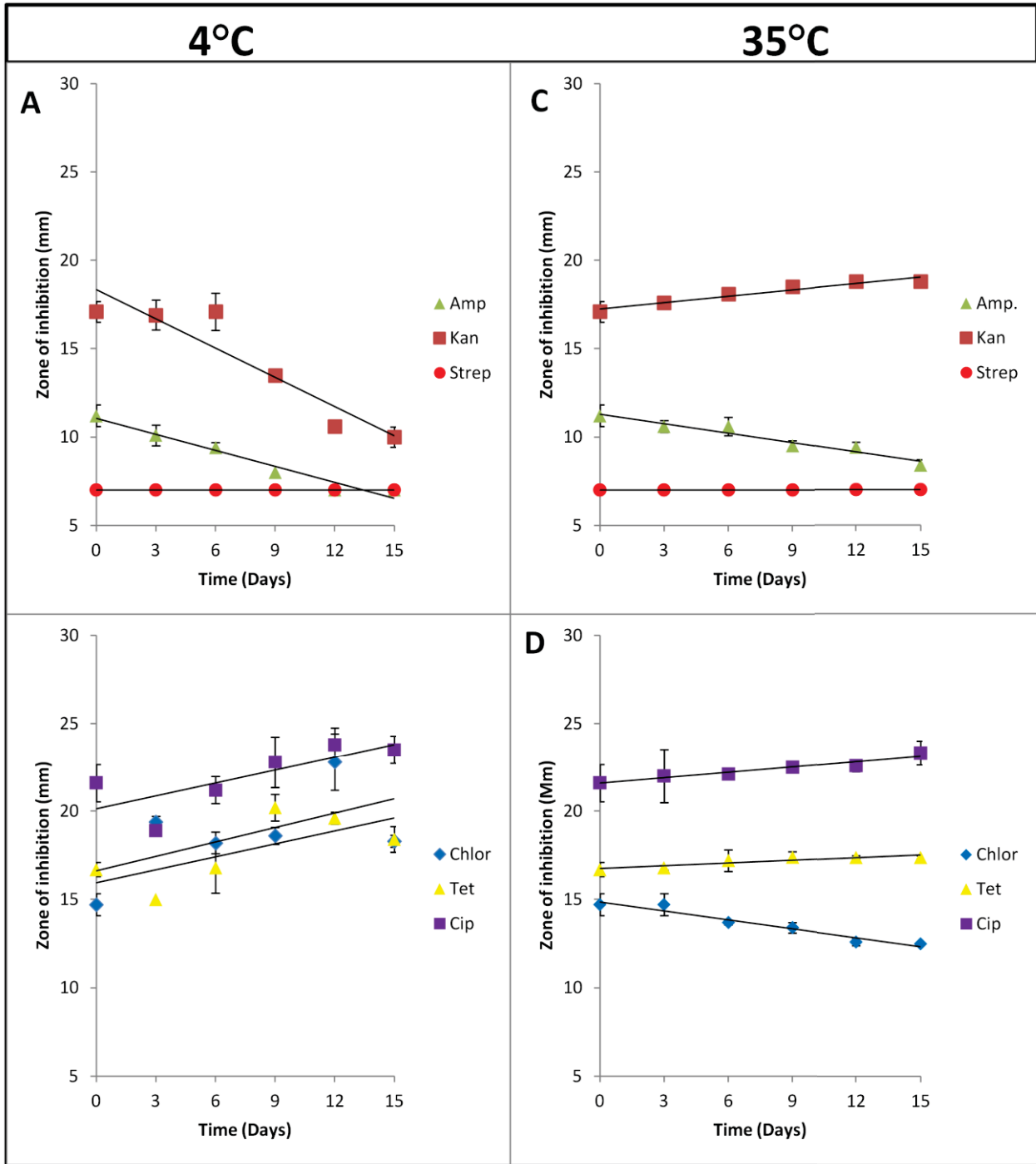
Understanding the effects of cold-stress on *E.coli* is of importance because of its prevalence in the environment as a result of being shed into the environment from its many mammalian hosts (CDC, 2012; Robeiro et al., 2012; Scherer et al., 2013). Wang and Doyle (1998) reported detection of *E.coli* O157:H7 on TSA in water samples 91 days after contamination at temperatures varying from 8 to 25°C, with lower temperatures favoring extended survival. The findings from Wang and Doyle (1998) and the current study indicate the importance of gaining a better understanding of the impact of environmental stress on this bacterium because it is often used as an indicator bacterium for fecal contamination.

## **5. Summary**

The changes in resistance and susceptibility to six antibiotics for both test organisms under stressed and unstressed conditions are summarized in Table 1. These findings suggest that physiological changes do occur when *C.sakazakii* and *E.coli* are exposed to cold-stressed, low-nutrient aquatic environments, including modifications to resistance/susceptibility to antibiotics. These observations have at least two potential applications. First, antibiotics are sometimes added to bacteriological media in order to increase the selectivity of the detection method. Based on the current findings, determining which antibiotic is most suitable to add to a selective medium can be more adequately evaluated. Second, the results from this study indicate the two most commonly administered antibiotics (ampicillin and chloramphenicol) to neonates with diagnosed *C.sakazakii* infections may need to be further examined to determine the best course of treatment as cold stress according to the results of this study may alter their effectiveness.



**Fig 1.** Responses of *C. sakazakii* to six antibiotics during 15 days of exposure to filter-sterilized groundwater held at 4°C (panels A and B) as compared to optimal growth conditions in TSB at 35°C (panels C and D). Data points are average of three experiments, performed with triplicate spread plates at day 0 and 3,6,9,12,15 days thereafter. Bars represent the standard error.



**Fig 2.** Responses of *E. coli* to six antibiotics during 15 days of exposure to filter-sterilized groundwater held at 4°C (panels A and B) as compared to optimal growth conditions in TSB at 35°C (panels C and D). Data points are average of three experiments, performed with triplicate spread plates at day 0 and 3,6,9,12,15 days thereafter. Bars represent the standard error.

**Table 1.** Resistance (R) and susceptibility (S) of *C.sakazakii* and *E.coli* following a 15 day exposure to non-growth conditions in nutrient-deplete groundwater at 4°C or optimal growth conditions in TSB at 35°C. Resistance (R) and susceptibility (S) determined with a modified Kirby-Bauer disk diffusion assay by measuring zones of growth inhibition.

Antibiotic	<i>C.sakazakii</i>		<i>E.coli</i>	
	Test Conditions			
	4°C/ND <sup>a</sup>	35°C/TSB <sup>b</sup>	4°C/ND <sup>a</sup>	35°C/TSB <sup>b</sup>
Chlor.	R*	S*	S*	R*
Kan.	R*	S*	R*	S*
Amp.	R*	S	R*	R*
Strep.	S*	S*	— <sup>nc</sup>	— <sup>nc</sup>
Cip.	S*	S*	S	S
Tet.	S	S	S	S

<sup>a</sup> Exposed 4°C nutrient-deplete filter sterilized groundwater.

<sup>b</sup> Grown at 35°C in TSB.

\*Statistically significant at  $p < 0.05$ .

<sup>nc</sup> No change in resistance (growth extending to edge of antibiotic disk).

## **6. APPENDIX**



**Table 2.** Regression equations and R<sup>2</sup> values for *C.sakazakii* and *E.coli* following a 15 day exposure to non-growth conditions in nutrient-deplete groundwater at 4°C or optimal growth conditions in TSB at 35°C.

Antibiotic	<i>C.sakazakii</i>		<i>E.coli</i>	
	Test Conditions			
	4°C/ND <sup>a</sup>	35°C/TSB <sup>b</sup>	4°C/ND <sup>a</sup>	35°C/TSB <sup>b</sup>
Chlor.	y = -0.7741x + 20.686 R <sup>2</sup> = 0.9897	y = 0.2686x + 21.186 R <sup>2</sup> = 0.9727	y = 0.2724x + 16.624 R <sup>2</sup> = 0.3466	y = -0.1676x + 14.857 R <sup>2</sup> = 0.9455
Kan.	y = -0.4686x + 20.114 R <sup>2</sup> = 0.9226	y = 0.5724x + 17.924 R <sup>2</sup> = 0.9067	y = -0.5524x + 18.343 R <sup>2</sup> = 0.8706	y = 0.119x + 17.257 R <sup>2</sup> = 0.9398
Amp.	y = -0.3257x + 10.743 R <sup>2</sup> = 0.6615	y = 0.0695x + 12.695 R <sup>2</sup> = 0.9418	y = -0.3019x + 11.048 R <sup>2</sup> = 0.9616	y = -0.1781x + 11.286 R <sup>2</sup> = 0.9399
Strep.	y = 0.6352x + 9.9524 R <sup>2</sup> = 0.9434	y = 0.4895x + 10.362 R <sup>2</sup> = 0.861	y = 7 R <sup>2</sup> = #N/A	y = 7 R <sup>2</sup> = #N/A
Cip.	y = 0.2514x + 20.248 R <sup>2</sup> = 0.833	y = 0.221x + 20.21 R <sup>2</sup> = 0.9385	y = 0.2457x + 20.124 R <sup>2</sup> = 0.5751	y = 0.1019x + 21.586 R <sup>2</sup> = 0.9427
Tet.	y = 0.3476x + 11.343 R <sup>2</sup> = 0.8156	y = 0.1867x + 12.4 R <sup>2</sup> = 0.9495	y = 0.2448x + 15.948 R <sup>2</sup> = 0.4862	y = 0.0524x + 16.757 R <sup>2</sup> = 0.8391

<sup>a</sup> Exposed 4°C nutrient-deplete filter sterilized groundwater.

<sup>b</sup> Grown at 35°C in TSB.

#N/A = No change in resistance (growth extending to edge of antibiotic disk).

## Literature Cited

- Aertsen, A., and Michiels, C.** 2004. Stress and how bacteria cope with death and survival. *Crit. Rev. Microbiol.* **30**: 263-273.
- Al-Nabulsi, A.A., Osaili, T.M., Zain Elabedeen, N.A., Jaradat, Z.W., Shaker, R.R., Kheirallah, K.A., Tarazi, Y.H., and Holley, R.A.** 2010. Impact of environmental stress desiccation, acidity, alkalinity, heat or cold on antibiotic susceptibility of *Cronobacter sakazakii*. *International Food Microbiol.* **146**: 137-143.
- Arcangioli, M.A., Leroy-Setrin, S., Martel, J.L., and Chaslus-Dancla, E.** 1999. A new chloramphenicol and chloramphenicol resistance gene flanked by two integron structures in *Salmonella typhimurium* DT104. *FEMS Microbiol. Lett.* **174**: 327-332.
- Arocena, G.M., Zorreguieta, A., and Sieira, R.** 2012. Expression of VjbR under nutrient limitation conditions is regulated at the post-transcriptional level by specific acidic pH values and urocanic acid. *pLoS One.* **7**: e35394.
- Bauer, A.W., Perry D.M., and Kirby, W. M. M.** 1959. Single disk antibiotic sensitivity testing of staphylococci. *A.M.A. Arch. Intern. Med.* **104**: 208-216.
- Beauchamp, C.J., Simao-Beaunoir, A.M., Beaulieu, C., and Chalifour, F.P.** 2006. Confirmation of *E.coli* among other thermotolerant coliform bacteria in paper mill effluents, wood chips screening rejects and paper sludges. *Water Res.* **40**: 2452-2462.
- Beuchat, L.R., Hoikyung, K., Gurtler, J.B., Lin, L., Rye, J., and Richards, G.M.** 2009. *Cronobacter sakazakii* in foods and factors affecting its survival, growth, and inactivation. *Internat. J. Food Microbiol.* **136**: 204-213.
- Bogosian, G., Sammons, L. E., Morris, P. J. L., O'Neil, J. P., Heitkamp, M. A., and Weber, D. B.** 1996. Death of the *Escherichia coli* K-12 strain W3110 in soil and water. *Appl. Environ. Microbiol.* **62**: 4114-4120.
- Brenner, K., Ranker, C., Sivaganessan, M., and Scarpino P.** 1996. Comparison of the recoveries of *Escherichia coli* and total coliform from drinking water by the MI agar method and the U.S. Environmental Protection agency-approved membrane filter method. *Appl. Environ. Microbiol.* **62**:203-208.
- Breeuwer, P., Lardeau, A., Peterz, M., and Joosten, H.** 2003. Desiccation and heat tolerance of *Enterobacter sakazakii*. *J. Appl. Microbiol.* **95**: 967-973.
- Cao-Hoang, L., F. Dumont, P.A. Marechal, M. Le-Thand, and P. Gervais.** 2008. Rates of chilling to 0°C: implications for the survival of microorganisms and relationship with membrane fluidity modifications. *Environ. Biotech.* **77**: 1379-1387
- Caspi, R., Foester, H., Fulcher, C., Hopkinson, R., Ingraham, J., Kaipa, P., Krummenacher, M., Paley, S., Pick, J., Rhee, S., Tissier, C., Zhang, P., and Karp, P.** 2006. MetaCyc: a multiorganism database of metabolic pathways and enzymes. *Nucleic Acids Res.* **34**: D511-D516.

- Centers for Disease Control and Prevention (CDC).** 2012. *Escherichia coli*. (*E. coli*). 29 March, 2013. Web. 15 May, 2013.< <http://www.cdc.gov/ecoli/>>
- Centers for Disease Control and Prevention (CDC).** 2012. *Cronobacter sakazakii*. 17 December, 2012. Web. 15 August, 2013.< <http://www.cdc.gov/cronobacter/technical.html>>
- CDR weekly.** 2004. Withdrawal of baby milk Pregestimil worldwide following possible link with fatal *Enterobacter sakazakii* infection in infants in France. **14**:4.
- Chambers, H.F., and Sande, M.A.** 1995. Antimicrobial agents: the aminoglycosides. in the pharmacological basis of therapeutics. eds Jardman J. G., Limbird, L. E., Molinoff P.B. Ruddon R. W., and Goodman Gilman A. (McGraw-Hill, New York, NY), pp. 1103-1121.
- Commission Regulations (EC).** 2005. No 2073/2005 of 15 November 2005 (and as amended) on microbiological criteria for foodstuffs. Official J. of the E.U. **L338** : 1-26.
- Connell, S., Tracz, D.M., Nierhaus, K.H., and Taylor, D.E.** 2003. Ribosomal protection proteins and their mechanism of tetracycline resistance. *Anti. Ag. Chemo.* **12**: 3675-3681.
- Dancer, G.I., Mah, J.H., Rhee, M.S., Hwang, I.G. and Kang, D. H.** 2009. Resistance of *Enterobacter sakazakii* (*Cronobacter* spp.) to environmental stresses. *J. Appl. Microbiol.* **107**: 1606-1614.
- D’Costa V.M., King, C.E., Kalan, L., Morar, M., Sung, W.W., Schwartz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G.B., Poinar, H.N., and Wright, G.D.** 2011. Antibiotic resistance is ancient. *Nature.* **477**: 457-461.
- De Palois, M.R. and Lippi, D.** 2008. Use of metabolic and molecular methods for the identification of *Bacillus* strain isolated from paper affected by foxing. *Microbiol. Res.* **163**: 121-131.
- Drudy, D., O’Rourke, M., Murphy, M., Mullane, N.R., O’Mahony, R., Kelly, L., Fischer, M., Sanjaq, S., Shannon, P., and Wall, P.** 2006. Characterization of a collection of *Enterobacter sakazakii* isolates from environmental and food sources. *Int. J. Food Microbiol.* **110**: 127-134.
- Van Elsas, J. D., Semorov, A.V., Costa, R., and Trevors, J.T.** 2011. Survival of *Escherichia coli* in the environment: fundamental and public health aspects. *ISME J.* **2**: 173-183.
- Donadio, S., Maffioli, S., Monciardini, P., Sosio, M., and Jabes, D.** 2010. Antibiotic discovery in the twenty-first century: Current trends and future perspectives. *J. Antibio.* **63**: 423–430.
- Eckburg P.B., Bik E.M., Bernstein C.N., Purdom E., Dethlefsen L., Sargent M., Gill S.R., Nelson K.E., and Relman D.A.** 2005. Diversity of the human intestinal microbial flora. *Science.* **308**: 1635–1638.
- Ermolenko, D.N., and Makhatadze, G.I.** 2002. Bacterial cold-shock proteins. *Cell. Mol. Life Sci.* **59**: 1902-1913

**Falagas, M.S., Grammatikos, A.P., and Michalopoulos, A.** 2008. Potential of old-generation antibiotics to address current need for new antibiotics. *Expert Review of Anti Infective Therapy* **6**: 593-600.

**Farber, J.M., and Forsythe, S.J.** 2008. *Enterobacter sakazakii*. ASM Press.

**Farmer, J.J., Davis, B.R., Hickman-Brenner, F.W., Mcwhorter, A., Huntley-Fanning, G.R., Steigerwalt, A.G., Ohara, C.M., Morris, G.K, Smith, P.B., and Brenner, D.J.** 1985. Biochemical identification of new species and biogroups of *Enterobacteriaceae* isolated from clinical specimens. *J. Clin. Microbiol.* **21**: 46-76.

**FAO/WHO.** 2006. *Enterobacter sakazakii* and *Salmonella* in powdered infant formula: meeting report. Microbial. Risk Assessment Series. **10**.

**FDA and CDC Update:** 2011. Investigation of *Cronobacter* bacteria illness in infants. CDC Media Relations.

**Feng P., Weagant S., and Grant, M.** 2002. Enumeration of *Escherichia coli* and the Coliform Bacteria. Bacteriological Analytical Manual (8th ed.). FDA/Center for Food Safety & Applied Nutrition.

**Finberg, R. and Guharoy, R.** 2012. Clinical use of anti-infective agents. Springer New York. 75-78.

**Finkel, S.E.** 2006. Long-term survival during stationary phase evolution and the GASP phenotype. *Nat. Rev. Microbiol.* **4**: 113-120.

**Friedman M. S., M. Hossain, T.H. Hasson, and A. Kawamura.** 2005. Gene expression profiling of intrinsic thermotolerance in *Escherichia coli*. *Current Microbiol.* **52**: 50-54.

**Friedemann, M.** 2007. *Enterobacter sakazakii* in food and beverages (other than infant formula and milk powder). *Internat. J. Food Microbio.* **116**: 1-10.

**Gakuya, F.M., Kyule, M.N.m Garthurs, P.B., and Kariuki, S.** 1991. Antimicrobial resistance of bacterial organisms isolated from rats. *East Afr. Med. J.* **78**: 646-649.

**Garland, J., and Mills, A.** 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community level sole carbon source utilization. *Appl. Environ. Microbiol.* **57**: 2351-2359.

**Graves, A., Weaver, R., and Entry, J.** 2009. Characterization of enterococci populations in livestock manure using BIOLOG. *Microb. Res.* **164**: 260-266

**Gore, J, Stone, B.Z. MD, Nollmann, M., Cozzarelli N.R., and Bustamant,e C.** 2006. Mechanochemical analysis of DNA gyrase using rotor bead tracking. *Nature* **439**: 100-104.

**Gurtler J, Kornacki J, and Beuchat L.** 2005. *Enterobacter sakazakii*: A coliform of increased concern to infant health. *Internat. J. Food Microbiol.* **104**:1-34

**Hawkey, P.M. and Jones, A.M.** 2009. The changing epidemiology of resistance. *J.Antimicrob. Chemother.* **64 Suppl 1**: i3-10.

- Hengge-Aronis R.** 1993. Survival of hunger and stress: The role of *rpoS* in early stationary phase gene regulation in *E. coli*. *Cell*. **72**: 165-168.
- Hengge-Aronis R.** 2002. Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase. *Microbiol. Mol. Biol. Rev.* **66**: 373-395.
- Hooper, D.C.** 2000. Mechanisms of action and resistance of older and newer fluoroquinolones. *Clin. Infect. Dis.* **31** (Suppl. 2): S24-S28.
- Ihssen, J., and T. Egli.** 2005. Global physiological analysis of carbon energy limited growing *Escherichia coli* confirms a high degree of catabolic diversity and preparedness of mixed substrate utilization. *Environ. Microbiol.* **7**: 1568-1581.
- Ihssen J., Grasselli, E., Bassin, C., Francois, P., Piffarett, J.C., Köster, W., Schrezel, J., and Egli, T.** 2007. Comparative genomic hybridization and physiological characterization of environmental isolates indicate that significant (eco-) physiological properties are highly conserved in the species *Escherichia coli*. *Micro.* **153**: 2052-2066.
- Ishii S., and Sadowsky M.J.** (2008). *Escherichia coli* in the Environment: Implications for Water Quality and Human Health. *Microbes Environ.* **23**: 101–108.
- Iversen, C, and Forsythe, S.** 2004. Isolation of *Enterobacter sakazakii* and other *Enterobacteriaceae* from powdered infant formula and related products. *Food Microbiol.* **21**: 771-776.
- Iversen C, Lehner A, Mullane N, Bidlas, E., Cleenwerk, I., Marugg, J., Fanning, S., Stephan, R. and Joosten, H.** (2007). The taxonomy of *Enterobacter sakazakii*: proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. nov., *Cronobacter sakazakii* subsp. *sakazakii*, comb. nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter mytjensii* sp. nov., *Cronobacter dublinensis* sp. nov. and *Cronobacter genomospecies* 1. *BMC Evol. Biol.* **7**: 64.
- Iversen C, Mullane N, Barbara McCardell, B., Tall B. D., Lehner A., Fanning S., Stephan R., and Joosten H.** (2008). *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov. comb. nov., *C. malonaticus* sp. nov., *C. turicensis* sp. nov., *C. mytjensii* sp. nov., *C. dublinensis* sp. nov., *Cronobacter genomospecies* 1, and of three subspecies, *C. dublinensis* sp. nov. subsp. *dublinensis* subsp. nov., *C. dublinensis* sp. nov. subsp. *lausannensis* subsp. nov., and *C. dublinensis* sp. nov. subsp. *lactaridi* subsp. nov. *Int. J. Syst. Evol. Microbiol.* **58**: 1442-1447.
- Joseph, S., Cetinkaya, E., Drahovska, H., and Levican A** 2011. *Cronobacter condimentis* sp. nov., isolated from spiced meat and *Cronobacter universalis* sp. nov., a novel species designation for *Cronobacter* sp. genomospecies 1, recovered from a leg infection, water, and food ingredients. *Intl. J. Syst. Evol. Microbiol.* **11**: 204.
- Kandror, O., DeLeon, A., and Goldberg, A.L.** 2002. Trehalase synthesis is induced upon exposure of *Escherichia coli* to cold and is essential for viability at low temperatures. *Proc. Natl. Acad. Sci. USA.* **99**: 9727-9732.

- Kandhai, M.C. Rehi, M.W., Gorris L.G., Guillaume-Gentil, O, van Schothorst M.** 2004. Occurrence of *Enterobacter sakazakii* in food production environments and households. *Lancet*. **363**: 39-40.
- Karlowsky, J.A., Hoban, D.J., Zelenitsky, S.A., and Zhanel, G.G.** 1997. Altered denA and anr gene expression in aminoglycoside adaptive resistance in *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* **40**: 371-376.
- Kepner, R., and Pratt, J.** 1994. Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. *Microbiol. Rev.* **58**:603-615.
- Kessler, I., Collado-Vides, J., Gama-Castro, S., Ingraham, J., Paley, S., Paulsen, I., Peralta-Gil, M., and Karp, P.** 2005. EsoCyc: a comprehensive database resource for *Escherichia coli*. *Nucleic Acid Res.* **33**: D334-D337.
- Ki, X., and Nikadio, H.** 2009. Efflux-mediated drug resistance in bacteria: an update. *Drug.* **69**: 1555-1623.
- Kolter, R., Siegele, D.A., and Tirmo A.** 1993. The stationary phase of the bacterial life cycle. *Annu. Rev. Microbiol.* **47**: 855-874.
- Kuzina, L.V., Peloquin, J.J., Vacek, D.C., and Miller, T.A.** 2001. Isolation and identification of bacteria associated with adult laboratory Mexican fruit flies, *Anastrepha ludens* (Diptera: Tephritidae). *Curr. Microbiol.* **42**: 290-294.
- Laurence, B., Lazo, J., and Parker, K.** 2005. Goodman & Gilman's the pharmacological basis of therapeutics. ( McGraw-Hill Prof Med/Tech., New York, NY) 11<sup>th</sup> ed. Retrieved 9 May 2013.
- Lee, D.G., and Kim, S.J.** 2003. Bacterial species in biofilm cultivated from the end of the Seoul water distribution system. *J. Appl. Microbiol.* **95**: 317-324.
- Levy, S.B., and Nelson, M.** 1998. Reversing tetracycline resistance: A renaissance for the tetracycline family of antibiotics. *Adv. Exp. Med. Biol.* **456**: 17-25.
- Leuscher, R., Baird, F., Donald, B., and Cox, I.** 2004. A medium for the presumptive detection of *Enterobacter sakazakii*. *Appl. Environ. Microbiol.* **21**: 604-613.
- Lindstrom, J., Barry, R., and Braddock, J.** 1998. Microbial community analysis: a kinetic approach to constructing potential C source utilization patterns. *Soil Biol. Biochem.* **30**: 231-239.
- Liu, Y., Cai, X., Zhang, X., Gao, Q., Yang, X., Zheng, Z., Luo, M., and Huang, X.** 2006. Real time PCR using TaqMan and SYBR Green for detection of *Enterobacter sakazakii* in infant formula. *J. Microbiol. Methods.* **65**: 21-31.
- Llorens, N., María, J., Antonio, T., and Esteban, M.G.** 2010. Stationary phase in gram-negative bacteria. *FEMS Microbiol. Reviews.* **34**: 476-495.

- Loken, M.R.** 1990. Immunofluorescence techniques in flow cytometry and sorting. (2<sup>nd</sup> Edition). Wiley. 341-353.
- Lu, X., Liu, Q., Di Wu, D., Al-Qadiri, H.M., Al-Alami, N.I., Kang, D.H., Shin, J.H., Tang, J., Jabal, J.M.F., Aston, E.D., and Rasco, B.A.** 2011. Using of infrared spectroscopy to study the survival and injury of *Escherichia coli* O157:H7, *Campylobacter jejuni* and *Pseudomonas aeruginosa* under cold stress in low nutrient media. *Food Microbiol.* **28**:537-546.
- MacConkey, A.T.** 1905. Lactose-fermenting bacteria in faeces. *J. Hyd. (Lond).* **5**: 333-379.
- MacConkey, A.T.** 1908. Bile salt media and their advantages in some bacteriological examinations. *J. Hyd. (Lond).* **8**: 322-334.
- Maiti, S.M., R.P. Kamalesh Babu, and R. Shan.** 2006. Overcoming bacterial resistance: role of  $\beta$ -lactamase inhibitors. *Top. Heterocycl. Chem.* **2**: 207-246.
- Marr, A.G., and Ingraham, J.L.** 1962 Effect of temperature on the composition of fatty acids in *Escherichia coli*. *J Bacteriol* **84**:1260–1267
- McCarroll, M.** 2008. Application of the BIOLOG microplate system to monitor the physiological response of heat-stressed bacteria. M.S. Thesis. West Virginia University, West Virginia.
- Miller, J.M., and Rhoden, D.L.** 1991. Preliminary evaluation of BIOLOG, a carbon source utilization method for bacterial identification. *J. Clin Microbiol.* **29**: 1143-1147.
- Mine, T., Morita, Y., Kataoka, A., Mizushima, T., and Tsuchiya, T.** 1998. Evidence for chloramphenicol/H<sup>+</sup> antiporter in Cmr(MdfA) system of *Escherichia coli* and properties of the antiporter. *J. Biochem. (Tokyo).* **124**: 187-193.
- Mingeot-Leclercq, MP., Glupczynski, Y., and Tulkerns, P.M.** 1999. Aminoglycosides: activity and resistance. *Antimicrob. Agents. Chemother.* **43**: 727-737.
- Mullane, N.R., Iverson C., Healy, B., Walsh C., Whyte, P. and Wall P.G.** 2007. *Enterobacter sakazakii* an emerging bacterial pathogen with implications for infant health. *Minerva. Padiatr.* **59**: 137-148.
- Murray, I.A., and Shaw, W. V.** 1997. O-Acetyltransferases for chloramphenicol and other natural products. *Antimicrob. Agents Chemother.* **41**:1-6.
- Muytjens, H., Roelogs-Willemse, H., and Jaspar, G.** 1988. Quality of powdered substitutes for breast milk with regard to members of the family *Enterobacteriaceae*. *J. Clin. Microbiol.* **26**: 743-746.
- Nair, M.K.M., and Venkitanarayanan, K.S.** 2006. Cloning and sequencing of the ompA gene of *Enterobacter sakazakii* and development of an ompA-targeted PCR for rapid detection of *Enterobacter sakazakii* in infant formula. *Appl. Env. Microbiol.* **72**: 2539-2546.

**Neu, H.C., and Gootz T.D.**, 1996. Antimicrobial chemotherapy:antimicrobial inhibitors of ribosome function In :Baron's Medical Microbiology(Baron S. editor.) 4<sup>th</sup> ed. Chapter 11. Univ of Texas Medical Branch.

**Nikaido, H.** 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* **264** 382-388.

**O'Connel, S., Lawson, R., Watwood, M., and Lehman, R.** 2000. Basic program for reduction of data from community-level physiological profiling using BIOLOG microplates: rationale and critical interpretation of data. *J Microbiol Methods*. **40**: 213-220.

**Piqueres, P., Moreno, Y., Alonse, J.L. and Ferrús, M.A.** 2006. A combination of direct viable count and fluorescent in situ hybridization for estimating *Helicobacter pylori* cell viability. *Research in Microbiol.* **157**: 345-349

**Poole, K.** 2001. Overcoming antimicrobial resistance by targeting resistance mechanisms. *J Pharm Pharmacol.* **53**: 283-294.

**Prisco, G., Giordano, D., Russo, R., and Verde, C.** 2012. Adaptation and evolution in marine environments: The challenges of low temperature in the evolution of bacteria. Springer-Verlag Berlin Heidelberg. **1**: 183-195.

**Restaino, L., Frampton, E.W., Loinberg, W.C., and Becker, R.J.** 2006. A chromogenic plating medium for the isolation and identification of *Enterobacter sakazakii* from foods, food ingredients, and environmental sources. *J Food Prot.* **69**: 315-322.

**Ribeiro, A.F, Laroche, E., Hanin,G., Fournier, M., Quillet, L., Depont, J.P., and Pawlak, B.** 2012. Antibiotic-resistant *Escherichia coli* in karstic systems: a biological indicator of the origin of fecal contamination? *FEMS Microbiol. Ecol.* **81**:267-280.

**Roberts, M.C.** 1996. Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. *FEMS Microbiol. Lett.* **19**: 1-24.

**Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. *Microbiol. Rev.* **51**:365-379.

**Sampson, R.W., Swiatnicki, S.A, Osinga, Supita, J.L, McDermott, C.M., and Kleinheinz, G.T.** 2006. Effects of temperature and sand on *E.coli* survival in a northern lake water microcosm. *J. Water and Health.* **4.3**:389-393.

**Sapkota, A.R., Lefferts, L.Y., McKenzie, S., and Walker, P.** 2007. What Do We Feed to Food-Production Animals? A Review of Animal Feed Ingredients and Their Potential Impacts on Human Health. *Environ. Health Perspect.* **115**: 663–670.

**Scherer, S., Vogt, V.R., Vilei, E.M., Frey, J., and Perreten, V.** 2013. Enhanced antibiotic multi-resistance in nasal and faecal bacteria after agricultural use of streptomycin. *Environ. Microbiol.* **15**:297-304.



- Schindler, P.R. G., and Metz, H.** 1990. Coliform bacteria in rinsed mugs-identification with the API 20E system and resistance behavior. *Offentliche Gesundheitswesn* **52**: 592-597.
- Schindler, P.R.G.,** 1994. Enterobacteria in various kinds of mineral water, spring water and table water. *Gesundheitswesen* **56**: 690-693.
- Shivaji, S., Jogadhenu, S., and Prakash, S.** 2010. How do bacteria sense and respond to low temperature? *Arch. Microbiol.* **192**: 85-95.
- Sinha, A.K., Hofmann, M.G., RöMer, U., Köckenberger, W., Elling, L., and Roitsch, T.** 2002. Metabolizable and non-metabolizable sugar activate different signal transduction pathways in tomato. *Plant Physiol.* **128**: 1480-1489.
- Song, S.H., and Vieille, C.** 2009. Recent advances in the biological production of mannitol. *Appl. Microbiol. Biotechnol.* **84**: 55–62
- Stewart, M., and Olson, B.** 1992. Impact of growth conditions on resistance of *Klebsiella pneumoniae* to chloramines. *Appl. Environ. Microbiol.* **58**: 2649-2653.
- Stock I, and Wiedemann B.** 2002. Natural antibiotic susceptibility of *Enterobacter amnigenus*, *Enterobacter cancerogenus*, *Enterobacter gergoviae* and *Enterobacter sakazakii* strains. *Clin. Microbiol. & Infect.* **8**:564-578.
- Tadesse D.A., Zhao S., Tong E., Ayers S., Singh A., Bartholomew M.J., McDermott, P.F.** 2012. Antimicrobial drug resistance in *Escherichia coli* from humans and food animals, United States, 1950–2002. *Emerg. Infect. Dis.* **18**: 741-749.
- Tenover, F.C.** 2006. Mechanisms of antimicrobial resistance in bacteria. *Am. J. Med.* **119 (6 Suppl 1)**: S3-10; discusion S62-70.
- Thunberg, R., Sextstone, A., Calabrese, J., and Bissonette, G.** 2001. Effects of antecedent fermentative and respiratory growth on the detection of chloramines-stressed *Escherichia coli* and *Salmonella typhimurium*. *Can. J. Microbiol.* **47**: 777-781.
- Tintinalli, J.E, Krome R.L, and Ruiz E.** *Emergency medicine: a comprehensive study guide.* 4<sup>th</sup> ed. McGraw-Hill, 1995.
- Tufariello, J.M., Mi, K., Xu, J., Manabe, Y.C., Kesavan, A.K., Drumm, J., Tanaka, K., Jacobs, W.R., and Chan, J.** 2006. Deletion of the *Mycobacterium tuberculosis* resuscitation-promoting factor Rv1009 gene results in delayed reactivation from chronic tuberculosis. *Infect. Immun.* **74**:2985-2995.
- Turcovský, I., Kuniková, K., Drahovská, H., and Kacliková, E.** 2011. Biochemical and molecular characterization of *Cronobacter* spp. (formerly *Enterobacter sakazakii*) isolated from foods. *Antonie van Leeuwenhoek.* **99**: 257-269.
- Umbreit, W.W.** 1970. *Advances in Applied Microbiology.* Academic Press. **11**:80.

- Verschuere, L., Fievez, V., Van Vooren, L. and Verstraete, W.** 1997. The contribution of individual populations to the BIOLOG pattern of model microbial communities. *FEMS Microbiol. Ecol.* **24**:353-362.
- Virta, M., Lineri, S., Kankaanpää, P., Jaro, M., Peltonen, K., Nuutila, J., and Lilius, E.** 1998. Determination of complement-mediated killing bacteria by viability staining and bioluminescence. *Appl. Environ. Microbiol.* **64**: 515-519.
- Vital, M., Hammes, F., and Egli, T.** 2008. *Escherichia coli* O157 can grow in natural freshwater at low carbon concentrations. *Environment. Micro.* **10**:2387-2396.
- Walsh, T.** 2006. Combinatorial genetic evolution of multiresistance. *Curr. Opin. Microbiol.* **9**: 476-482.
- Wang, G., and Doyle, M.P.** 1998. Survival of enterohemorrhagic *Escherichia coli* O157:H7 in water. *J. Food Prot.* **6**: 662-667.
- Young-Duck, L., Jong-Hyun, P., and Hyoihl, C.** 2012. Detection, antibiotic susceptibility and biofilm formation of *Cronobacter* spp. from various foods in Korea. *Food Control.* **24**: 225-230.
- Zak, J., Willig, M., Moorhead, D., and Wildman H.** 1994. Functional diversity of microbial communities: a quantitative approach. *Soil Biol. Biochem.* **26**: 1101-1108.

## VITA

Alisha L. Dailey was born February 12, 1986 in Wheeling, WV. She is the daughter of Keith A. Isner and Glenna L. Isner. She graduated from Shadyside High School (OH) in 2004. In the spring of 2008 she earned her bachelors of biology degree from West Virginia University. Following graduation, she married Corey Dailey and moved to Norwich, CT. where he was stationed in the Navy and became a substitute teacher there for two years. As Corey's six years of military service were completed, they decided to move back to West Virginia and she began pursuing a Master of Science degree in Applied and Environmental Microbiology. Throughout her time at WVU, she oversaw independent study students and assisted in the undergraduate general microbiology laboratory course as a teaching assistant.