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Transport and Pathogenicity of *Salmonella enterica* Subspecies in Groundwater:
In vitro, in vivo, and in silico

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of the requirements for the degree of

Doctor of Philosophy

in

Chemical and Environmental Engineering

by

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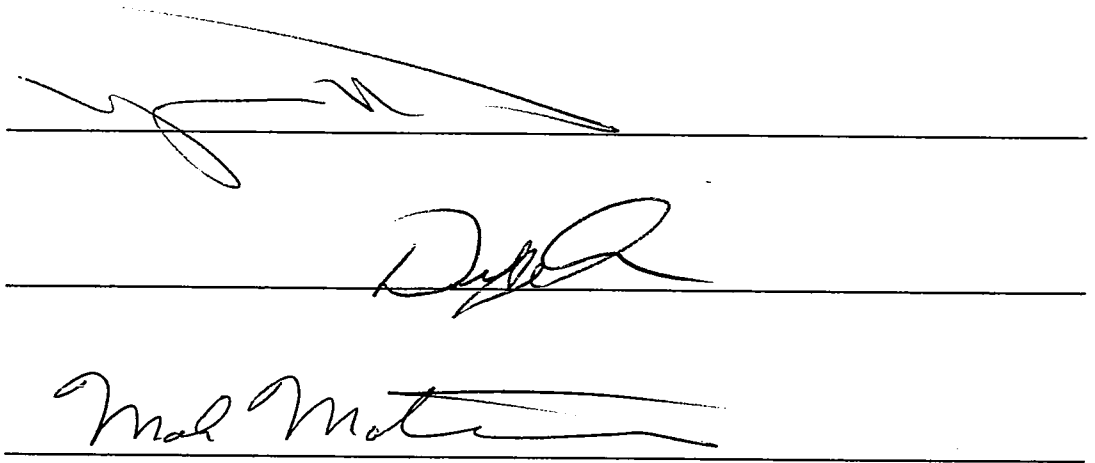
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Dedication

This dissertation is dedicated to my loving family, to my fiancée, Ece Hartevioğlu,
and to my dear classmate Yan “Jennie” Liu whose soul is among the angels. -BZH



ABSTRACT OF THE DISSERTATION

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by

Berat Zeki Haznedaroğlu

Doctor of Philosophy, Graduate Program in Chemical and Environmental Engineering
University of California, Riverside, March 2010
Dr. Mark R. Matsumoto, Chairperson

The fate of pathogens upon excretion to natural environments from initial hosts is not fully understood. Often overlooked is the fact that pathogens either cannot survive or lose their virulent characteristics upon exposure to environmental stress. However, problematic pathogens such as *Salmonella* are very resilient and acclimate well in secondary habitats; therefore requiring comprehensive studies to understand the mechanisms of fitness and survival in external environments. Additionally, despite the widespread knowledge of *Salmonella* and its interaction with targeted hosts, the deposition and transport behavior of this important food- and waterborne pathogen is not commonly studied.

The objective of this research was to understand the fate and transport of *Salmonella* under a framework of environmentally relevant groundwater conditions. Therefore several *Salmonella enterica* serovars with different phenotypes were selected and the depositions of these pathogens onto solid surfaces were examined over a range of

solution chemistries. Packed bed column and radial stagnation point flow (RSPF) systems were utilized for the transport experiments. Several batch experiments were designed to simulate the stress conditions in aquatic environments to understand the fate of *Salmonella* in terms of fitness, survival and virulence. These experiments were complemented by a wide range of cellular characterization techniques.

Transport experiments of *Salmonella* in comparison to another important pathogen, *E. coli* O157:H7 has shown that *Salmonella* can be transported further than *E. coli* in saturated packed bed column systems. Additionally, this transport behavior was shown to be affected by different solution chemistries and cell injection concentrations. Deposition and transport behavior of three *Salmonella enterica* serovars with different motility phenotypes were studied in packed bed column and RSPF systems. Results have shown that flagellated strains deposit more than nonflagellated strains, concluding that nonmotile cells can be transported further than motile cells.

Several batch experiments were designed to simulate a wide range of groundwater conditions to study the fate of *Salmonella* upon short and long term exposure to environmental stress. Both phenotypical (antibiotic resistance, *in vitro* and *in vivo* virulence) and genotypical (gene screening *in silico*) evaluations of *Salmonella* fitness, survival, and pathogenicity were conducted. The findings of this research suggest that *Salmonella* successfully remains viable and virulent under a wide range of groundwater environments.

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Chapter 1

Introduction

1.1 Motivation and Background

Pathogenic organisms impact the health of both humans and livestock, as well as the economy of the U.S. While emerging waterborne pathogens threaten the health of millions of individuals each year, the spectrum of diseases is expanding as well (1). Infections caused by these pathogens may be transmitted by contaminated recreational waters, surface water, and groundwater, putting all communities into risk (2). One of the most problematic food- and waterborne pathogens, *Salmonella*, for example, is responsible for 1.5 million estimated cases of acute gastrointestinal illness (AGI) each year in the U.S. alone (3). The U.S. Center for Disease Control and Prevention (CDC) reports approximately 400 deaths each year due to acute Salmonellosis, the infection caused by *Salmonella*, in the U.S. (4). The situation is worse in the developing world, where gastrointestinal diseases are the second most common cause of childhood mortality, and typhoid (caused by *Salmonella enterica* serovar Typhi) alone is estimated to result in 500,000 deaths per year (5).

AGI associated with microbial contamination of water systems results in typical symptoms such as diarrhea, nausea, vomiting, abdominal pain, abdominal cramps, and fever (6). *Salmonella* as an agent of AGI is primarily transmitted by the fecal-oral route, as a result of direct contact between water and human-animal fecal pollution. Among the possible source water environments vulnerable to fecal contamination, protection of groundwater is one of the top priorities, since half of all Americans and more than 95% of rural Americans get their house-hold water supplies from groundwater, as well as half

of the nation's agricultural irrigation, and nearly one-third of the industrial water needs are supplied from groundwater (7).

Despite the efforts to control the problem, recent studies have shown that half of all the U.S. drinking water wells tested had fecal pollution, causing an estimated 750,000 to 5.9 million illnesses per year (8). This is not surprising considering the fact that there are 238,000 animal feeding operations in the U.S. producing 317 million tones of manure annually, including liquid and solids discharged from the animals along with bedding animals, excluding the manure from the grazing animals (9). In order to address the problem of bacterial pollution in groundwater environments, there is a significant and immediate need for comprehensive knowledge understanding of fate and transport of pathogens of concern.

This Ph.D. study aims to create a framework to study this issue: Firstly, via the transport of model bacteria, *Salmonella spp.*, through experimental systems simulating the groundwater environment under the influence of various parameters, such as solution chemistry, cell concentration, and cell motility, which are suspected to play important role in bacterial transport. Secondly, the fate of pathogenicity is investigated in groundwater by determining the extent of antibiotic resistance, *in vitro* and *in vivo* virulence, and *in silico* gene regulation. The ultimate goal is to determine the mobility behavior and identify the changes in phenotypic and genotypic characteristics of *Salmonella spp.*, notably controlling its virulence, when they reach source waters and eventually human or animals coming in contact with contaminated water. The Ph.D. work is unique and novel in this respect, since comprehensive fate of pathogenicity and

transport of *Salmonella spp.* in groundwater environments has not been studied in literature, to the best of author's knowledge.

1.2 Model Bacteria

One of the most considerable threats to human and animal health are *Salmonella spp.*, which are ~0.9 µm long gram negative facultative anaerobic, rod-shaped bacteria, and contains over 2,500 serovars being of the most important pathogens in the family *Enterobacteriaceae* (10, 11). *Salmonella spp.* infections range from gastrointestinal infections that are accompanied by inflammation of intestinal epithelia, diarrhea and vomiting, to typhoid fever, a life threatening systemic infection (12). Although *Salmonella spp.* are very closely related to one another, different species may have drastically different biological features in terms of diseases they cause and infected range of hosts (13, 14). Among these serovars, *Salmonella enterica* serovar Typhimurium, an invasive type that causes gastroenteritis and systemic infection in both humans and animals, is the most frequently reported one with 714 cases (human) in a recent outbreak (March 2009) in U.S. (15). *Salmonella enterica* serovar Pullorum and Gallinarum are both nonflagellated and nonpathogenic to human whereas they are common pathogens to chicken and other avian species, mostly poultry (16-20). Being avian-specific pathogens, *S. pullorum* and *S. gallinarum* cause economic losses due to increasing problems of antibiotic resistance, and long-term persistence in chickens after infection (13, 21-27). Diseases caused by *Salmonella spp.* are depicted in Table 1.1.

Table 1.1. Diseases caused by *Salmonella enterica* serovars in humans and some higher vertebrates [Adopted from (28)]

Host species	Disease	<i>S. enterica</i> serovar most frequently encountered	Most susceptible age group
Humans	Typhoid fever Paratyphoid fever	typhimurium, enteritidis typhi ^a sendai; paratyphi A, B, and C ^a	Children (<4 yrs) Children and adults
Cattle	Salmonellosis Pullorum disease Fowl typhoid	typhimurium dublin pullorum ^{a,b} gallinarum ^{a,b}	Calves (<8 wks) Calves and adult cattle Newly hatched birds
Poultry	Avian paratyphoid	enteritidis, typhimurium	Growing stock and adults Newly hatched birds
Sheep	Salmonellosis Pig paratyphoid	abortusovis ^a typhimurium choleraesuis ^a	Adult sheep, Lambs Lambs
Pigs	Salmonellosis Chronic paratyphoid	typhimurium typhisuis	Weaned and adult pigs Weaned pigs (<4 mos)
Horses	Salmonellosis	abortusequi ^a typhimurium	Adult horses, Foals Foals
Wild rodents	Murine typhoid	typhimurium, enteritidis	

^aThese serotypes have been most frequently associated with illness in the preantibiotic era but are now rare or have been eradicated in most developed countries.

^bGallinarum and pullorum are considered biotypes that belong to the same serotype.

1.3 Fate and Transport

Transport of individual bacterial cells in natural and engineered systems is governed by a combination of physical, chemical, and biological factors, such as cell type (29), growth phase (30, 31), extent of hydrophobic interactions, surface charge characteristics (32, 33), flagellar motion (34, 35), and solution chemistry (36, 37). The majority of previous studies on cell fate and transport have focused on Gram-negative bacteria since this cell type comprises the majority of cultivable cells found in aquatic environments (38). Additionally, some cells of this type are indicators of fecal contamination (38) and are capable of degrading contaminants in the subsurface via bioremediation (39, 40). Many Gram-negative cells exude extracellular polymeric substances (EPS), which consist

of a variable combination of proteins, polysaccharides, and nucleic acids (41, 42). Several studies have addressed the role of these macromolecules on cell adhesion; specifically, the adhesive nature of bacteria has been attributed to LPS (29, 43-45), outer membrane proteins (46-49), fimbriae (47, 50), flagella (51, 52), and EPS (42, 53, 54). However, there exists a significant gap of knowledge in literature to understand the comprehensive nature of fate and transport of pathogens. Often overlooked is the fact that, many bacteria e.g., *Salmonella* spp., are motile and this motility affects the extent of their transport as well as their attachment to surfaces. In addition, during its transport the extent of changes in *Salmonella* phenotype and genotype remains unclear.

1.4 Aims and Scope

In this Ph.D. study, *Salmonella enterica* spp. were used in systematic transport experiments to capture the influence of two critical factors controlling pathogen transport: adhesion and mobility. A methodology was proposed utilizing controlled column and radial stagnation point flow systems (under a range of environmentally relevant chemistries) utilizing motile and nonmotile, outbreak causing *Salmonella* strains. Additionally, this study investigated the fate of *Salmonella* pathogenicity in extent to which environmental factors alter the genotypic state and phenotypic nature of the cells. The project aims, as noted above, were developed based upon the following working hypotheses and objectives presented in next section which comprised this study.

1.4.1 Hypotheses and Specific Objectives

To achieve the overall objective of this dissertation work, several working hypotheses and specific objectives were set and these are presented below (Note that each specific objective has been addressed in a chapter of this dissertation):

1) *Pathogen transport and deposition in saturated porous media is dependent on the injection concentration and species of pathogens and the background solution chemistry.*

The specific objective of this part of the study was to understand the role of species type and concentration of pathogens in transport and deposition of *Salmonella* in different solution chemistries. For this purpose, transport experiments were carried out in saturated packed bed columns over a range of cell concentrations ($5 \times 10^6/\text{mL}$ to $10^8/\text{mL}$) and ionic strength (IS) (1–100 mM KCl) representative of groundwater conditions (55, 56). To understand the role of species type in pathogen transport, *E. coli* O157:H7 cells were used in comparison to *S. pullorum* in the packed bed (Chapter 2).

2) *Motile Salmonella strains would be deposited and transported differently than nonmotile strains, considering the fact that there exist several surface irregularities and three dimensional pore spaces in porous media that is only accessible to motile bacteria.*

The specific objective of this part of the study was to determine the role of flagellar motion in *Salmonella* transport and adhesion. In order to achieve this objective, a packed bed column and a radial stagnation point flow systems were employed to investigate bacterial deposition kinetics onto quartz surfaces under chemically unfavorable and

favorable conditions. To better understand the role of flagellar motion, three *Salmonella* strains with different motility phenotypes were used. Surface properties of these strains were extensively characterized and compared via electrophoretic mobility, hydrophobicity, potentiometric titration, and theoretical Derjaguin-Landau-Vervey-Overbeek (DLVO) theory (57-61). In addition, the influence of growth phase (mid and late exponential) on cellular characteristics was also investigated (Chapter 3).

3) *Ion rich groundwater matrix would influence the energy-driven active efflux pumps of Salmonella and eventually alter the mechanisms of antibiotic resistance and virulence upon exposure to groundwater stress conditions.*

The specific objective of this part of the study was to understand the effects of groundwater exposure on the antibiotic resistance and invasiveness of *Salmonella*. For this purpose, three *Salmonella* strains with different morphologies (in terms of serovar type and motility) were exposed to artificial groundwater (AGW) with varying IS (3.33–30 mM) for 6, 12, 18, and 24 hours in batch culture flasks. The antibiotic resistance profiles of stressed organisms were determined and compared to control groups that no stress conditions were applied. In addition, the invasiveness of AGW-exposed *Salmonella* was tested with *in vitro* exposure to human epithelial cells (HEp2) (Chapter 4).

4) *Residual antibiotics found in groundwater environments would induce gained resistance for Salmonella upon long term exposure and alter its virulence.*

The specific objective of this part of the study was to determine if *Salmonella* gains antibiotic resistance or alters its virulence when exposed to groundwater supplemented with residual antibiotics over a long period of time. For this purpose, a wild type, *S. typhimurium* outbreak strain was exposed to AGW supplemented with representative concentrations (0.05, 1, and 100 µg/L) of amoxicillin, tetracycline, and a mixture of nine antibiotics (1 µg/L each) for four weeks. Antibiotic susceptibility analyses were conducted weekly to determine any changes in the resistance of *S. typhimurium* against tested antibiotics. In addition, the changes in the virulence of *S. typhimurium* upon exposure to antibiotic supplemented AGW were tested by *in vitro* invasion assays (with HEp2 cells) and *in vivo* virulence assays (with *Caenorhabditis elegans* nematodes) (Chapter 5).

5) *Under different environmental stress conditions, Salmonella relies on different genes for fitness and survival to remain viable and virulent.*

The specific objective of this part of the study was to identify the essential genes of importance in the fitness and survival of *Salmonella* exposed to varying environmental stress conditions that the bacteria experiences upon excretion from its host. For this purpose, a *S. typhimurium* strain 14028s derivative and its 90,000 independent mutant library was exposed to several stress conditions such as artificial seawater (ASW), AGW with high pH, low temperature, organic matter, and in a static environment. The genotypic screening of all annotated genes under these conditions was determined by

microarray analysis, and genes required for fitness and survival were identified (Chapter 6).

1.4.2 Experimental Approach

Two representative pathogens, *Escherichia coli* O157:H7 and *Salmonella enterica*, were selected for systematic transport experiments to capture the influence of species type, cell concentration, solution chemistry, and motility on the pathogen transport. *E. coli* O157:H7/pGFP strain 72 was obtained from the laboratory of Dr. Pina Fratamico (USDA-ARS-ERRC, Wyndmoor, PA) and *Salmonella enterica* serovars were purchased from the *Salmonella* Genetic Stock Centre (SGSC) at the University of Calgary, Canada. The specific objectives mentioned in Section 1.4.1 were achieved by comprehensive characterization, batch tests, and transport analyses. A packed bed column and radial stagnation point flow systems were utilized to conduct adhesion and transport experiments under a range of groundwater solution chemistries and velocity. Batch exposure experiments were used to understand the fate of *Salmonella* in groundwater conditions. Several macroscopic and microscopic characterization tools (e.g., size, shape, extracellular polymeric substances, electrophoretic mobility, hydrophobicity, and surface charge density measurements, and electron and light microscopy analyses, etc.) were utilized to characterize surface properties of the model bacteria. The regulation of genes in groundwater and seawater environments was determined *in silico*.

1.5 Organization of the Dissertation

Following the Introduction, Chapter 2 describes the transport and retention behavior of *S. pullorum* and *E. coli* O157:H7 in a saturated packed bed column under relevant groundwater solution chemistries. It was found that *E. coli* O157:H7 is more adhesive than *S. pullorum* and higher injection concentration resulted in decreased retention in the column. Cell surface characterizations showed that two pathogens' size and hydrophobicity values were not statistically different whereas their acidity and corresponding surface densities varied. The combination of cell characterization and column experiments showed that *S. pullorum* can be transported to further distances than *E. coli* O157:H7. This observation is described thoroughly in Chapter 2 entitled, "Relative Transport Behavior of *Salmonella enterica* serovar Pullorum and *Escherichia coli* O157:H7 in Packed Bed Column Systems: Influence of Solution Chemistry and Cell Concentration".

Chapter 3, entitled "Identifying the Role of Flagella in the Transport of Motile and Nonmotile *Salmonella enterica* Serovars" investigates the influence of flagellar motion on the transport and adhesion behavior of *Salmonella* in packed bed column and RSPF systems. This work compares three *Salmonella enterica* serovars with different motility phenotypes. Flagellar components clearly demonstrated the limitations of classic DLVO theory in accounting for biological factors affecting the transport and adhesion of pathogens. This work has shown that nonmotile cells can be transported further than motile cells and the growth phase plays role in deposition mechanisms.

Chapter 4, entitled “Changes in Antibiotic Resistance and Invasiveness of *Salmonella* under Short Term Exposure to Artificial Groundwater”, highlights the fact that functioning of energy-driven active efflux pumps of *Salmonella* could be affected due to exposure to ion rich groundwater environments. The influence of groundwater exposure on the invasiveness of *Salmonella* to human epithelia cell lines was also discussed. In complement to Chapter 2 and 3, three *Salmonella enterica* serovars with different morphologies were used in this part. The results have shown that *Salmonella typhimurium* serovars could enhance their resistance to the some of the antibiotic classes that are mediated with active efflux pumps which was not observed for *Salmonella pullorum*, suggesting that the increased resistance found in AGW-exposed organisms could be strain dependent. Invasion to human epithelial cell lines resulted in higher degree of virulence following an exposure to AGW for 24 hours; however, lower levels of invasiveness were observed for short duration periods (6, 12, and 18 hours). This work demonstrated the possibility of increased virulence in *Salmonella* against human and animal target hosts upon experiencing groundwater environments.

Chapter 5, entitled “Effects of Long Term Exposure to Antibiotic Containing Groundwater in *Salmonella enterica* serovar Typhimurium: Changes in Antibiotic Resistance, *in vivo* and *in vitro* Pathogenicity” presents the results of antibiotic susceptibility analysis and changes in the virulent characteristics of *S. typhimurium* exposed to residual antibiotic containing AGW for the duration of four weeks. It has been showed that *S. typhimurium* can remain viable but not cultivable under long term exposure to AGW and posses hyper-virulence against human epithelial cells and earth

worms. It was also shown that, exposure to antibiotics containing AGW does not induce gained antibiotic resistance in *S. typhimurium*.

To complement the fate of pathogenicity in *Salmonella* work presented in Chapter 4 and 5, genotypic screenings by microarray analysis were conducted in Chapter 6, entitled “Survival and Fitness of Random Generated *Salmonella enterica* serovar Typhimurium Transposon Library under Long Term Environmental Stress: From *in vitro* to *in silico*”. This work describes experiments conducted to identity the genes of importance in the fitness and survival of *S. typhimurium* exposed to external stress upon excretion to aquatic environments. Experimental methodology was designed and followed through exposure of a uniquely constructed *S. typhimurium* mutant library to several AGW and ASW stress conditions, and the determination of required functional genes by microarray analysis. The results have shown that under different stress conditions *S. typhimurium* relies on same essential genes required for survival in aquatic environments. In addition, the genes that would provide advantage for fitness and survival for each tested stress conditions were identified and presented.

Finally, the appendices contain full list of annotated genes that were essential for survival in several stress conditions determined in Chapter 6 (Appendix A), and the representative images of experimental setups used throughout this Ph.D. work (Appendix B).

The findings from this doctoral research are summarized in Chapter 7, “Summary and Conclusions”. Some of the work in this dissertation has been published, while some

recent work is still under review or in preparation for publication in refereed journals.

Below is a list of manuscripts which have resulted from this research:

- 1) Haznedaroglu, B.Z., Kim, H.N., Bradford, S.A., and Walker, S.L., 2009. "Relative Transport Behavior of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Pullorum in Packed Bed Column Systems: Influence of Solution Chemistry and Cell Concentration", *Environmental Science and Technology*, 43(6), 1838-1844.
- 2) Haznedaroglu, B.Z., Zorlu, O., Hill, J.E., and Walker, S.L., 2009. "Identifying the Role of Flagella in the Transport of Motile and Nonmotile *Salmonella enterica* Serovars", *Environmental Science and Technology*, (submitted).
- 3) Haznedaroglu, B.Z., Yates, M.V., and Walker, S.L., 2009. "Changes in Antibiotic Resistance and Invasiveness of *Salmonella* under Short Term Exposure to Artificial Groundwater", *Applied Microbiology and Biotechnology*, (in review).
- 4) Haznedaroglu, B.Z., Yates, M.V., Maduro, M.F., and Walker, S.L., 2009. "Effects of Long Term Exposure to Antibiotic Containing Groundwater in *Salmonella enterica* serovar Typhimurium: Changes in Antibiotic Resistance, in vivo and in vitro Pathogenicity", *Applied and Environmental Microbiology*, (in preparation).
- 5) Haznedaroglu, B.Z., Porwollik, S., McClelland, M., Cheng, P., Ahmer, B.M., and Walker, S.L., 2009. "Survival and Fitness of Random Generated *Salmonella*

typhimurium Transposon Library under Long Term Environmental Stress: From *in vitro* to *in silico*”, *Applied and Environmental Microbiology*, (in preparation).

Although not specifically mentioned in the dissertation, the following publications have contributed to the development of this research and helped to expand my knowledge in the bacterial fate and transport field:

- 6) Bradford, S.A., Kim, H.N., Haznedaroglu, B.Z., Torkzaban, S., and Walker, S.L., 2009. “Coupled Factors Influencing Concentration Dependent Colloid Transport and Retention in Saturated Porous Media”, *Environmental Science and Technology*, 43(18), 6996-7002.
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Chapter 2

Relative Transport Behavior of *Salmonella enterica* serovar Pullorum and *Escherichia coli* O157:H7 in Packed Bed Column Systems: Influence of Solution Chemistry and Cell Concentration

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Haznedaroglu, B. Z., Kim, H. N., S. A. Bradford, and S. L. Walker. 2009. Relative Transport Behavior of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Pullorum in Packed Bed Column Systems: Influence of Solution Chemistry and Cell Concentration *Environmental Science and Technology* **43**:1838-1844.

Abstract

The influence of solution chemistry and cell concentration on bacterial transport has been examined using *Salmonella pullorum* SA1685 and *Escherichia coli* O157:H7. A column was employed to determine the transport behavior and deposition kinetics with aquifer sand over a range of ionic strength and cell concentration. O157:H7 was found to be more adhesive than SA1685, with calculated deposition rate coefficients higher than SA1685. Comprehensive cell surface characterization techniques including size, surface charge density, extracellular polymeric substance content, electrophoretic mobility, and hydrophobicity analyses were conducted to explain observed transport trends. The pathogens' size and hydrophobicity were not significantly different; whereas they varied in acidity, for which O157:H7 had 19 times higher surface charge density than SA1685. Electrophoretic mobilities, in general agreement with titration analysis and column experiments, revealed SA1685 to be more negative than O157:H7. This combination of column and characterization experiments indicates that SA1685 can be transported to a greater extent than O157:H7 in groundwater environments. This study is the first comprehensive work comparing the transport behavior of two important pathogens in aquifer systems.

2.1 Introduction

In the U.S. there are 238,000 animal feeding operations producing 317 million tonnes of liquid and solid manure annually (1), which contains a variety of pathogenic microorganisms (2). For example, the shedding rate of *Salmonella* in poultry may be as high as 10^5 to 10^6 CFUs/g (3), and up to 10^2 to 10^4 CFUs/g of *Escherichia coli* O157:H7 in calf manure (4). Precipitation and surface water flow can cause pathogens to leach from manure to flowing water (5). Concentrations of *E. coli* O157:H7 from land applied animal manure can reach levels of 10^7 CFUs/ml within three hours in silty clay and sandy loam soil (6). Hence, improperly treated manure and manure-contaminated water can pose a risk to human and animal health when used as a source for irrigation or groundwater recharge (1,7).

Recent studies have shown that half of all the U.S. drinking water wells tested had fecal pollution, causing an estimated 750,000 to 5.9 million illnesses per year (8). Drinking water outbreaks in the U.S. have primarily been associated with inadequately disinfected groundwater and distribution system contamination, with a significant number of these outbreaks being caused by *E. coli* O157:H7 and *Salmonella* spp. (9). *E. coli* O157:H7 causes an estimated 73,000 cases of infection and 60 deaths in the U.S. annually (10). Waterborne outbreaks associated with various subspecies of *Salmonella* have been traced to contaminated wells and water storage; and has been estimated to cause 1,412,498 cases of acute gastroenteritis illnesses in the U.S. annually (11).

Salmonella pullorum is an especially serious avian pathogen causing “pullorum disease” in infected chickens and turkeys (12). Usually newly hatched birds are the

primary victims (13), although there are reports of infection to other animals (14). *S. pullorum* is also of interest due to increasing development of antibiotic resistance among zoonotic pathogens (15).

To address bacterial pollution in groundwater environments and to prevent the spread of infectious diseases to humans via this means of transport, there is an immediate need for a comprehensive understanding of the fate and transport of pathogens of concern. Therefore, this study was designed to investigate the transport behavior of *S. pullorum* and *E. coli* O157:H7 under a variety of solution chemistry conditions and cell concentrations, consistent with the variability of natural environments. Extensive cell surface characteristics were compared to provide an explanation for the calculated differences in pathogen travel distances under test conditions.

2.2 Materials and Methods

2.2.1 Bacterial Cell Selection and Preparation

S. pullorum strain SA1685 obtained from the Salmonella Genetic Stock Centre, University of Calgary, Canada, is a non-motile, non-flagellated avian pathogen originally isolated from an infected turkey. *E. coli* O157:H7 strain p72 was also non-flagellated and non-motile under the utilized cell growth conditions, and was obtained from the USDA (Pina Fratamico, USDA-ARS-ERRC, Wyndmoor, PA). SA1685 was pre-cultured in Luria-Bertani (LB) broth (Fisher Scientific, Fair Lawn, NJ) at 37 °C overnight while shaken continuously at 200 rpm. On the day of experimental analysis, overnight pre-cultures were used to inoculate fresh LB broth (1:100 dilution) and harvested at mid-

exponential growth phase. O157:H7 cells were prepared following the same protocol, except the growth medium was Tryptic Soy Broth (Becton, Dickinson and Company, Sparks, MD) supplemented with ampicillin (50 µg/mL final concentration). Electron microscopy images were taken to confirm that the cells were non-flagellated (data not shown). A refrigerated bench-top centrifuge (5804R; Eppendorf, Hamburg, Germany) equipped with fixed angle rotor (F-34-6-38; Eppendorf) was used to pellet the cells with an applied 3,700*g* for 15 min at 4 °C. Growth medium was decanted and the pellet was resuspended in 10 mM KCl solution. The process was repeated twice in order to ensure complete removal of the growth medium. All electrolyte solutions used in cell preparation and experiments were prepared with deionized (DI) water (Millipore, Billerica, MA) and reagent-grade KCl (Fisher Scientific) with no pH adjustment (pH 5.6–5.8). Concentrations of cell solutions were determined by using a cell counting hemocytometer (Bürker-Turk, Germany) and light microscope (Fisher Scientific).

2.2.2 Bacterial Cell Characterization

Viability tests were conducted on SA1685 and O157:H7 using the Live/Dead BacLight[®] kit (L-7012; Molecular Probes, Eugene, OR) in 10 mM KCl solution. Direct counting of the stained cells was done using an inverted microscope (IX70; Olympus, Japan) and the appropriate fluorescence filter (Chroma Technology Corp., Brattleboro, VT).

In order to measure the dimensions of SA1685 and O157:H7, phase contrast images were taken using an inverted microscope (IX70, Olympus) and a camera (Retiga 1300; Qimaging, Canada) at $\sim 10^8$ cells/mL in 10 mM KCl. Images of cells ($n > 60$) were

processed (SimplePCI, Precision Instruments Inc., Minneapolis, MN) and individual cell lengths and widths were determined. Measured values were used to calculate the effective cell radius and corresponding bacterial surface area.

Hydrophobicity analysis of the cells was done using the microbial adhesion to hydrocarbons (MATH) test (16). Briefly, the partitioning of cells was measured between a hydrocarbon (n-dodecane; laboratory grade, Fisher Scientific, Fairlawn, NJ) and electrolyte solution (10 mM KCl) as the percent of total cells partitioned into the hydrocarbon from the aqueous phase.

Potentiometric titrations of cells were conducted to determine the relative acidity and charge density of the bacterial surfaces. An auto-titrator (798 MPT Titrino; Metrohm, Switzerland) was used to determine the amount of base consumed by suspended cells during titration between pH 4 and 10. Bacterial suspensions with concentrations of $3\text{--}5.3 \times 10^8$ cells/mL in 10 mM KCl were titrated by 0.3 N NaOH. The resulting acidity and corresponding surface charge density were calculated (17,18).

Extracellular polymeric substance (EPS) composition, specifically total protein and sugar content, was analyzed based on an established extraction method (19). The pellet of harvested bacterial cells was suspended in formaldehyde-NaCl solution followed by various centrifugation steps and final exposure to ethanol-KCl solution. The analysis of protein was performed using the Lowry method (20) with Bovine Serum Albumin (BSA, 20 mg/mL) (Fisher Scientific) as the standard and measured spectroscopically (BioSpec-mini, Shimadzu, Kyoto, Japan) at a wavelength of 500 nm. The analysis of total sugars was performed using the phenol-sulfuric acid method described previously (21). Xanthan

gum (Practical Grade, Fisher Scientific) was used as the standard and samples measured spectroscopically at 480 nm (BioSpec-mini, Shimadzu).

2.2.3 Electrokinetic Properties and DLVO Calculations

Electrophoretic mobility of bacterial cells was determined by diluting the cell pellet to an optical density of 0.2-0.25 in 1, 10, and 100 mM KCl solution at 546 nm (BioSpec-mini, Shimadzu). Electrophoretic mobility measurements were conducted at 25°C using a ZetaPALS analyzer (Brookhaven Instruments Corporation, Holtsville, NY), repeated five times, and converted to zeta potentials using Smoluchowski equation (22).

Electrokinetic properties of sand were determined using a streaming potential analyzer (EKA, Anton Paar GmbH, Graz, Austria) with a cylindrical powder cell. Measurements were obtained in KCl over the range of ionic strength (IS) used in the transport experiments. The instrument was first rinsed with 2 L of deionized (DI) water followed by 0.5 L of the electrolyte solution used in the measurement. Prior to the streaming potential measurements, the cylindrical powder cell packed to a bed depth of 3 cm was equilibrated with the fresh electrolyte solution for 40 min. Zeta potential values were calculated from the streaming potentials as described elsewhere (23,24).

Derjaguin-Landau-Verwey-Overbeek (DLVO) theory was utilized to predict interaction energy between the cell and collector surfaces (25). Total interaction energies existing between the pathogen and sand particle were quantified as the sum of van der Waals and electrostatic interactions, both of which decay with separation distance (22).

2.2.4 Porous Medium Preparation

American Society for Testing and Materials 20/30 unground silica sand (U.S. Silica, Ottawa, IL) was used as packing material for the column experiments. Average sand diameter (d_{50}) was 710 μm . In order to remove metal oxide coatings and organic matter associated, the sand was shaken vigorously in a 2-L flask containing 12 M hydrochloric acid (Fisher Scientific) twice and kept in acid overnight (61). The mixture was then rinsed with DI water until the rinse water pH was equal to that of the DI water. Prior to column packing the sand was sterilized by autoclaving at 121 °C and 15 psi for 30 min. Porosity was gravimetrically determined to be 0.33 ± 0.02 .

2.2.5 Column experiments

2.5 cm diameter and 60 cm long Chromaflex™ chromatography columns (Kontes Glass Co., Vineland, NJ) were wet packed using autoclaved quartz sand (710 μm). After packing, columns were operated in a downward direction using a peristaltic pump (Cole-Palmer, Vernon Hills, IL). Approximately 20-25 pore volumes (PV) of DI water, followed by 3 PV of electrolyte solution were passed through column to equilibrate the sand. Columns were operated at 15 mL/min to achieve a Darcian velocity of ~ 0.05 cm/s. Calculated Reynolds (22) and Peclet (22,26) numbers for the packed bed were 1.10 and 0.21, respectively. Three PVs of bacteria (concentrations of 5×10^6 , 10^7 or 10^8 cells/mL) were injected in the column followed by 5 PV of bacteria-free electrolyte solution. Effluent was collected every minute using a fraction collector (RTRV II, Isco Inc, Lincoln, NE). Bacterial concentrations were determined via optical density at 546 nm using a UV – visible spectrophotometer (BioSpec-mini, Shimadzu).

2.2.6 Deposition Profile and Extent of Kinetics

To determine the deposition profile of bacteria across the length of the column, sand was excavated after the experiment (27). The inlet fitting was removed and saturated porous media were transferred to 50 mL Falcon tubes containing 10 to 20 mL of KCl solution with the same IS used during the conducted transport experiment. Starting at the top of the column, sand was recovered every 1 cm section with each tube containing approximately 10 g of sand. From the 10th cm to the bottom section of the column, sand was collected in 5 cm increments (approximately 40 g of sand was recovered). Tubes were shaken gently for 5 min and the supernatant was spread onto LB agar plates. Spread-plating procedure was followed for the bacterial stock solution as well to quantify the number of colony forming units (CFU) in the stock solution. CFUs forming on the overnight incubated (37 °C) plates were counted and normalized by the CFUs counted from the cell stock solution. Liquid and sand filled tubes were placed in a hot oven (105°C) and incubated at least 48 h in order to volatilize remaining KCl solution. The total percentage (M_{total}) of cells recovered was determined from the sum of the percentages of cells recovered from the column effluent (M_{eff}) and the aquifer sand (M_{sand}) by following a protocol described previously (62,63).

The bacterial deposition rate coefficient, k_d , was determined by (28)

$$k_d = -\frac{U}{\varepsilon L} \ln \left[\frac{C}{C_0} \right] \quad (2-1)$$

where C/C_0 is normalized concentration exiting the column, U is superficial velocity, ε is porosity, and L is length of the packed bed. The average value of C/C_0 was obtained

between 1.8 and 2.0 PV, which represents “clean-bed” conditions (29). Approximate travel distances of SA1685 and O157:H7 were estimated from measured values of k_d .

2.2.7 Travel Distance Calculations

Approximate travel distances of both SA1685 and O157:H7 were estimated based on described colloid travel distance equations given below (62,63).

$$L_T = -\ln(C_L / C_0) \left(\frac{4a_c}{3(1-\varepsilon)\alpha\eta_0} \right) \quad (2-2)$$

In this equation, L_T is denoted as vertical travel distance, a_c is the radius of sand particle, ε is the porosity of the column, α is the collision efficiency, and η_0 is the single collector efficiency. For the calculation of collision efficiency, experimental C/C_0 values were first used to determine filter coefficient (λ) as follows:

$$\frac{dC}{dx} = -\lambda C \quad (2-3)$$

In this equation, x denotes the direction parallel to flow in the column. The single collector efficiency was calculated based on the R-T model by Rajagopalan and Tien (61). Calculated filter coefficient values in eq (2-3) and single collector efficiencies by R-T model were incorporated into eq. (2-2) developed by Yao and co-workers (60).

$$\lambda = \left(\frac{3}{2} \right) \times \left[\left(\frac{1-\varepsilon}{d_c} \right) \right] \times \alpha \times \eta_0 \quad (2-4)$$

In this equation, d_c is denoted as the diameter of the sand particle. Remaining parameters calculated from equations (2-3) and (2-4) were also incorporated into equation (2-2) where 99.9% removal was considered with C_L/C_0 value of 0.001.

2.3 Results

2.3.1 Bacterial Transport

Results of transport experiments are presented in terms of influence of solution chemistry and injection concentrations (C_0). Breakthrough curves (BTC) of SA1685 and O157:H7 showing the effects of solution chemistry are presented in Figure 2.1 when $C_0 = 10^7$ cells/mL. As observed in Figure 2.1a, SA1685 retention in the column increased with IS, approximately 27, 64 and 69% at 1, 10 and 100 mM respectively. Retention of O157:H7 was independent of IS and approximately 80% of bacteria were retained in as shown in Figure 2.1b.

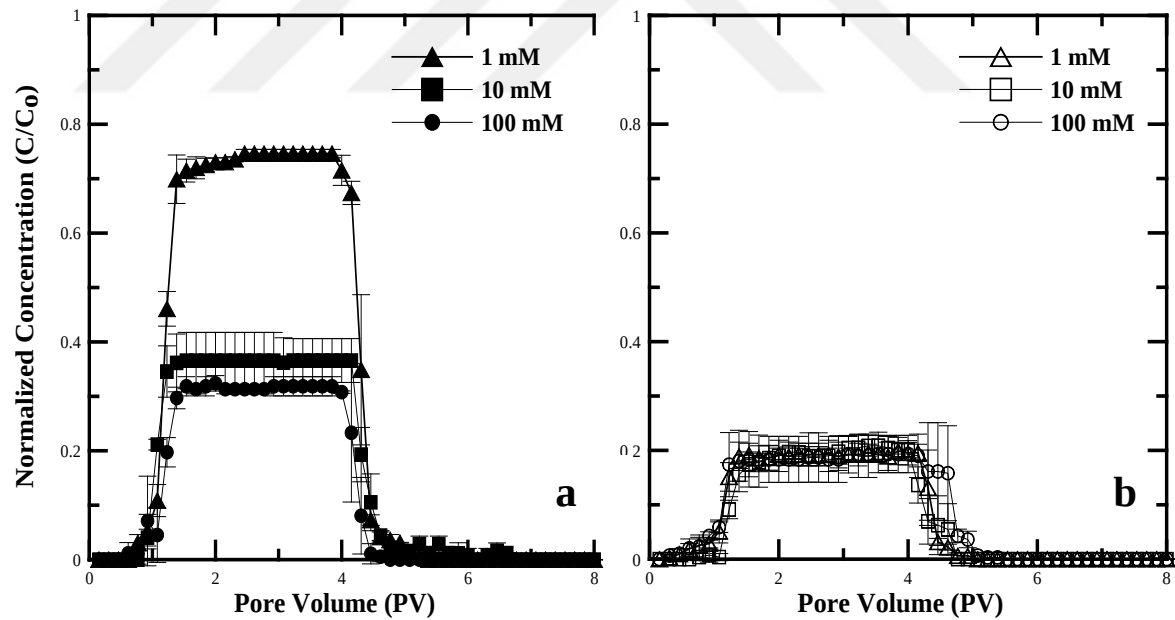


Figure 2.1. BTCs of SA1685 (a) and O157:H7 (b) tested at 1mM, 10mM, and 100 mM IS ($C_0 = 10^7$ cells/mL). Error bars indicate average of three runs.

The effect of cell concentration (10 mM KCl) is depicted in Figure 2.2. Cell retention was nearly identical when C_0 was 5×10^6 and 10^7 cells/mL, with an average retention of

63% for SA1685 (Figure 2.2a) and 80% for O157:H7 (Figure 2.2b). At $C_0 = 10^8$ cells/mL, values of C/C_0 in BTCs increased $\sim 20\%$ between 1-4 PV for both cell types.

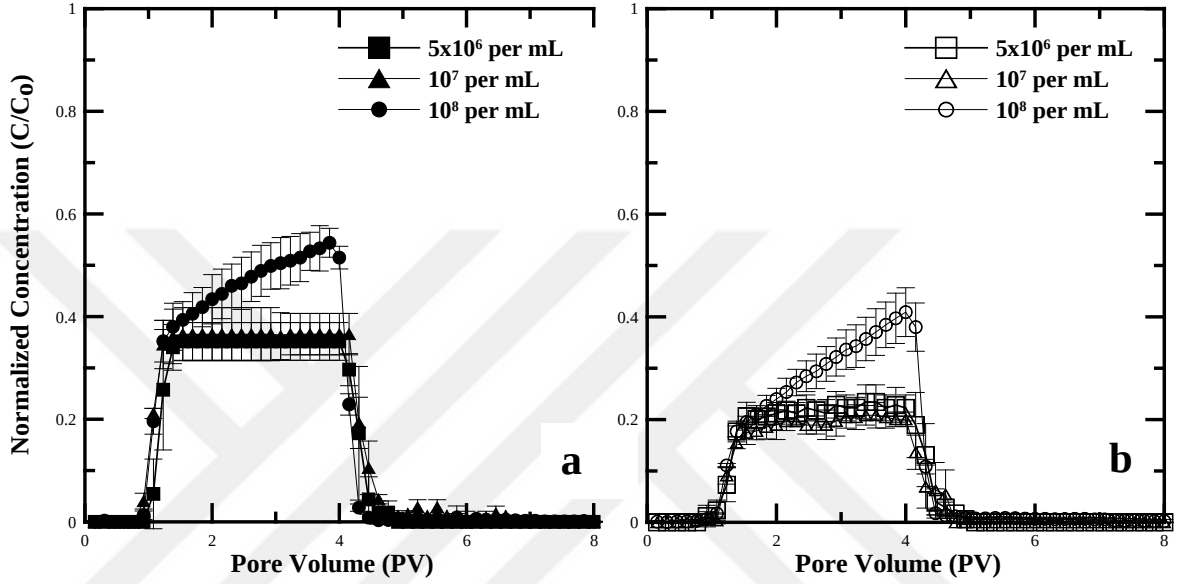


Figure 2.2. BTCs of SA1685 (a) and O157:H7 (b) tested at different C_0 (10mM KCl). Error bars indicate average of three runs.

Figure 2.3 presents k_d as a function of IS and C_0 . As expected, k_d values follow the same trend as cell retention. Namely, when 10^7 cells/ mL were injected k_d values were virtually identical at all IS for O157:H7, whereas k_d values increased with IS for SA1685. The k_d values of both SA1685 and O157:H7 in 10 mM KCl were essentially independent of C_0 at 5×10^6 and 10^7 cells/mL, but slightly decreased at $C_0 = 10^8$ cells/mL.

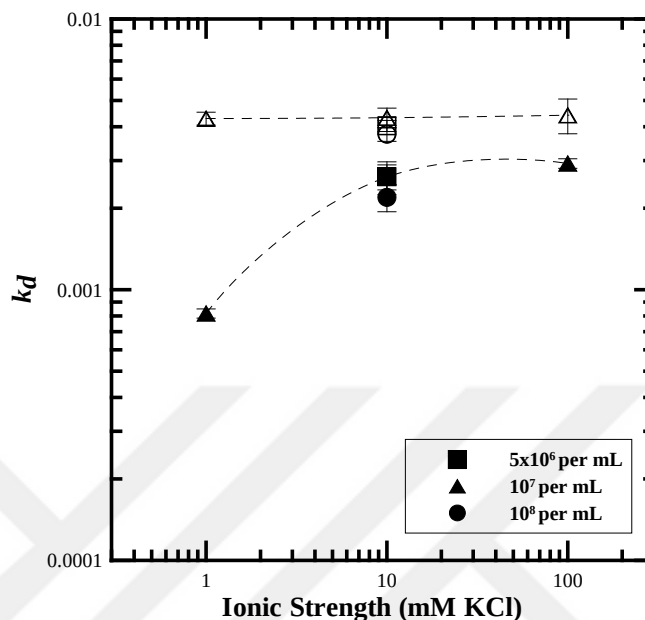


Figure 2.3. Deposition rate coefficient (k_d) versus IS for SA1685 (closed shapes) and O157:H7 (open shapes) injected at 5×10^6 cells/mL (squares), 10^7 cells/mL (triangles), and 10^8 cells/mL (circles). Dashed line drawn to provide eye guide to indicate trends in changing k_d . Error bars indicate one standard deviation.

2.3.2 Deposition Profiles and Extent of Kinetics

Deposition profiles were determined following completion of transport experiments and reported in Supporting Information. Figure 2.4 shows deposition profiles for SA1685 (Figure 2.4a,b) and O157:H7 (Figure 2.4c,d) with respect to the influence of solution chemistry and C_0 . The observed number of deposited bacteria was normalized by the total number injected to the column. Figure 2.4 shows the normalized concentration; number of CFU (N_C) divided by the total number of CFU injected to column (N_T) per gram of dry sand (X-axis) plotted as a function of dimensionless distance from the column inlet divided by the column length (Y-axis). When 10^7 cells/mL of SA1685 was injected, the bacterial concentration retained in the column normalized by the mass of

sand increased with IS (Figure 2.4a), similar to observed BTCs. In the case of O157:H7 there were no significant changes observed with IS (Figure 2.4c) like observed BTCs. In terms of C_0 the number of SA1685 retained in the column was similar when 5×10^6 and 10^7 cells/mL; however, a decrease was observed at $C_0 = 10^8$ cells/ml (Figure 2.4b). Amounts of O157:H7 retained in the column at all C_0 were similar (Figure 2.4d), and no trend with injection concentration was observed. Table 2.1 indicates that reasonable mass balance was achieved in the column experiments (78-127%).

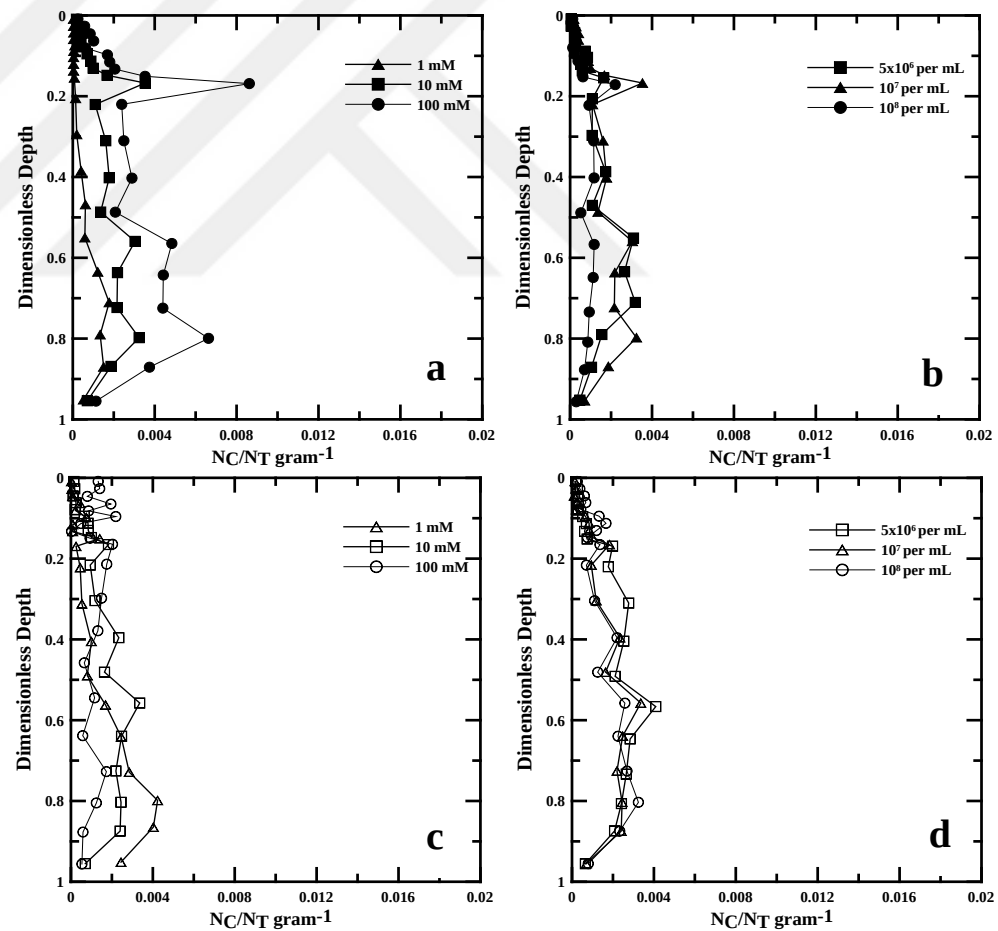


Figure 2.4. Deposition profiles of SA1685 (a,b) and O157:H7 (c,d) tested at different IS and C_0 . $C_0=10^7$ cells/mL in experiments reported in (a) and (c). IS=10 mM KCl for experiments are reported in (b) and (d).

Mass balance based on CFU numbers were conducted for each transport experiment using the influent concentration and final deposition profiles and presented in Table 2.1. The cells were fully recovered at all conditions except SA1685 transport experiment with $C_0=10^8$ cells/mL at 10 mM KCl where $M_{total}=95\%$.

Table 2.1. The effluent (M_{eff}), retained (M_{sand}), and total (M_{total}) percentage of *Salmonella enterica* SA1685 and *E. coli* O157:H7 cells recovered from column experiments. All values represent the relative percentage of the cells by number.

		Mass Balances (%)								
Strain	IS (mM KCl)	5×10^6 (cells/mL)			10^7 (cells/mL)			10^8 (cells/mL)		
		M_{eff}	M_{sand}	M_{total}	M_{eff}	M_{sand}	M_{total}	M_{eff}	M_{sand}	M_{total}
SA1685	1				73	37	110			
	10	37	73	110	36	74	110	55	40	95
	100				31	47	78			
O157:H7	1				20	82	102			
	10	20	107	127	20	82	102	26	76	102
	100				20	58	78			

2.3.3 Travel Distances

Based on experimental results, travel distances were calculated at which 99.9% of cells would be removed. SA1685 was transported further than O157:H7 at comparable IS and C_0 conditions (Figure 2.5). The longest travel distance calculated for SA1685 was 11.8 m at 1 mM and C_0 of 10^7 cells/mL. O157:H7 travel distances were similar under all conditions, with 3.7 m as the longest travel distance at 10 mM and $C_0=10^8$ cells /mL. It should be mentioned that these are conservative estimates of travel distance, as k_d was assumed independent of concentration whereas k_d was slightly greater at lower concentrations (Figure 2.3).

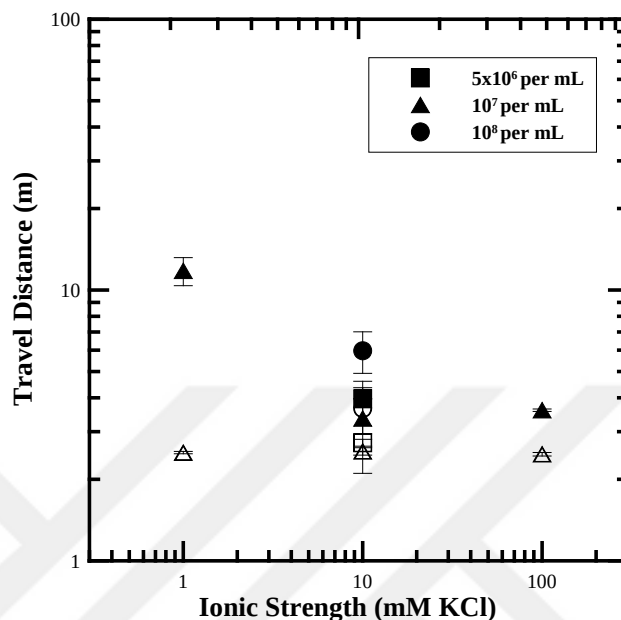


Figure 2.5. Calculated travel distance versus IS for SA1685 (closed shapes) and O157:H7 (open shapes) injected at 5×10^6 cells/mL (squares), 10^7 cells/mL (triangles), and 10^8 cells/mL (circles). Error bars indicate one standard deviation.

2.3.4 Electrokinetic Properties and DLVO Profiles

The electrophoretic mobilities of SA1685 and O157:H7 were negatively charged under all conditions (Table 2.2), with SA1685 being more negative than O157:H7. Increased IS caused a reduction in mobility for SA1685 due to compression of the electric double layer (30). In contrast, differences in O157:H7 mobility with IS were not statistically significant.

Measured zeta potentials of sand particles are reported in Table 2.3. These values were used in lieu of surface potentials to calculate interaction energy existing between cells and sand particles using DLVO theory (25). DLVO theory predicts favorable attachment conditions for O157:H7 at 10 and 100 mM, and for SA1685 at 100 mM. Conversely, unfavorable attachment conditions are predicted for these bacteria at the other IS

conditions (1 mM for O157:H7, 1 and 10 mM for SA1685). At 1 mM, cells experience energy barriers greater than 3.16 kT to attachment to the collector surface. At 10 mM, an energy barrier of 9.87 kT exists between SA1685 and sand. Additionally, the predicted secondary minima depth for SA1685 and O157:H7 were negligible at all IS.

2.3.5 Bacterial Surface Characteristics

Cell characteristics of SA1685 and O157:H7 are presented in Table 2.2. Size measurements showed no significant difference between the two pathogens. MATH test results indicate both were similarly hydrophobic (16.74-26.72%). Potentiometric titration revealed that O157:H7 was more acidic than SA1685, with more dissociable functional groups and greater charge density. Measured values of acidity were 0.47×10^{-5} , 0.19×10^{-5} , and 0.18×10^{-5} meq/ 10^8 cells for SA1685 at 1, 10, and 100 mM respectively. O157:H7 acidity values were measured as 1.49×10^{-5} , 8.48×10^{-5} , and 3×10^{-5} at 1, 10, and 100 mM, respectively. From these values, the surface charge densities were calculated (18) as 180, 75 and 68 $\mu\text{C}/\text{cm}^2$ for SA1685 and 512, 2840, and 1027 $\mu\text{C}/\text{cm}^2$ for O157:H7 at 1, 10, and 100 mM respectively. EPS analysis revealed SA1685 had almost 4 times more sugar content than O157:H7, with a similar trend for protein. Viability test results revealed that greater than 90% viability at all conditions.

Table 2.2. Key cell characteristics of SA1685 and O157:H7.

Strain	IS (mM KCl)	Radius (μm) ^a	Acidity (meq/ 10^8 cell) ^b	Surface Charge Density ($\mu\text{C}/\text{cm}^2$) ^c	MATH (%) ^d	Sugar ($\mu\text{g}/\text{ml}$) ^e	Protein ($\mu\text{g}/\text{ml}$) ^f	Mobility [($\mu\text{m}/\text{sec}$)/(V/cm)]	Zeta Potential (mV)	Viability (%) ^g
SA1685	1	0.455 $\pm$ 0.08	0.47 $\times 10^{-5}$	180	26.19 $\pm$ 2.20	6.75 $\pm$ 0.84	289.30 $\pm$ 24.7	-0.87 $\pm$ 0.09	-11.11 $\pm$ 1.16	95 $\pm$ 1.21
	10		0.19 $\times 10^{-5}$	75	17.74 $\pm$ 5.05			-0.65 $\pm$ 0.04	-8.23 $\pm$ 0.70	92 $\pm$ 2.34
	100		0.18 $\times 10^{-5}$	68	26.45 $\pm$ 9.96			-0.59 $\pm$ 0.21	-7.54 $\pm$ 2.71	91 $\pm$ 3.23
O157:H7	1	0.480 $\pm$ 0.06	1.49 $\times 10^{-5}$	512	26.72 $\pm$ 9.33	1.75 $\pm$ 0.16	139.61 $\pm$ 12.7	-0.18 $\pm$ 0.28	-2.23 $\pm$ 3.57	94 $\pm$ 2.27
	10		8.48 $\times 10^{-5}$	2840	18.83 $\pm$ 1.92			-0.14 $\pm$ 0.13	-1.84 $\pm$ 1.62	91 $\pm$ 1.92
	100		3.0 $\times 10^{-5}$	1027	16.74 $\pm$ 6.70			-0.12 $\pm$ 0.16	-1.60 $\pm$ 2.04	93 $\pm$ 0.83

^a Value for equivalent spherical radius calculated from measured length and width of individual cells.

^b Acidity determined from amount of NaOH consumed during titration between pH 4-10.

^c Indicates density of charged functional groups across cell surface. Value determined from measured acidity, and accounting for exposed surface area of the cells (calculated for a spherical cell) and Faraday's constant= 96,485 C/mol.

^d Microbial adhesion to hydrocarbon (MATH) indicates the relative hydrophobicity of the cell as the percent of cells partitioned in dodecane versus electrolyte.

^e Based on Phenol-sulfuric acid method and Xanthan gum as standard at 10^8 cells/ml.

^f Based on Lowry method and Bovine Serum Albumin (BSA) as standard at 10^8 cells/ml.

^g Percent of cell population viable based on the Live/Dead BacLight[®] kit. Values are average of measured viability across the range of IS utilized in transport experiments.

Table 2.3. Zeta potential of sand particles, energy barrier heights, and secondary energy minimum depths as a function of IS as calculated by DLVO theory^a for SA1685 and O157:H7.

Strain	IS (mM KCl)	Sand particle	Cell-sand interactions	
		Zeta potential (mV)	1 ⁰ max (kT)	2 ⁰ min (kT)
SA1685	1	-26.02±1.48	94.83	NB ^b
	10	-15.30±0.73	9.87	NB
	100	-1.81±4.7	NB	NB
O157:H7	1	-26.02±1.48	3.16	NB
	10	-15.30±0.73	NB	NB
	100	-1.81±4.7	NB	NB

^a Calculations done assuming Hamaker constant of 6.5×10^{-21} J (60) and using bacterial sizes reported in Table 2.2.

^b NB denotes no energy barrier.

2.4 Discussion

2.4.1. Effect of IS

Electrophoretic mobilities indicate SA1685 to be more negatively charged than O157:H7. Conversely, titration results indicate that higher acidity and surface charge density for SA1685 compared with O157:H7. This apparent discrepancy in cell charge results can be explained by differences in the measurement techniques. Electrophoretic mobility reflects the net surface charge, whereas titration captures the density of dissociable functional groups on the membrane surface and within the macromolecular (EPS) complex. Furthermore, titration results may be sensitive to cell size and interspecies variation. Hence, electrophoretic mobility provides a better indication of cell-cell and cell-quartz interactions than titration results. Indeed O157:H7 had a higher (less negative) electrophoretic mobility (Table 2.2) and greater cell retention than

SA1685 (Figure 2.1) at a given IS, a result consistent with DLVO theory. The deposition rate coefficients were also constantly higher for O157:H7 than SA1685 (Figure 2.3).

DLVO theory was also able to qualitatively explain the transport and adhesion trends for SA1685. As observed in Figure 2.1a, SA1685 retention in the column increased with IS. This was an expected trend due to double layer compression occurring with increasing IS. Electrophoretic mobility and zeta potential trends also confirmed this with decreased values with increasing IS. As SA1685 experienced the highest primary energy barrier that must be overcome for attachment at 1 mM, retention in the column was much less at 1 mM than 10 mM and 100 mM conditions where SA1685 experienced a small energy barrier (9.87 kT) and no barrier, respectively. SA1685 therefore achieved its longest transport distance at 1 mM when the pathogen had the highest zeta potential.

Retention of O157:H7 was independent of IS (Figure 2.1b). It was observed that the k_d values of O157:H7 were similar at all conditions with ~20% of the pathogen breaking through. This is attributed to the surface being relatively insensitive to solution chemistry as observed by the electrophoretic mobility and hydrophobicity values overlapping at 1, 10, and 100 mM. Travel distances for O157:H7 were therefore virtually identical and independent of IS for 10^7 cells/mL.

It is interesting to note that DLVO theory predicts favorable (10 and 100 mM of O157:H7 and 100 mM for SA1685) and/or near favorable conditions (a minor energy barrier of 3.16 kT occurred at 1 mM for O157:H7, hardly more than Brownian motion could overcome (39)) occurred for some of the experiments. Classic filtration theory would predict very limited transport under such favorable conditions. The observed

significant transport behavior (Figures 2.1-2.5) and the reasonable mass balance results (Table 2.1) however, suggest the cells were not irreversibly retained in the primary minimum. Recent literature indicates that steric stabilization of macromolecules on the surface of microorganisms may hinder attachment in the primary minimum (54), and that hydrodynamic forces may remove weakly associated cells from collector surfaces (55).

2.4.2. Effect of Cell Concentration

C_0 of 5×10^6 and 10^7 cells/mL did not change retention values for either pathogen; however, at 10^8 cells/mL, BTCs displayed increasing C/C_0 values for both O157:H7 and SA1685 (Figure 2.2). This trend has been explained in the literature by “blocking”, (31-35), simultaneous bacterial deposition and release (36-38), or colloid/microbial collisions and its effect on the filling of small pore spaces that are associated with enhanced retention (56). Blocking implies a limited number of favorable attachment sites, and that deposited cells provide less favorable surfaces for subsequent cell attachment. Higher concentrations are expected to “block” favorable attachment sites more rapidly than lower concentrations, and therefore exhibit less of a time dependency. This hypothesis is not consistent with the time dependent shape of the BTC at the highest C_0 value shown in Figure 2.2. Furthermore, simultaneous cell deposition and release implies a high release rate, yet significant concentration tailing that was not observed in the BTCs. The later mechanism -cell-cell collisions- seems most plausible collisions, which increase with concentration, may hinder retention by knocking weakly associated cells off the solid surface. This hypothesis is consistent with the observation that 10^7 cells/mL of either pathogen resulted in increased retention as compared to 10^8 cells/mL. The time

dependency of the filling of the small pore spaces can also logically be expected to be a function of cell concentration, due to plugging of small pore spaces at higher concentrations. In this study, the no time dependency of the rising portion of the BTC was observed when C_o was less than 10^8 cells/mL, whereas when C_o was greater than 10^8 cells/mL a time dependency in the BTC was observed, presumably due to filling of the smallest pore spaces.

2.4.3. Deposition Profiles

Deposition profiles for SA1685 (Figure 2.4a) showed that the number of bacteria retained in the column increased with IS. The influence of concentration was also confirmed with deposition profiles (Figure 2.4b) where bacteria numbers retained were lower at 10^8 than 5×10^6 and 10^7 cells/mL cases. Deposition profiles for O157:H7 were similar at all tested IS and C_o conditions.

Deposition profiles for many types of colloids (i.e. viruses and bacteria) have been reported to follow hyper-exponential profile distributions, implying a decreasing rate of deposition with increasing travel distance (40-49). However, both SA1685 and O157:H7 showed a tendency to be retained in lower portions of the column, from a dimensionless depth of 0.5 to 1 (Figure 2.4). Similar trends have been reported in the literature by Tong (50) and Bradford (27). Possible mechanisms to explain observed non-exponential deposition behavior may include the time-dependent deposition (32,51,52) or particle detachment both of which are implicated by the concentration dependent trends discussed already (53). There are other explanations for non-exponential deposition profiles such as physical factors not included in filtration theory including “straining”, referred to as

retention of bacterial cells in smaller pores and at grain-grain junctions (27,29,4458), surface roughness (47,54), hydrodynamic drag (57,59), and cell aggregation (27). Others have related the non-exponential behavior to heterogeneity within the bacterial suspension or population (50,53), which produces a distribution of cells that interact with each other or the sand. In addition to these, hydrodynamic forces during the elution step might also displace cells and push them towards the lower regions of the column. This may be contributing, considering the column was flushed with 5 PV of electrolyte before column excavation at a high Darcy water velocity of 0.05 cm/s. The observed deposition trends are most likely a combination of these aforementioned phenomena.

2.5 Conclusions

Relative transport trends of SA1685 and O157:H7 were investigated with a focus on the influence of solution chemistry and cell injection concentration. Both experimental results and theoretical calculations were employed to predict these two important pathogen's interactions and transport behavior in representative groundwater conditions. Cell surface characterization experiments were conducted along with classical DLVO theoretical calculations.

Electrophoretic mobility analysis showed that SA1685 was more negative than O157:H7. Column experiments results were consistent with these observations as O157:H7 was retained more than SA1685. Interestingly, at higher injection concentration (10^8 cells/mL), both cell types showed increasing C/C_0 values and time dependency in the rising portion of the BTC. Travel distance calculations concluded that

both pathogens can move long distances under possible groundwater conditions in terms of flow velocities, cell concentrations and IS. These calculations are based upon k_d values as determined for aquifer sand which are highly unfavorable and where maximum transport is anticipated. Top distances calculated were 3.7 m for O157:H7 and 11.8 m for SA1685 for viable cells within a period of a day, even when achieving a 3-log removal.

This study shows that outbreak causing pathogens such as SA1685 and O157:H7 may follow complex transport behavior in groundwater conditions, more so than straightforward trends of non-pathogenic species (35,48), indicating additional mechanisms may be involved in their fate. In addition, other factors such as steric stabilization of cell surface macromolecules, cell collisions and interactions, cell heterogeneity, hydrodynamic drag forces, and considerations of pore structure will likely have to be considered to predict the fate of such pathogens. Additionally, relatively low and high cell concentrations may lead to considerable travel distances and pose water quality hazards. This study documents the first comparative transport behavior of *Salmonella spp.* and *E. coli* O157:H7 in a wide range of potential groundwater conditions.

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Chapter 3

Identifying the Role of Flagella in the Transport of Motile and Nonmotile *Salmonella enterica* Serovars

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Abstract

The influence of bacterial flagella on cell transport and adhesion has been examined using three *Salmonella enterica* serovars with different motility phenotypes. Two experimental setups, a packed bed column and a radial stagnation point flow system (RSPF), were employed to investigate bacterial adhesion kinetics onto quartz surfaces over a range of chemically unfavorable and favorable conditions. The aim of this study was to better understand the role of flagellar motion on the deposition behavior of *Salmonella*, an important food- and waterborne pathogen. Our results indicated that theoretical Derjaguin-Landau-Verwey-Overbeek (DLVO) theory cannot be used to explain the deposition behavior due to the virtually neutrally charged nature of the cells. In both the packed bed column and RSPF systems, flagellated strains showed higher deposition, indicating the nonmotile strain can actually be transported further than a motile strain. At late exponential growth phase, the deposition of the functional flagellated motile strain was enhanced, much more so than the dysfunctionally flagellated nonmotile and nonflagellated strains, highlighting the role of flagellar motion in cellular deposition and retention through available pore spaces and irregularities on quartz surfaces.

3.1 Introduction

Recent outbreaks due to *Salmonella* contaminated water and fresh produce confirms the role of waterborne *Salmonella* in human illnesses (62-65) and water as a major reservoir of *Salmonella* transmission (66, 67). Considering the widespread occurrence of *Salmonella* in aquatic systems, it is important to understand this pathogen's transport behavior in groundwater due to the very probable transmission via direct well water consumption, human contact, or consumption of food irrigated with contaminated water (62, 68-70).

Although several studies investigating the transport behavior of motile (71), nonmotile (35), or both type of bacteria in comparison (34, 72, 73) were documented in literature; this phenomena in *Salmonella* has not well studied (74). Besides, there is still considerable discrepancy in the literature whether motile cells can be transported further (75, 76) or attach to surfaces more so than nonmotile cells (77, 78). Nevertheless, it is widely accepted that flagellar motion and growth phase affect the bacterial deposition in well characterized porous media and radial stagnation point flow systems (30, 31, 34, 72, 79).

In this chapter, the deposition dynamics of motile and nonmotile *Salmonella* was studied to determine the impact of flagellar motion on the overall transport of these pathogenic bacteria. Three *Salmonella enterica* serovars with different motility features were selected; a flagellated and motile cell, a flagellated and nonmotile cell, and a nonflagellated, nonmotile cell. By working with these three strains, the contribution of flagellar presence and motility could be extrapolated. Additionally, cells were worked

with at both mid exponential and late exponential phases, as the motile cells only gained flagellar motion at the later growth stage.

A packed bed column and RSPF system was utilized for this study. The data presented below demonstrates that flagellated strains deposit more than their nonflagellated counterpart in both experimental systems, and flagellar motion enhances retention in the column via swimming towards available pore spaces, and/or collector surface irregularities. It was also concluded that nonmotile strain can be transported further than motile strain under the experimental conditions tested.

3.2 Materials and Methods

3.2.1 Bacterial Cell Preparation

All *Salmonella enterica* serovars were obtained from the *Salmonella* Genetic Stock Centre (SGSC), University of Calgary, Canada. Experiments were conducted with three *Salmonella enterica* serovars, two of which are outbreak causing strains. The first organism, *Salmonella enterica* serovar Typhimurium (ST5383) was a wild type, flagellated strain originally isolated from an interprovincial outbreak that infected more than 1700 people (80). The unique feature of the strain ST5383 was it gained flagellar motion only when harvested at late-exponential growth phase; otherwise non-motile at mid exponential. The second organism *Salmonella typhimurium* (SGSC1512) was a dysfunctional flagellated nonmotile mutant of *S. typhimurium* TM2 (81). The last organism used in this work was *Salmonella enterica* serovar Gallinarum (SGSC2478), a wild type, nonflagellated, avian pathogen (82). To visualize the cells in RSPF system

(described below), a plasmid coding for an enhanced green fluorescing protein (GFP) and ampicillin resistance was introduced to native *Salmonella* cells via standard electroporation protocols (83). The transformed cells were referred as ST5383g, SGSC1512g, and SGSC2478g, respectively. All non-GFP labeled *Salmonella* cells were precultured in Luria-Bertani (LB) broth (Fisher Scientific, Fair Lawn, NJ) at 37°C overnight while shaken continuously at 200 rpm. On the day of experimental analysis, overnight precultures were used to inoculate fresh LB broth (1:100 dilution) and harvested at mid (~2.5 hrs) and late exponential (~6 hrs) growth phase. GFP labeled *Salmonella* cells were prepared following the same protocol, except the growth media was supplemented with ampicillin (100 µg/mL final concentration). Following the cultivation of bacteria until desired growth phase, a refrigerated bench-top centrifuge (5804R; Eppendorf, Hamburg, Germany) equipped with fixed angle rotor (F-34-6-38; Eppendorf) was used to pellet the cells with an applied 3,700g for 15 min at 4 °C. Growth medium was decanted and the pellet was resuspended in respective IS KCl solution. The process was repeated twice in order to ensure complete removal of the growth medium. All electrolyte solutions used in cell preparation and experiments were prepared with deionized (DI) water (Millipore, Billerica, MA) and reagent-grade KCl (Fisher Scientific) with no pH adjustment (pH 5.6–5.8). Concentrations of cell solutions were determined by using a cell counting hemocytometer (Bürker-Turk, Germany) and light microscope (Fisher Scientific).

3.2.2 Bacterial Cell Characterization

Viability tests were conducted using the Live/Dead BacLight kit (L-7012, Molecular Probes, Eugene, OR) in respective ionic strength (IS) KCl solutions. Direct counting of the stained cells was done using an inverted microscope (IX70, Olympus, Japan) and the appropriate fluorescence filter set (Chroma Technology Corp., Brattleboro, VT).

To measure the dimensions of cells, phase contrast images were taken using an inverted microscope (IX70, Olympus) and camera (Retiga 1300, Qimaging, Canada) at $\sim 10^8$ cells/mL in 10 mM KCl. Images of cells ($n > 60$) were processed (SimplePCI, Precision Instruments Inc., Minneapolis, MN) and individual cell lengths and widths were determined. Measured values were used to calculate the effective cell radius and corresponding bacterial surface area.

Hydrophobicity analysis of the cells was done using the microbial adhesion to hydrocarbons (MATH) test (84). Briefly, the partitioning of cells was measured between a hydrocarbon (n-dodecane, Fisher Scientific, Fairlawn, NJ) and respective electrolyte solution and reported as the percentage of total cells partitioned into the hydrocarbon from the aqueous phase.

Potentiometric titrations of cells were conducted to determine the relative acidity and charge density of the bacterial surfaces. An autotitrator (798 MPT Titrino, Metrohm, Switzerland) was used to determine the amount of base consumed by suspended cells during titration between pH 4 and 10. Bacterial suspensions with concentrations of $\sim 10^8$ cells/mL in electrolyte solutions were titrated with 0.3 N NaOH. The resulting acidity and corresponding surface charge density were calculated (31, 85).

The extracellular polymeric substance (EPS) composition, specifically the total protein and sugar content, was extracted based on an established method (86). The analysis of protein was performed using the Lowry method (87) with bovine serum albumin (Fisher Scientific) as the standard and measured spectroscopically (BioSpec-mini, Shimadzu, Kyoto, Japan) at a wavelength of 500 nm. Total sugar analysis was performed using the phenol-sulfuric acid method (88). Xanthan gum (Fisher Scientific) was used as the standard and samples measured spectroscopically at 480 nm (BioSpec-mini, Shimadzu).

3.2.3 Scanning Electron Microscopy Imaging

SEM images were taken to confirm the flagella structure of all strains. The images were taken with a Phillips XL30-FEG scanning electron microscope operated at 3-10 kV. Bacteria were prepared by standard procedures described previously, diluted with DI water onto a microscopy slide, air-dried at 37°C for 20 minutes, and finally sputter-coated for 20 seconds with Au/Pd. SEM images of the sand particles were taken with Hitachi Tabletop Microscope (TM-1000; Tokyo, Japan).

3.2.4 Motility Assays

To determine the motility profiles of *Salmonella* strains both qualitative and quantitative assays were performed. A modified version of soft agar motility assay was performed as follows (89). A single line of bacterial inoculation was streaked on Difco motility test medium (Becton, Dickinson and Company, NJ) plates and the growth spreading out from the line of inoculation was monitored for three to eight hours. Additional motility assays were performed based on a previously described swarm

motility assay (90). In this method, 200 μ l of overnight grown bacteria were dropped onto Difco motility GI medium (Becton, Dickinson and Company) plates supplemented with Tween 80 (Fisher Scientific) to a final surfactant concentration of 0.02% (91). The changes in the dimensions of the initial drop were monitored for three to eight hours. Finally, direct visual observation of bacteria grown until both mid and late exponential phase, and prepared as previously described was observed under light microscopy (Fisher Scientific).

3.2.5 Electrokinetic Properties and DLVO Calculations

Electrophoretic mobility of bacterial cells was determined by diluting the cell pellet to an optical density of 0.2-0.25 in 10, and 100 mM KCl at 546 nm (BioSpec-mini, Shimadzu). Electrophoretic mobility measurements were conducted at 25°C using a ZetaPALS analyzer (Brookhaven Instruments Corporation, Holtsville, NY), repeated five times, and converted to zeta potentials using Smoluchowski equation (92).

Electrokinetic properties of coated sand were determined using a streaming potential analyzer (EKA, Anton Paar GmbH, Graz, Austria) with a cylindrical powder cell. Measurements were obtained in KCl over the range of IS used in the transport experiments. The instrument was first rinsed with 2 L of DI water followed by 0.5 L of the electrolyte solution used in the measurement. Prior to the streaming potential measurements, the cylindrical powder cell packed to a bed depth of 3 cm was equilibrated with the fresh electrolyte solution for 40 min. Zeta potential values were calculated from the streaming potentials as described elsewhere (93, 94).

Derjaguin-Landau-Verwey-Overbeek (DLVO) theory was utilized to predict interaction energy between the cell and collector surfaces (60). Total interaction energies existing between the pathogen and sand particle (assuming sphere-plate interaction) were quantified as the sum of van der Waals (95) and electrostatic interactions with constant surface potential (96), both of which decay with separation distance (92).

3.2.6 Porous Medium Preparation

American Society for Testing and Materials 20/30 unground silica sand (U.S. Silica, Ottawa, IL) was used as packing material for the column experiments. Average sand diameter (d_{50}) was 710 μm . In order to remove metal oxide coatings and organic matter associated, the sand was shaken vigorously in a 2-L flask containing 12 M hydrochloric acid (Fisher Scientific) twice and kept in acid overnight (97). The mixture was then rinsed with DI water until the rinse water pH was equal to that of the DI water. Prior to column packing the sand was sterilized by autoclaving at 121 °C and 15 psi for 30 min. Porosity was gravimetrically determined to be 0.34 ± 0.02 . For transport experiments under favorable (nonrepulsive) electrostatic conditions, the sand was treated with a solution of 1% (vol/vol) 3-aminopropyl-triethoxysilane (Sigma) for 5 min, rinsed in DI water, and finally cured at 80 °C for 24 (97, 98).

3.2.7 Packed Bed Column Experiments

Chromaflex chromatography columns (Kontes Glass Co., Vineland, NJ), 2.5 cm diameter and 30 cm long, were wet packed using autoclaved quartz sand (710 μm diameter). After packing, columns were operated in a downward direction using a peristaltic pump (Cole-Palmer, Vernon Hills, IL). Approximately 20-25 pore volumes

(PV) of deionized (DI) water, followed by 3 PV of electrolyte solution (10 mM and 100 mM KCl) were passed through column to equilibrate the sand. Columns were operated at 2.5 mL/min to achieve a Darcian velocity of ~ 7.33 m/d. Calculated Reynolds (92) and Peclet (29, 92) numbers for the column were 0.19 and 0.01, respectively. Three PVs of bacteria (concentration of 10^7 cells/mL) were injected in the column followed by 5 PV of bacteria-free electrolyte solution. Effluent was collected every minute using a fraction collector (RTRV II, Isco Inc, Lincoln, NE). Bacterial concentrations were determined via optical density at 546 nm using a UV–visible spectrophotometer (BioSpec-mini, Shimadzu).

3.2.8 Radial Stagnation Point Flow Experiments

To better understand bacterial adhesion mechanisms and transport behavior, deposition experiments were also conducted in well controlled RSPF system (29, 97, 99) consisting of a customized glass chamber installed on the stage of an inverted fluorescent microscope (IX70, Olympus). Fluid stream entered the chamber from a capillary tube (2 mm inner diameter) with a flow rate of 1.5 mL/min and impinged upon a quartz microscope cover slip (located 2 mm from the end of the tube) at a right angle and flowed away in all directions. Fluorescently labeled cells depositing on the quartz were imaged by a 40 $\times$ objective (UPlanFI, Olympus) focused on the inner surface of the cover slip and using a fluorescent filter set with an excitation wavelength of 480 nm and emission wavelength of 510 nm (Chroma Technology Corp., Brattleboro, VT).

All quartz microscope cover slips (Electron Microscopy Sciences, Ft. Washington, PA) were cleaned by a surfactant, ethanol, and deionized water rinse, followed by

submersion in NOCHROMIX solution (Godax Laboratories, Inc., Takoma Park, MD). After removal from NOCHROMIX solution and rinsing with deionized water, cover slips were mounted in the RSPF flow cell. To achieve favorable, non-repulsive electrostatic conditions, the slides were chemically modified (93, 94) and exhibited a net positive zeta potential of +3.3 mV in 10 mM KCl measured previously (37). This was achieved by exposure of the quartz to a 0.2% (v/v) mixture of (aminoethylaminomethyl)–phenethyltrimethoxysilane (Gelest, Inc., Tullytown, PA) in ethanol for 3–5 min at room temperature and then curing for 90 min at 130 °C (100).

Deposition of fluorescently labeled bacterial cells was recorded with a digital camera (Retiga 1300, QImaging) capturing images every 20 seconds over the course of a 20 min injection and analyzed with the supplied software (SimplePCI, Precision Instruments, Inc., Minneapolis, MN). The number of deposited bacteria was determined for each time interval by comparing the changes between successive images. Inlet concentrations for experiments were maintained at 10^7 cells/mL, same as column system.

To ensure that both systems are comparable, hydrodynamic conditions in the RSPF were designed to match the column system. Calculated Reynolds (92) and Peclet (29, 92) numbers for the RSPF system were 7.96 and 0.01, respectively.

3.2.9 Deposition Profiles and Extent of Kinetics

To determine the deposition profile of bacteria across the length of the column, sand was excavated after each experiment (101). The inlet fitting was removed and saturated porous media were transferred to 50 mL Falcon tubes containing 10 to 20 mL of KCl solution with the same IS used during the conducted transport experiment. Starting at the

top of the column, sand was recovered every 5 cm section with each tube containing approximately 45 g of sand. Tubes were shaken gently for 5 min and the supernatant was spread onto LB agar plates. Spread-plating procedure was followed for the bacterial stock solution as well to quantify the number of colony forming units (CFU) in the stock solution. CFUs forming on the overnight incubated (37°C) plates were counted and normalized by the CFUs counted from the cell stock solution. Liquid and sand filled tubes were placed in a hot oven (105°C) and incubated at least 48 h in order to volatilize remaining KCl solution for gravimetric measurement of the sand particles.

The total percentage (M_{total}) of cells recovered was determined from the sum of the percentages of cells recovered from the column effluent (M_{eff}) and the aquifer sand (M_{sand}) by following a protocol described previously (102, 103).

The bacterial deposition rate coefficient, k_d , was determined by (104)

$$k_d = -\frac{U}{\varepsilon L} \ln \left[\frac{C}{C_0} \right] \quad (3-1)$$

where C/C_0 is the normalized concentration exiting the column, U is the superficial velocity, ε is the porosity, and L is length of the packed bed. The average value of C/C_0 was obtained between 1.8 and 2.0 pore volumes (PV), which represents “clean-bed” conditions (105). Approximate travel distances of all *Salmonella* strains grown until mid and late exponential phase were estimated from measured values of k_d . Details of the deposition profile, mass balance, and travel distance equations are provided in the SI.

The kinetics of bacterial adhesion in the RSPF system was quantified by calculating the bacterial transfer rate coefficient, k_{RSPF} , which is related to the bacterial deposition

flux (number of cells per area per time), J , and the bulk bacterial cell concentration, C_0 , by

$$k_{RSPF} = \frac{J}{C_0} \quad (3-2)$$

Here, the deposition flux (J) was determined by normalizing the initial slope of the number of deposited cells versus time curve by the microscope viewing area ($210 \mu\text{m} \times 168 \mu\text{m}$).

In order to minimize the role of flagellar motion and correlate the deposition behavior among the two systems, “favorable” experiments were conducted (described in the SI). Since the theoretical deposition rates under chemically favorable, transport-limited conditions can only be predicted for spherical particles (106), values for the favorable deposition rate in the RSPF system ($k_{RSPF, fav}$) were determined experimentally for the ST5383g, SGSC1512g, and SGSC2478g in 10mM KCl.

The corresponding adhesion (attachment) efficiency in the RSPF system, α , was calculated by normalizing the bacterial transfer rate coefficient at each IS by the transfer rate coefficient determined under favorable electrostatic conditions by

$$\alpha_{RSPF} = \frac{k_{RSPF}}{k_{RSPF, fav}} \quad (3-3)$$

Similarly, attachment efficiency in the packed bed column system was calculated by

$$\alpha_{Column} = \frac{k_{sd}}{k_{sd, fav}} \quad (3-4)$$

α is indicative of the success of a cell attaching to the quartz surface. Theoretically, α values should be between 0 and 1 (106). The values of α_{RSPF} and α_{column} were compared to provide a mechanistic means of analysis (29).

3.2.10 Travel Distance Calculations

Approximate travel distances for all *Salmonella* strains were estimated based on described colloid travel distance equations given below (103, 107)

$$L_T = -\ln(C_L / C_0) \left(\frac{4a_c}{3(1-\varepsilon)\alpha\eta_0} \right) \quad (3-5)$$

In this equation, L_T is denoted as vertical travel distance, a_c is the radius of sand particle, ε is the porosity of the column, α is the collision efficiency, and η_0 is the single collector efficiency. For the calculation of collision efficiency, experimental C/C_0 values were first used to determine filter coefficient (λ) as follows:

$$\frac{dC}{dx} = -\lambda C \quad (3-6)$$

In this equation, x denotes the direction parallel to flow in the column. The single collector efficiency was calculated based on the R-T model by Rajagopalan and Tien (103, 107). Calculated filter coefficient values in eq. (3-6) and single collector efficiencies by R-T model were incorporated into eq. (3-7) developed by Yao and co-workers (108).

$$\lambda = \left(\frac{3}{2} \right) \times \left[\left(\frac{1-\varepsilon}{d_c} \right) \right] \times \alpha \times \eta_0 \quad (3-7)$$

In this equation, d_c is denoted as the diameter of the sand particle. Remaining parameters calculated from equations (3-6) and (3-7) were also incorporated into eq. (3-5) where 99.99% removal was considered with C_L/C_0 value of 0.001.

3.3 Results and Discussion

3.3.1 Effect of Growth Phase on Cell Characteristics

Comprehensive cellular characteristics were evaluated for all three strains harvested at both mid and late exponential phases across the range of experimental IS (Table 3.1). Cells grown until late exponential phase were slightly smaller in dimension, with approximate volumetric spherical radii of $\sim 0.4\text{--}0.5\text{ }\mu\text{m}$. Titration experiments showed that the nonflagellated SGSC2478 strain had a higher level of acidity ($\sim 5 \times 10^{-5}\text{ meq}/10^8$ cell and $\sim 1 \times 10^{-5}\text{ meq}/10^8$ cell at mid and late exponential phases, respectively) than the two flagellated strains. Corresponding surface charge densities were calculated based on the measured dimensions (37) and indicated a similar trend that SGSC2478 had higher cell surface charge density than ST5383 and SGSC1512, with the exception of 100 mM, at which ST5383 had a slightly higher surface charge density ($2511\text{ }\mu\text{C}/\text{cm}^2$) compared to SGSC2478 ($2418\text{ }\mu\text{C}/\text{cm}^2$) which is attributed to the slightly shorter dimensions of ST5383 occurring at the higher IS condition.

EPS quantification results confirmed the titration data as the nonflagellated wild type SGSC2478 had more sugar and protein content in the EPS as compared to the flagellated strains. This observation might be due to the fact that SGSC2478 as a pathogen does not have the “advantage” of possessing flagella as a mediator of virulence (109). It is

proposed that this “disadvantage” requires more complex surface antigens (as indicated by high protein mass) to initiate the virulence response of the target host. However, this hypothesis requires further investigation with controlled virulence assays which was not in the scope of this study. Cells harvested at late exponential phase indicated a major decrease in the extracellular protein content as bacteria enter to the onset of starvation and synthesize less protein as compared to their mid exponential phase counterparts. Sugar content was increased slightly for the flagellated strains, ST5383 and SGSC1512 (dysfunctional), whereas a significant decrease was observed for SGSC2478. Both sugar and protein content of SGSC2478 were lower than ST5383 and SGSC1512 at late exponential phase which suggest that nonflagellated strain was more vulnerable to starvation effects than flagellated strains.

MATH test results showed that all *Salmonella* strains are quite hydrophilic at both IS and growth phases except hydrophobicity of ST5383 increased from ~14% at mid exponential growth phase to ~82% at late exponential phase. It is hypothesized that ST5383 may in fact swim away from the hydrocarbon phase to the water phase which resulted in higher optical absorbance used to quantify the relative hydrophobicity as described earlier.

Electrophoretic motility measurements and calculated zeta potential values of strains are presented in Table 3.1. The data indicate there was no significant difference among the zeta potentials of all strains. At mid exponential phase, calculated zeta potential values ranged approximately from -1 mV to -6.4 mV and from -4.8 mV to +1.9 mV at late exponential phase. As IS was increased from 10 mM to 100 mM, zeta potential

values became less negative as anticipated due to the compression of electrostatic double layer. Zeta potential values reported in this study were close to neutral and comparable to pathogens, i.e. *Salmonella pullorum* and *E. coli* O157:H7, measured previously (74); however, distinctly different from other various nonpathogenic organisms previously studied (29, 97, 99).

Viability measurements were taken at prior to transport and characterization experiments. Results have shown that all strains were >90% alive at both growth phases across the range of tested IS.

SEM images were taken at both growth phases (only mid exponential phase images were shown) to present the detailed flagella features of motile and nonmotile *Salmonella* strains (Figure 3.1). Figure 3.1a shows the functional flagella of ST5383, Figure 3.1b shows the dysfunctional flagellated structure of SGSC1512, and Figure 3.1c confirms the nonflagellated nature of SGSC2478.

To determine the motility profiles of *Salmonella* strains both qualitative and quantitative assays were performed. Table 3.2 shows the changes in the dimensions of the dropped bacterial inoculation circle monitored for three to eight hours. The results show that functional flagellated strain ST5383 swarms much faster at late exponential phase than mid exponential phase where minor swarming motion was observed. Dysfunctional, flagellated, and nonmotile SGSC1512 strain's, motion was limited at both mid and late exponential phase. Nonflagellated strain SGSC2478's motion was similar to SGSC1512. Similar motility trends were observed in the soft agar line motility assay (data not shown). Light microscopy observation confirmed no motility for ST5383 (mid

exponential phase), SGSC1512 (at both mid and late exponential phase), and SGSC2478 (at both mid and late exponential phase); whereas active motility was observed for ST5383 grown until late exponential phase. Minor swarming motion of SGSC1512 and SGSC2478 on motility medium assays was attributed to the nature of the soft agar plates.



Table 3.1. Key cell characteristics of ST5383, SGSC1512, and SGSC2478 grown until mid and late exponential phase.

MID EXPONENTIAL PHASE										
Strain	IS (mM KCl)	Radius (μm) ^a	Acidity (meq/10 ⁸ cell) ^b	Surface Charge Density (μC/cm ²) ^c	MATH (%) ^d	Sugar (μg/ml) ^e	Protein (μg/ml) ^f	Mobility [(μm/sec)/(V/cm)]	Zeta Potential (mV)	Viability (%) ^g
ST5383	10	0.415±0.10	1.10×10 ⁻⁵	506	14.60±5.95	0.43±0.01	21.46±1.88	-0.17±0.34	-2.17±4.39	92±3.23
	100		4.49×10 ⁻⁵	2511	13.36±4.57			-0.07±1.32	-0.97±17.00	92±4.36
SGSC1512	10	0.563±0.12	0.56×10 ⁻⁵	140	22.76±8.28	0.76±0.01	42.35±1.13	-0.50±0.25	-6.40±3.18	95±1.27
	100		3.54×10 ⁻⁵	881	39.06±1.42			-0.32±0.36	-4.13±4.55	94±3.40
SGSC2478	10	0.435±0.10	5.35×10 ⁻⁵	2226	18.23±2.61	2.94±0.01	294.22±61.94	-0.19±0.28	-2.45±3.51	94±3.56
	100		5.81×10 ⁻⁵	2418	20.35±3.54			-0.13±0.84	-1.64±10.69	92±1.89
LATE EXPONENTIAL PHASE										
ST5383	10	0.377±0.08	0.24×10 ⁻⁵	135	81.40±9.88	0.62±0.05	8.08±0.70	-0.32±0.24	-4.04±3.08	96±2.5
	100		0.27×10 ⁻⁵	152	85.37±3.61			0.08±0.82	0.97±10.53	94±2.8
SGSC1512	10	0.489±0.06	0.15×10 ⁻⁵	50	18.02±5.14	0.79±0.06	10.34±0.20	-0.38±0.31	-4.81±3.98	93±4.9
	100		0.19×10 ⁻⁵	63	13.36±6.33			-0.19±0.85	-2.37±10.83	92±6.3
SGSC2478	10	0.417±0.07	1.12×10 ⁻⁵	508	22.99±6.71	0.45±0.01	6.12±0.25	-0.22±0.30	-2.76±3.85	95±1.2
	100		1.27×10 ⁻⁵	574	24.72±1.85			0.15±0.35	1.89±4.48	97±1.8

^a Value for equivalent spherical radius calculated from measured length and width of individual cells.

^b Acidity determined from amount of NaOH consumed during titration between pH 4-10.

^c Indicates density of charged functional groups across cell surface. Value determined from measured acidity, and accounting for exposed surface area of the cells (calculated for a spherical cell) and Faraday's constant = 96,485 C/mol.

^d Microbial adhesion to hydrocarbon (MATH) indicates the relative hydrophobicity of the cell as the percent of cells partitioned in dodecane versus electrolyte.

^e Based on Phenol-sulfuric acid method and Xanthan gum as standard at 10^8 cells/ml.

^f Based on Lowry method and Bovine Serum Albumin (BSA) as standard at 10^8 cells/ml.

^g Percent of cell population viable based on the Live/Dead BacLight[®] kit. Values are average of measured viability across the range of IS utilized in transport experiments.

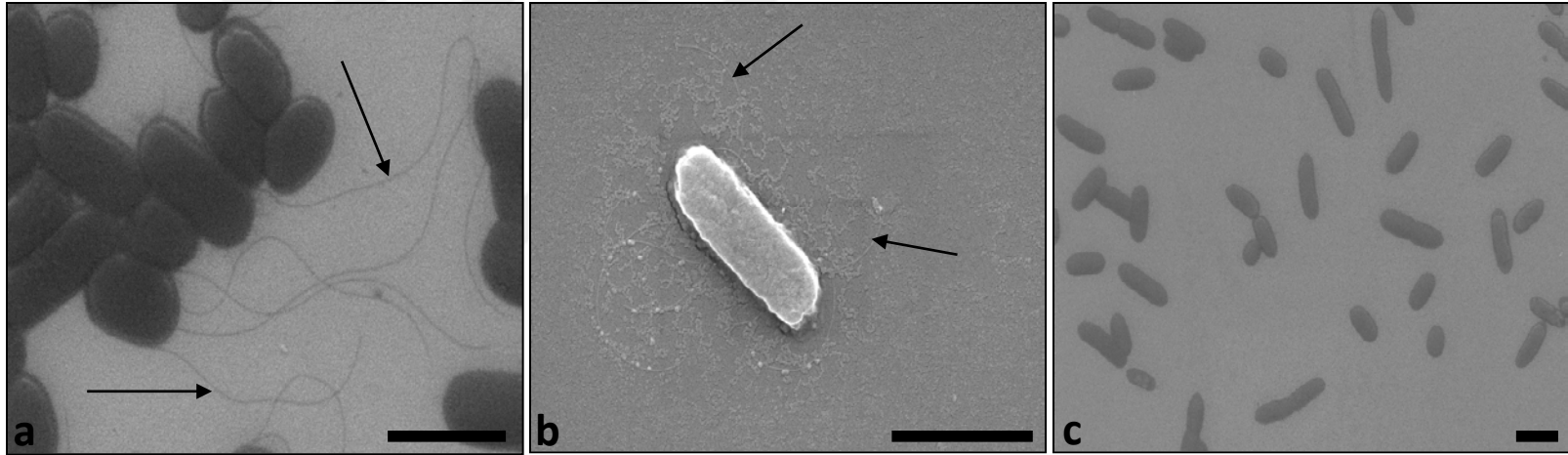


Figure 3.1. SEM images of fully functional flagellated, motile ST5383 (a); dysfunctional flagellated, nonmotile SGSC1512 (b); and nonflagellated, SGSC2478 (c). Black arrows highlight the flagella. Scale bars indicate the length of 2 μm .

Table 3.2. Results of soft agar swarming assay in terms of changes in the dimensions of the dropped bacterial inoculation circle measured with time.

Strain	0 Hours	3 Hours		8 Hours	
	Circle diameter (mm)	L (mm) ^a	W(mm) ^b	L (mm)	W(mm)
ST5383	20.35	28.35±0.12	38.29±0.16	53.4±0.12	56.34±0.12
SGSC1512		27.37±0.11	39.21±0.14	27.77±0.18	42.4±0.05
SGSC2478		25.4±0.13	33.25±0.09	25.59±0.12	39.48±0.10

^a Denotes the vertical length of the initial inoculation drop.

^b Denotes the horizontal length of the initial inoculation drop.

3.3.2 Bacterial Deposition in Packed Bed Column System

Bacterial deposition rates of motile and nonmotile strains were determined in the packed bed column system as a function of IS and growth phase (Figure 3.2). Both qualitative and quantitative motility assays (Table 3.2) revealed that the wild type flagellated strain ST5383 was only motile when harvested at late exponential growth phase. No flagellar motion was observed for this strain at mid exponential growth phase. Therefore, at mid exponential growth phase all three strains were nonmotile and their deposition kinetics were strongly dependent on the cell surface characteristics as well as physico-chemical properties of the column system. As can be seen on Figure 3.2a, at 10 mM only 10% of (total mass) nonflagellated SGSC2478 were retained in the column, whereas flagellated strains ST5383 and SGSC1512 (dysfunctional) were retained 25% and 45%, respectively. The deposition of all strains increased to 17%, 40%, and 60% for SGSC2478, ST5383, and SGSC1512 respectively (Figure 3.2c) with an increase in IS from 10 mM to 100mM due to the reduction in electrostatic repulsion forces between the cells and collector surface.

When the bacteria were harvested at late exponential phase, the functionally flagellated ST5383 strain gained flagellar motion and its deposition was increased substantially (by 66% and 50% at 10 mM and 100 mM, respectively) (Figure 3.2b, d). Compared to mid exponential phase cells; however, the difference in deposition behavior of late exponential phase SGSC1512 (dysfunctional flagella) and SGSC2478 (nonflagellated) was minor. The only exception was for SGSC2478, in which the extent of deposition changed only 10% – 20% with the longer growth period at 10 mM. However, as compared to the ST5383, the impact of growth phase was still considered minimal (Figure 3.2).

The BTCs obtained under chemically “favorable” conditions are presented in Figure 3.3. Similar to the “unfavorable” conditions, flagellated strains deposited more than the nonflagellated strain at both growth phases. Interestingly, at late exponential phase, when ST5383 is motile, SGSC1512’s deposition was highest; suggesting that the electrostatically “favorable” condition achieved by the positively charged aminosilane coating on the quartz surface may overwhelm the effect of flagellar motion.

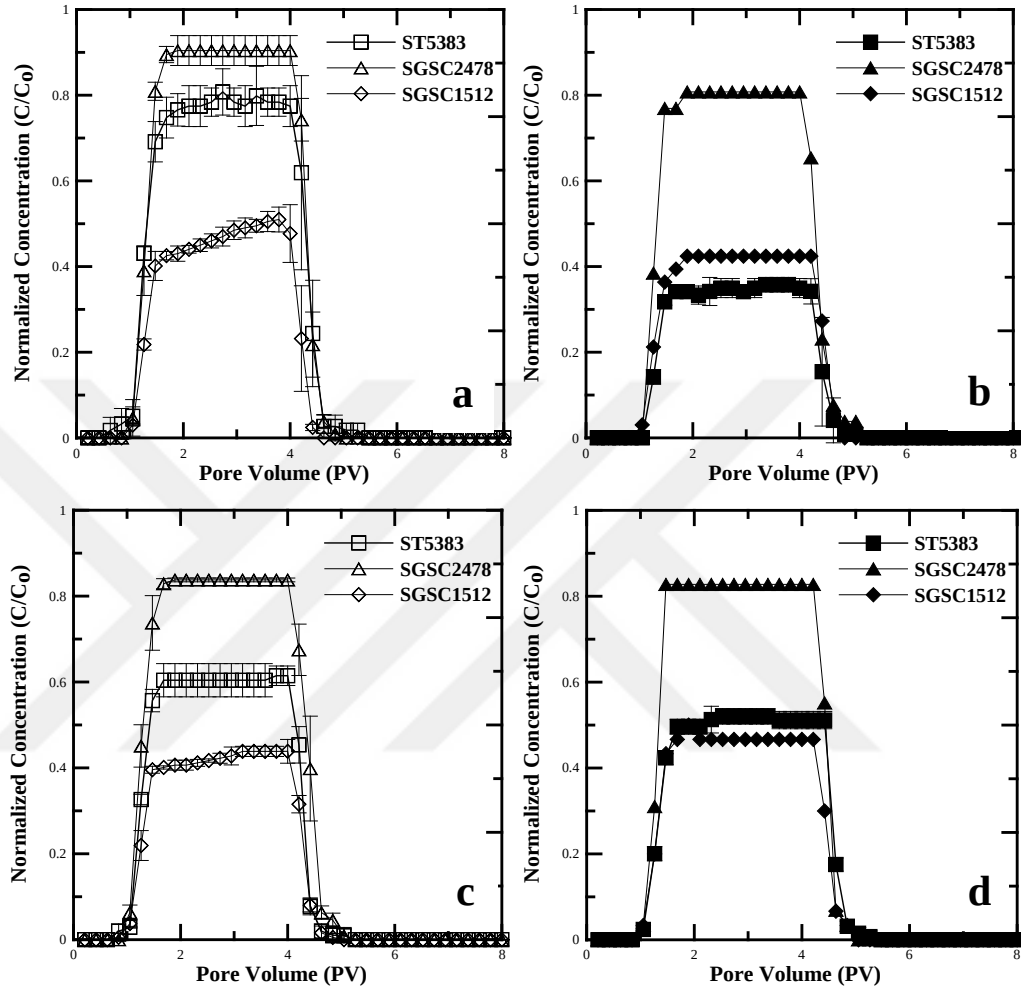


Figure 3.2. BTCs of ST5383, SGSC2478, and SGSC1512 tested at 10mM (a), and 100 mM (c) IS, grown until mid exponential phase; 10 mM (b), and 100 mM (d) IS, grown until late exponential phase ($C_0=10^7$ cells/mL). Error bars indicate average of three runs.

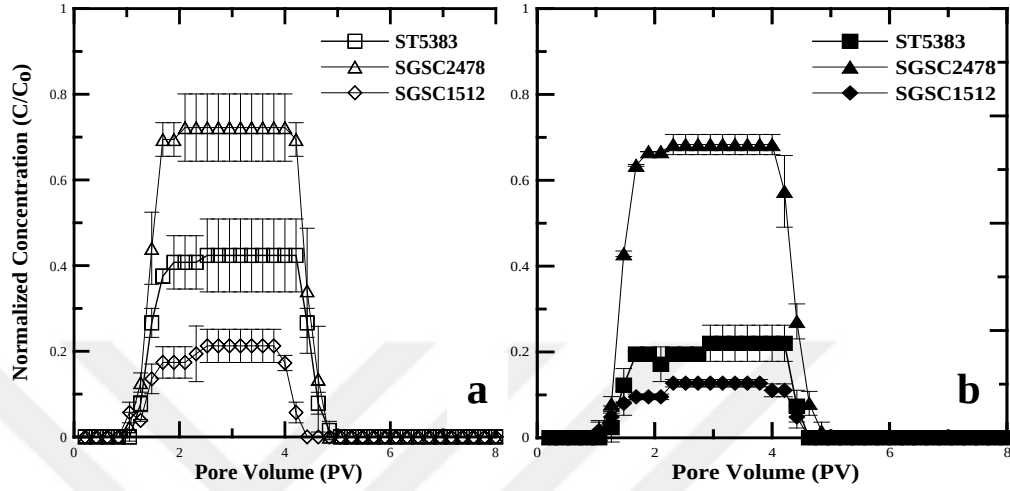


Figure 3.3. Favorable condition BTCs of ST5383, SGSC2478, and SGSC1512 tested at 10 mM with aminosilane coated sand grown until mid exponential phase (a); grown until late exponential phase (b) ($C_0=10^7$ cells/mL). Error bars indicate average of two runs.

3.3.3 Bacterial Deposition in RSPF System

In an effort to better understand the effect of motility in the absence of the three dimensional pore structures, deposition kinetics was investigated in the RSPF system as well, to complement the column system (Figure 3.4). As ST5383g was motile at late exponential growth phase, its bacterial transfer coefficient (k_{RSPF}) was ~ 4 times more at 10 mM, and ~ 6 times more at 100 mM compared to k_{RSPF} values for the identical, but nonmotile cells at mid exponential phase (Table 3.3). The effect of growth phase on the deposition of SGSC1512g and SGSC2478g strains was observed only as a slight increase at both IS; except SGSC2478 deposition at 100 mM in late exponential phase increased significantly compared to in the mid exponential phase cells. Overall, the nonflagellated strain SGSC2478g deposited less than flagellated strains at both growth phases and IS. The bacterial transfer rate coefficients for favorable conditions ($k_{RSPF, fav}$) in the RSPF

system are presented in Table 3.3. There was an enhanced deposition for all three strains as compared to the unfavorable conditions due to mass transfer of bacterial cells under transport limited conditions. Table 3.3 shows that the deposition of SGSC2478g and SGSC1512g was much more substantial than ST5383g under favorable conditions. The deposition of ST5383g was enhanced ~ 5 and 4 times for mid and late exponential phase cells, respectively; even though the strain was motile at the late exponential condition. The deposition of SGSC1512g was enhanced ~ 4.5 and ~ 45 times at mid and late exponential phases respectively. In case of non-flagellated SGSC2478g, the deposition was enhanced ~ 19.5 and ~ 7 times at mid and late exponential phases respectively, compared to unfavorable conditions at corresponding IS. This observation suggests that under unfavorable conditions, ST5383g has already reached mass transport limited conditions.

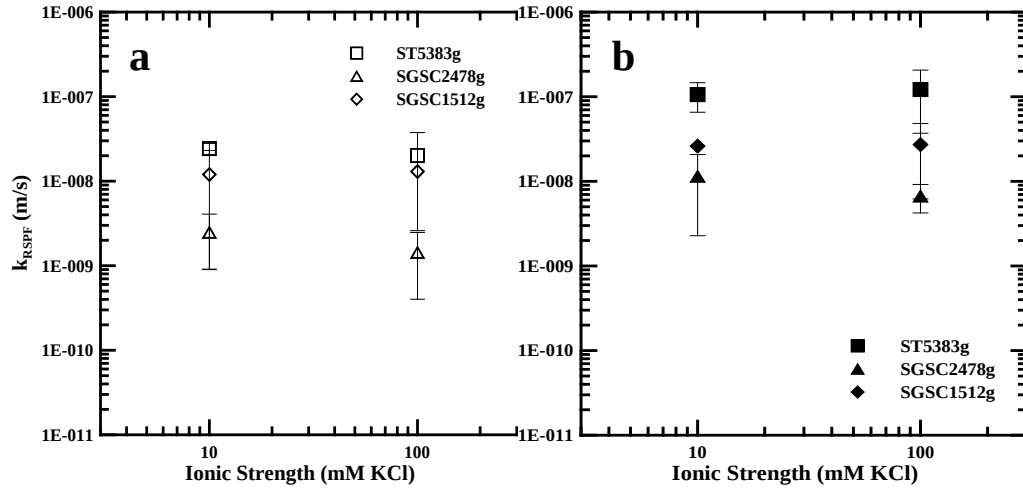


Figure 3.4. Bacterial transfer rate coefficient (k_{RSPF}) versus IS for ST5383g, SGSC2478g, and SGSC1512g grown until mid exponential phase (a); grown until late exponential phase (b) ($C_0 = 10^7$ cells/mL). Error bars indicate average of three runs.

Table 3.3. Comparison of bacterial deposition, transfer rate coefficients, and attachment efficiencies calculated from packed bed and RSPF system experiments.

Strain	IS	Packed bed			RSPF	
		Growth phase	k_d (s ⁻¹) ^a	α_{col}	k_{RSPF} (m/s)	α_{RSPF}
ST5383	10	Mid-exp	$2.32 \times 10^{-4} \pm 4.96 \times 10^{-5}$	0.29 ± 0.08	$2.43 \times 10^{-8} \pm 3.65 \times 10^{-9}$	0.19 ± 0.04
	100		$4.47 \times 10^{-4} \pm 5.71 \times 10^{-5}$	ND ^b	$2.01 \times 10^{-8} \pm 1.75 \times 10^{-8}$	ND
SGSC1512	10	Mid-exp	$7.36 \times 10^{-4} \pm 2.71 \times 10^{-5}$	0.47 ± 0.06	$1.20 \times 10^{-8} \pm 1.11 \times 10^{-8}$	0.22 ± 0.21
	100		$7.97 \times 10^{-4} \pm 2.52 \times 10^{-5}$	ND	$1.30 \times 10^{-8} \pm 1.76 \times 10^{-8}$	ND
SGSC2478	10	Mid-exp	$8.96 \times 10^{-5} \pm 3.38 \times 10^{-5}$	0.29 ± 0.13	$2.49 \times 10^{-9} \pm 1.58 \times 10^{-9}$	0.05 ± 0.03
	100		$1.57 \times 10^{-4} \pm 4.65 \times 10^{-6}$	ND	$1.44 \times 10^{-9} \pm 1.04 \times 10^{-9}$	ND
ST5383	10	Late-exp	$9.62 \times 10^{-4} \pm 3.93 \times 10^{-5}$	0.64 ± 0.06	$1.06 \times 10^{-7} \pm 4.04 \times 10^{-8}$	0.27 ± 0.10
	100		$6.20 \times 10^{-4} \pm 3.14 \times 10^{-5}$	ND	$1.22 \times 10^{-7} \pm 8.46 \times 10^{-8}$	ND
SGSC1512	10	Late-exp	$7.35 \times 10^{-4} \pm 2.01 \times 10^{-5}$	0.35 ± 0.01	$2.61 \times 10^{-8} \pm 3.08 \times 10^{-8}$	0.02 ± 0.02
	100		$7.01 \times 10^{-4} \pm 5.37 \times 10^{-5}$	ND	$2.72 \times 10^{-8} \pm 2.10 \times 10^{-8}$	ND
SGSC2478	10	Late-exp	$2.18 \times 10^{-4} \pm 2.72 \times 10^{-5}$	0.59 ± 0.13	$1.15 \times 10^{-8} \pm 9.23 \times 10^{-9}$	0.14 ± 0.11
	100		$1.74 \times 10^{-4} \pm 6.89 \times 10^{-6}$	ND	$6.71 \times 10^{-9} \pm 2.48 \times 10^{-9}$	ND
Favorable Experiments						
ST5383	10	Mid-exp	$7.99 \times 10^{-4} \pm 1.36 \times 10^{-4}$	–	$1.26 \times 10^{-7} \pm 2.18 \times 10^{-8}$	–
	10	Late-exp	$1.50 \times 10^{-3} \pm 1.14 \times 10^{-4}$	–	$4.00 \times 10^{-7} \pm 3.26 \times 10^{-8}$	–
SGSC1512	10	Mid-exp	$1.56 \times 10^{-3} \pm 1.88 \times 10^{-4}$	–	$5.38 \times 10^{-8} \pm 5.69 \times 10^{-9}$	–
	10	Late-exp	$2.08 \times 10^{-3} \pm 5.97 \times 10^{-5}$	–	$1.18 \times 10^{-6} \pm 4.78 \times 10^{-8}$	–
SGSC2478	10	Mid-exp	$3.07 \times 10^{-4} \pm 7.37 \times 10^{-5}$	–	$4.86 \times 10^{-8} \pm 1.10 \times 10^{-8}$	–
	10	Late-exp	$3.69 \times 10^{-4} \pm 1.43 \times 10^{-5}$	–	$8.00 \times 10^{-8} \pm 7.73 \times 10^{-9}$	–

^a Determined by averaging over pore volumes (1.8-2), as described in the text.

^b Not determined.

3.3.4 Comparison of the Packed Bed and RSPF Systems: Mechanisms of Bacterial Deposition

The comparison of the two systems is summarized in Table 3.3 with k_d and k_{RSPF} values. The enhanced deposition behavior of ST5383 at late exponential growth is attributed to the gained flagellar motion, allowing for the cells to access previously unavailable pore spaces and surface irregularities (Figure 3.5), and swim beyond the fluid streamlines towards to the quartz surface. This phenomenon has been reported previously, when the transport behavior of motile cells was compared to nonmotile cells in packed bed and RSPF systems (34, 72, 73). Additionally, under the laminar flow [$Re=7.96$ for RSPF (34), and $Re=0.19$ (110) for column] and diffusion dominating ($Pe=0.01$ for both systems) conditions of this study, cell motility is sufficient to allow for the upstream swimming of bacteria (111) and subsequent cell deposition at sites on the collector surface which would be otherwise inaccessible to non-motile cells due to the “shadow effect” as presented elsewhere (34, 72, 73). The deposition profiles (Figure 3.6) provide additional evidence to upstream swimming effect, as at the majority of ST5383 cells at late exponential growth phase were retained in higher sections of the column (Figure 3.6c) as compared to the mid exponential phase when the strain was nonmotile (Figure 3.6d). This trend was evident for 100mM, but not as pronounced at 10 mM (Figure 3.6a,b) likely due to the mass recovery of ST5383 being relatively low (42%) (Table 3.4). In the RSPF system, the deposition rates of dysfunctional flagellated SGSC1512g were lower than the functional flagellated ST5383g; however, greater than the nonflagellated SGSC2478g. In the column, SGSC1512’s retention was higher than

both SGSC2478 and ST5383, except for the 10mM condition with late exponential phase motile ST5383 cells. These trends can be explained by the presence of the flagella. The flagella, albeit dysfunctional, facilitate enhanced cell-surface interactions and eventually higher retention than nonflagellated strain. In addition, SGSC1512 had almost twice sugar and protein content, and percent hydrophobicity than ST5383 which could have facilitated the stronger initial deposition at the forward stagnation point of RSPF system compared to ST5383.

For both RSPF and column systems, SGSC2478 deposited less than flagellated strains. However, unlike ST5383 and SGSC1512, there appeared to be no link between the cell surface characteristics and observed lower deposition values of SGSC2478. DLVO theory (60) would suggest that there is no energy barrier to the nonflagellated SGSC2478 cell-surface interactions, as the zeta potential values were virtually neutral at these IS (Table 3.5). Similarly, the high sugar and protein content and corresponding high surface charge density would suggest a favorable condition for deposition; however, this was not the case with the observed BTCs (Figure 3.2). A possible explanation may include the fact the high surface charge density and EPS content may impart a relatively homogeneous distribution of charged functional groups across the cell surface (30). Greater surface charge heterogeneity has been linked to greater instability and subsequent deposition (31, 92, 93, 112-114), and perhaps under these conditions the SGSC2478 has been somewhat stabilized. The SGSC2478 deposition trends may also be due to this nonmotile strain experiencing simultaneous bacterial deposition and release differently than the nonflagellated strains (115-117).

Another potential mechanism to explain the deposition behavior would be the differences in cell hydrophobicity. At late exponential phase ST5383 became quite hydrophobic (~85% for both IS conditions, Table 3.1) as compared to the mid exponential phase cells; whereas the other two cell types remained hydrophilic. The hydrophobic ST5383 had the highest deposition rate of the three strains at late exponential phase. However, SGSC1512 remained hydrophilic regardless of growth phase similar to SGSC2478, and yet resulted in greater retention in the column. Hence, the deposition results for the three strains in both experimental systems are not attributed to this particular mechanism.

The attachment efficiencies for the column and RSPF systems are compared in Figure 3.7. Dashed line shown in Figure 3.7 represents a slope of unity, where experimental data would align if the mechanisms and extent of adhesion were identical in the column and RSPF. There is no substantial correlation between the two systems as both α_{Column} and α_{RSPF} points lie above the dashed line. It was hypothesized that this deviation was primarily due to the three dimensional pore structure (spherical and ellipsoidal sand particles) and surface irregularities in the packed bed column (Figure 3.5), potentially enhancing the deposition of flagellated strains. In addition, only cells that deposits in a primary energy minimum were enumerated for bacterial deposition in RSPF system whereas the column has the capacity to retain bacteria in both primary and secondary minima (30, 97).

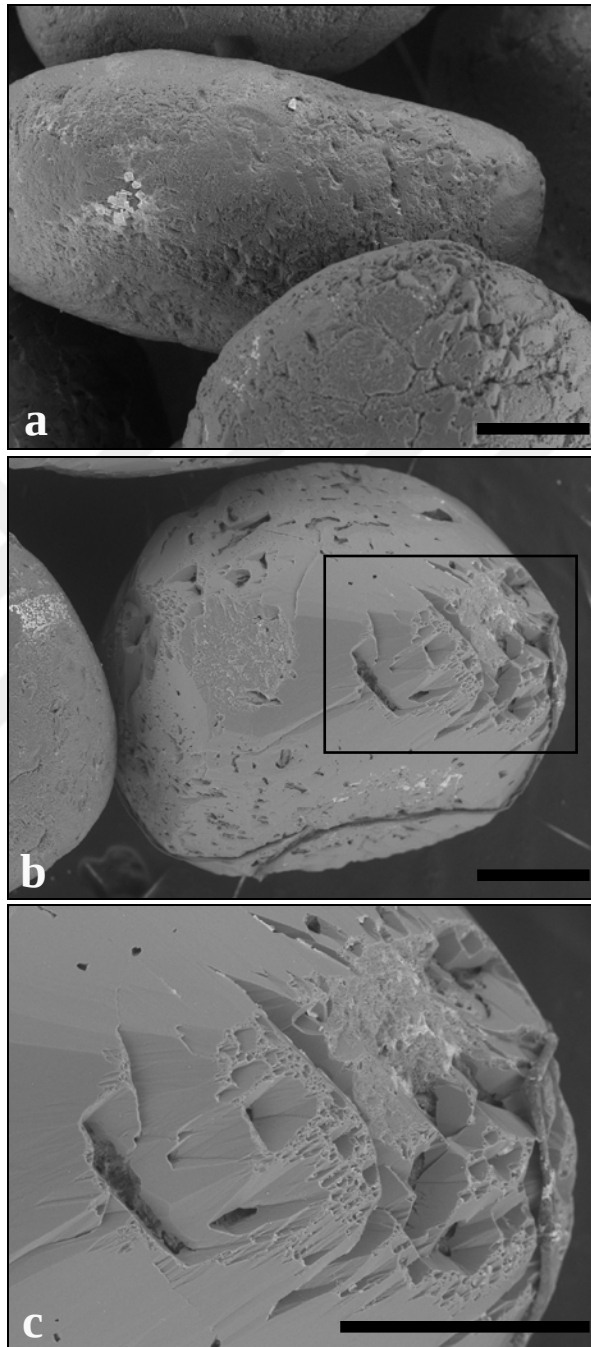


Figure 3.5. SEM images of quartz sand showing different shapes such as ellipsoidal (a) and spherical (b); and several surface irregularities. Image c shows a close up of the selected area in image b. Scale bars indicate the length of 200 μm .

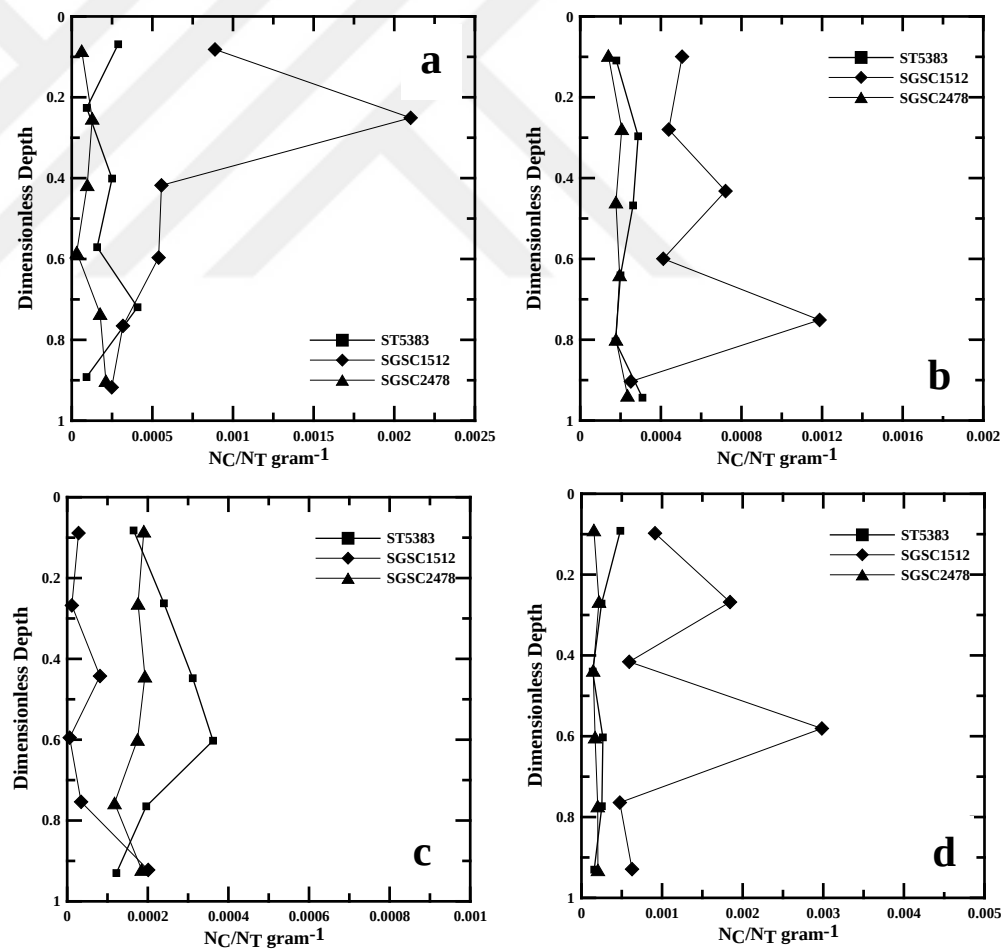


Figure 3.6. Deposition profiles of ST5383, SGSC1512, and SGSC2478 tested at 10 mM grown until midexponential phase (a), 10 mM grown until late exponential phase (b), 100 mM grown until mid exponential phase (c), and 100 mM grown until late exponential phase (d).

Table 3.4. The effluent (M_{eff}), retained (M_{sand}), and total (M_{total}) percentage of all *Salmonella* strains grown at mid- and late exponential phase recovered from column experiments. All values represent the relative percentage of the cells by number.

Strain	Growth Phase	IS (mM KCl)	Mass Balances (%)		
			M_{eff}	M_{sand}	M_{total}
ST5383	Mid-exp	10	81	5	86
		100	62	7	69
	Late-exp	10	36	6	42
		100	56	6	62
SGSC1512	Mid-exp	10	46	19	59
		100	48	31	79
	Late-exp	10	47	13	60
		100	48	1	49
SGSC2478	Mid-exp	10	92	3	95
		100	88	5	93
	Late-exp	10	81	5	86
		100	88	4	92

Table 3.5. Electrokinetic properties of bacteria and calculated DLVO interaction parameters^a in both packed bed column and RSPF systems.

Strain	IS	Growth phase	Packed bed ^b				RSPF ^c		
			Bacteria ζ (mV) ^d	Energy Barrier (kT)	2 ^o min depth (kT)	2 ^o min distance (nm)	Energy Barrier (kT)	2 ^o min depth (kT)	2 ^o min distance (nm)
ST5383	10	Mid-exp	-2.17	NB ^c	NS ^d	–	NB	NS	–
	100		-0.97	NB	NS	–	NB	NS	–
SGSC1512	10		-6.40	3.03	NS	–	NB	NS	–
	100		-4.13	NB	NS	–	NB	NS	–
SGSC2478	10		-2.45	NB	NS	–	NB	NS	–
	100		-1.64	NB	NS	–	NB	NS	–
ST5383	10	Late-exp	-4.04	NB	NS	–	NB	NS	–
	100		0.97	NB	NS	–	NB	NS	–
SGSC1512	10		-4.81	NB	NS	–	NB	NS	–
	100		-2.37	NB	NS	–	NB	NS	–
SGSC2478	10		-2.76	NB	NS	–	NB	NS	–
	100		1.89	NB	NS	–	NB	NS	–

^a Based on a Hamaker constant of 6.5×10^{-21} J.

^b Based on the previously measured value of quartz sand (74).

^c Based on the measured zeta potential value (+2.4 mV) of quartz slide.

^d No energy barrier.

^e No secondary energy minimum.

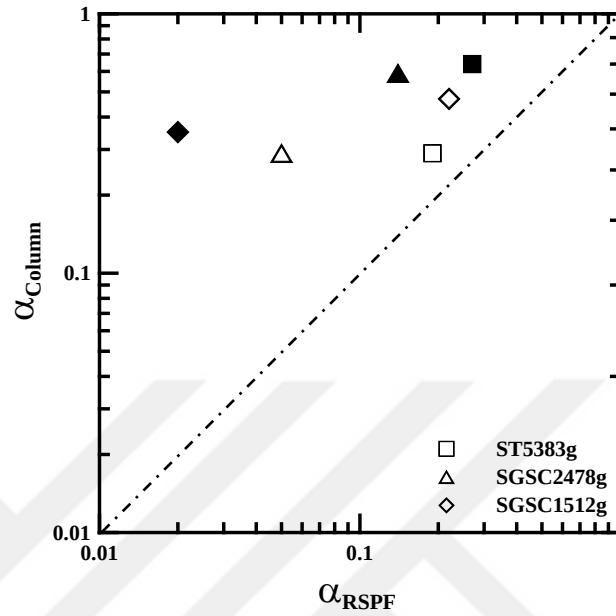


Figure 3.7. Correlation between attachment efficiencies of ST5383, SGSC2478, and SGSC1512 tested at 10mM, grown until mid exponential phase (open shapes); grown until late exponential phase (closed shape) in the packed bed column (α_{Column}), and RSPF (α_{RSPF}) systems. Dashed line indicates a perfect correlation.

Based on the k_d values obtained in the packed bed column system, travel distances were estimated (three log removal was assumed) and presented in Figure 3.8. As can be seen from the figure, nonmotile strain SGSC2478 can move the farthest distance (~23 m at 10 mM, mid exponential phase) compared to flagellated strains at all tested conditions. The functional flagellated motile strain ST5383 was the most effectively removed, with the shortest calculated transport distance (~2 m at 10 mM, at late exponential phase).

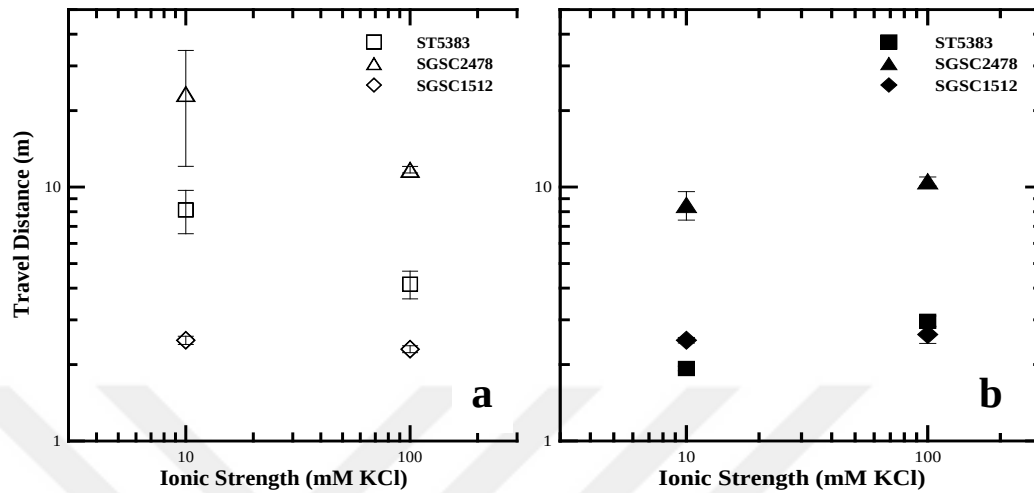


Figure 3.8. Calculated travel distances versus IS for ST5383, SGSC2478, and SGSC1512 grown until mid exponential phase (a); grown until late exponential phase (b). Error bars indicate average of three calculations.

3.4 Conclusions

Deposition behavior of motile and nonmotile strains of *Salmonella* was investigated in packed bed column and RSPF systems under the influence of flagella. Both experimental systems were designed to capture and predict the transport of these outbreak causing pathogens' in groundwater environments. Comprehensive cell surface characterization experiments showed that nonflagellated strain had more sugar and protein content than flagellated strains at mid exponential growth phase and vice versa in late exponential growth phase. Electrophoretic mobility calculations showed that all *Salmonella* strains' zeta potential values were close to neutral, resulting in no DLVO energy barrier predictions. In both systems, flagellated strains deposited more than nonflagellated strain, concluding that flagellar presence plays a major role in pathogen transport. Additionally, of the two flagellated strains, ST5383 was observed to gain flagellar motion only at late exponential phase. At this growth phase, ST5383's

deposition behavior was increased at both systems suggesting the bacteria use its flagella to swim towards the pore spaces, surface irregularities and quartz surfaces which were not accessible otherwise. Under favorable experiments where the role of flagellar motion was minimized, dysfunctional flagellated strain SGSC1512's deposition was highest in both systems.

This study shows that complex collector surface structures and bacterial appendages such as flagella may control the deposition kinetics and documents the first comparative transport behavior of motile and nonmotile *Salmonella enterica* serovars in column and RSPF systems.

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Chapter 4

Changes in Antibiotic Resistance and Invasiveness of *Salmonella* under Short Term Exposure to Artificial Groundwater

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Haznedaroglu, B. Z., Yates, M. V., and S. L. Walker. 2009.

Abstract

Energy-driven active efflux pumps are one of the major mechanisms of antibiotic resistance in bacteria. Environmental signals trigger the activation of these pumps that are energized by proton motive force that in turn excretes the drug out of the bacteria. This study investigates the effects of artificial groundwater (AGW) exposure, an ion-rich medium, on the antibiotic resistance profiles and invasiveness of three *Salmonella enterica* serovars with different morphologies. A wild type, flagellated *Salmonella typhimurium* outbreak strain; a mutant, dysfunctional flagellated (non-motile) *Salmonella typhimurium*; and a non-flagellated wild type avian disease causing *Salmonella pullorum* were exposed to 3.33, 10, and 30 mM ionic strength (IS) AGW for 6, 12, 18, and 24 hours. Stressed organisms' resistance against 10 common antibiotics were tested and compared to control groups that were not exposed to AGW. The invasiveness of AGW-exposed *Salmonella enterica* spp. was also tested by *in vitro* exposure to a human epithelial cell line (HEp2) and compared to control groups. The results show that *Salmonella typhimurium* serovars enhance their resistance to some of the antibiotic classes that are mediated with active efflux pumps. This trend was not observed for *Salmonella pullorum*, suggesting that the increased resistance found in AGW-exposed organisms might be strain-dependent. *In vitro* epithelial cell invasion assays showed that the number of bacteria that can invade HEp2 cells decreases with time [from 6, 12 to 18 hours (only at 3.33 and 10 mM IS)] and increases after 24 hours of AGW exposure. It is concluded that *Salmonella enterica* spp. reaching groundwater environments may exhibit

increased invasiveness against human or animal targets and develop antibiotic resistance by exposure to the ion-rich matrix of groundwater systems.



4.1 Introduction

Salmonella is a serious pathogen threatening both human and animal health at the global level. Non-typhoidal *Salmonella* infections in the U.S. are on the rise (1); with a recent outbreak caused by *Salmonella typhimurium* affecting 714 individuals in 46 states (2). Antibiotic resistance, including multidrug resistance, creates problems in clinically treating enteric fever and bacteremia caused by this infectious bacterium (3). Emergence of antibiotic resistance is inevitable due to selective pressure created by the use of specific antibacterial chemotherapy (3). According to Centers for Disease Control and Prevention (CDC), the proportion of *Salmonella* infections caused by antimicrobial-resistant strains has increased around the world and represents 20 to 40% of all isolates from human infections (4). The most recent report by National Antimicrobial Resistance Monitoring System (NARMS) mentions that 19.4% of 2052 non-typhoidal *Salmonella* isolates received by CDC showed resistance to at least one Clinical and Laboratory Standards Institute (CLSI) antibiotic subclass, and 7.6% of the isolates showed resistance to more than 5 antibiotic subclasses (5).

Antibiotic resistance is a complex mechanism successfully achieved in pathogenic bacteria by dynamic state of resistance that constantly being spread as more antibiotics are used that develop resistant populations among commensal bacteria found in humans and animals (6). *Salmonella enterica* is of particular concern with the increasing occurrence of strains with resistance to key antimicrobial chemicals (7). The mechanisms of resistance in *Salmonella* against antimicrobial chemicals are usually studied based on the antimicrobial drug groups such as aminoglycosides, β -lactams,

chloramphenicol, quinolones, tetracyclines, and sulfonamides (46). Primary mechanisms for bacterial resistance to aminoglycosides are drug modification, decreased uptake, and modification of the ribosomal protein target of the drug (8). The most common resistance mechanism against β -lactams is the secretion and release of β -lactamases, which results in limited or no antimicrobial activity (46). *Salmonella enterica* spp. have genes that encode for chloramphenicol O-acetyl transferase (9-11), which enzymatically inactivates the antibiotic and gains resistance. In terms of resistance to quinolones, *Salmonella* has developed two mechanisms mostly due to 1) mutations in the quinolone resistance determining regions of *gyrA* and *gyrB*, and *parC* subunit of topoisomerase IV (12-14) mutations in the genes encoding regulators of AcrAB-TolC efflux system resulting in the overexpression (15, 16). Resistance to tetracyclines in *Salmonella* is mainly due to the production of an energy-dependent efflux pump that removes the drug from the cell and enzymatic inactivation of tetracycline (46). The resistance against the sulfanamide group of antibiotics in *Salmonella* has been attributed to the presence of an extra *sul* gene, which expresses an insensitive form of dihydropteroate synthetase, an enzyme involved in the synthesis of tetrahydrofolic acid (17). A wide variety of *Salmonella* isolates are reported to carry *sul1* and *sul2* genes (46).

As mentioned briefly, the impact of bacterial efflux pumps on antimicrobial resistance is large, especially against aminoglycosides (18), tetracyclines (19) and quinolones (20). Working mechanisms of these bacterial efflux pumps are directly and/or indirectly linked to the ionic components in the external environment. For example, the insertion of aminoglycosides into bacterial LPS (18) follows a proton

gradient (energy) dependent transport across the cytoplasmic membrane. Additionally, a tetracycline efflux pump system is induced under the presence of Mg^{2+} in the external environment (21-23), which results in the excretion of the antibiotic in an energy-dependent manner with proton exchange for a tetracycline-cation complex (24). The majority of efflux pumps work in this manner, *i.e.*, compound-ion antiporter, and are reviewed in detail elsewhere (25).

Efflux pump systems in *Salmonella* also assist the bacteria in resistance to antibiotics such as ciprofloxacin (26), β -lactams (27), as well as creating simultaneous resistance to multiple drugs (28-30). Resistance to β -lactams in *Salmonella* is mediated by the AcrAB efflux system and AcrB is also required for virulence of *Salmonella typhimurium* (31), suggesting that drug transporters may contribute not only to antibiotic resistance but also to virulence (32). This has been observed for the *macAB* drug efflux system gene which harbors a binding site for the response regulator PhoP (32). The PhoP/PhoQ two-component system is also a major regulator of *Salmonella* virulence (33). Additional studies showing the enhanced virulence of *Salmonella* by increased invasion to HeLa cell lines in vitro with increased Ca^{2+} concentration exist (34, 35).

One of the exposure routes of pathogenic bacteria excreted from infected individuals occurs via groundwater (36). Pathogens released from various point and non-point sources of human waste may eventually reach groundwater and create health related issues in individuals who are exposed to the groundwater (37). Approximately 26% of total freshwater demand in the U.S. is supplied through groundwater (38). Therefore, it is

essential to understand if groundwater environments act as a matrix where pathogens may develop resistance to certain antibiotics and/or gain virulence.

This study aims to investigate whether the antibiotic resistance profile and virulence features of *Salmonella enterica* spp. are affected when exposed to ion-rich groundwater conditions in the external environment. Therefore, an outbreak-causing wild type *Salmonella typhimurium*, a non-motile mutant phenotype of *Salmonella typhimurium*, and *Salmonella pullorum*, an important avian outbreak-causing strain, are selected as model organisms. These particular strains are chosen due to their different genotypic and phenotypic variation and their low level susceptibility to tested antibiotics as control groups to monitor any suspected changes in their profiles.

4.2 Materials and Methods

4.2.1 Bacterial Strain Selection and Preparation

All *Salmonella* strains used in this study were obtained from the Salmonella Genetic Stock Centre (SGSC) of University of Calgary, Alberta, Canada. *S. typhimurium* designated as strain ST5383 [Laboratory Center for Disease Control (LCDC) Classification number 84-5383], is a wild type strain and originally isolated from an interprovincial outbreak infecting more than 1700 persons (39). The strain designated as SGSC1512 is a non-motile mutant of *S. typhimurium* TM2 (40). The non-motile, non-flagellated avian pathogenic *S. pullorum* is designated as strain SA1685 (CDC number 2863-65) and was originally isolated from an infected turkey.

Salmonella used during the course of study was pre-cultured in Luria-Bertani (LB) broth, (Fisher Scientific, Fair Lawn, NJ) at 37°C overnight, and shaken continuously at 120 rpm. On the day of the experiment, overnight grown cultures were inoculated into fresh LB broth in 1:100 dilutions and harvested at the mid-exponential phase determined by growth curve analysis for each bacterium. A refrigerated bench-top centrifuge (5804R; Eppendorf, Hamburg, Germany) equipped with fixed angle rotor (F-34-6-38; Eppendorf) was used to pellet the cells with an applied $3,700\times g$ force for 15 min at 4°C. Growth medium was decanted and the pellet was resuspended in 10 mM KCl solution. The process was repeated twice in order to ensure complete removal of the growth medium. Electrolyte solutions used in cell preparation and other experiments were prepared with de-ionized water (DIW) (Millipore, Billerica, MA) and reagent-grade KCl (Fisher Scientific) with no pH adjustment (pH 5.6–5.8). Concentration of cell stock solution was determined by using a cell counting hemocytometer (Bürker-Turk, Germany) under light microscope (Fisher Scientific).

4.2.2 Application of Stress Conditions

Bacteria cultured and harvested as described above were exposed to stress conditions as defined by suspension at room temperature in artificial groundwater (AGW) for 6, 12, 18, and 24 hours. AGW is prepared with a slight modification to a previously used recipe (41) by dissolving following salts in 1 L of sterile DIW: $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ (36mg), $\text{CaSO}_4\cdot 2\text{H}_2\text{O}$ (25mg), KNO_3 (20mg), NaHCO_3 (36mg), $\text{Ca}(\text{NO}_3)_2\cdot 4\text{H}_2\text{O}$ (35mg), and $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ (60mg). The pH of solutions kept constant at 7.0 ± 0.2 . The ionic strength (IS) of solutions tested were 3.33 mM, 10 mM and 30 mM. Bacteria suspensions of 10^8

cells/mL were exposed to the aforementioned IS conditions in 100 mL tissue culture flasks with 0.2 μ m vented caps (Corning), placed on orbital shakers and mildly shaken (70 rpm) for the desired time period. Bacterial isolates not exposed to stress conditions (*i.e.*, characterized after harvesting) are referred as the control group.

At the end of stress exposure to AGW, viability of the cells was determined by using the Live/Dead BacLight® kit (L-7012; Molecular Probes, Eugene, OR) according to the manufacturer's directions. Direct counting of the stained live and dead cells was done using an inverted microscope (IX70; Olympus, Japan) operated under red/green fluorescence filter set (Chroma Technology Corp., Brattleboro, VT).

4.2.3 Antibiotic Susceptibility Analysis

Antibiotic susceptibility tests were employed to assay *Salmonella* isolates' resistance to 10 different antimicrobial chemicals commonly used by U.S. NARMS. Tests were performed in compliance with the CLSI / National Committee for Clinical Laboratory Standards (NCCLS) (42) for the following antimicrobial agents (numbers in parentheses denote the amount of antimicrobial chemical impregnated in 6 mm disks): amikacin (30 μ g), amoxicillin-clavulanic acid (30 μ g) cefoxitin (30 μ g), ceftriaxone (30 μ g), cephalothin (30 μ g), chloramphenicol (30 μ g), ciprofloxacin (5 μ g), nalidixic acid (30 μ g), tetracycline (30 μ g), trimethoprim-sulfamethoxazole (25 μ g).

4.2.4 Epithelial Cell Culture and Invasion Assays

The human epithelial cell line HEp-2 was obtained from American Type Culture Collection (ATCC) (CCL-23; ATCC, Manassas, VA) and was grown in Eagle's Minimum Essential Medium (EMEM) (ATCC) supplemented with 10% fetal bovine

serum (ATCC), and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO). Epithelial cells were incubated at 37 °C under 5% CO₂.

Invasion of *Salmonella* into epithelial cells was quantified with slight modifications to protocol as described elsewhere (43). Briefly, a monolayer of HEp-2 cells was grown until confluence in 24-well plates (Corning, Corning, NY). *Salmonella*, at a concentration 10⁵ cells/mL, was inoculated into cell-culture plates and incubated for 2 hours at 37 °C to allow for internalization. Following the incubation, each well was washed three times with phosphate buffered saline (PBS) to remove unbound bacteria. Bacteria that were bound to epithelial cells but had not been internalized were killed by applying fresh growth medium containing 5 µg penicillin/mL, and 100 µg gentamicin/mL, incubated for 2 hours at 37 °C. Following incubation, cells were washed with PBS, treated with trypsin-EDTA complex (ATCC), and lysed with 0.1 % Triton-X100 (Fisher Scientific). The lysates were spread onto LB agar plates and incubated for 18 hours at 37 °C. The number of colony forming units (CFU) was counted to quantify number of *Salmonella* that had successfully invaded the monolayer of epithelial cells.

4.2.5 Statistical Analysis of Data

Changes in the diameters of antibiotic inhibition zones and the number of CFUs of *Salmonella* infecting HEp2 cells were statistically compared using unpaired Student's t-test using Minitab[®] Version 14 (State College, PA). Differences between control and stressed *Salmonella* isolates were considered to be significant at 95% confidence interval ($P < 0.05$).

4.2.6 Light Microscopy Imaging of Epithelial Cells

Images of both uninfected and *Salmonella*-invaded HEp2 cells were obtained by using a light microscope (Micromaster, Fisher Scientific). Giemsa stain (Fisher Scientific), which differentially stains human and bacterial cells purple and pink respectively, was used. Chambered cover glass slides (Nunc Lab-Tek, Fisher Scientific) were used to grow HEp2 cells until desired confluence and infected with *Salmonella* as described in previous sections. After the chambered cover glasses were manually removed, the slides were placed in Giemsa stain (2.5%) for 45-60 minutes. Slides were rinsed by dipping in a buffer solution [0.59% (w/v) Na_2HPO_4 , 0.36% (w/v) $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$] three-four times and left for air drying. Stained slides were observed under light microscope and images were taken at 400 $\times$ magnification.

4.3 Results

4.3.1 Antibiotic Resistance Analysis

As presented in Table 4.1, the non-motile mutant SGSC1512 control group was susceptible to all antibiotics except cephalothin and nalidixic acid, for which the isolates showed intermediate level resistance. SGSC1512 exposed to AGW for 6, 12, 18 and 24 hours did not change in resistance to antibiotics except for cephalothin to which the isolates became susceptible after exposure periods of 18 and 24 hours at all IS. After 12 hours of exposure to AGW, the isolates became susceptible at 3.33 and 10 mM IS, with no change in intermediate resistance at 30 mM IS. For 6 hours of exposure, SGSC1512

showed decreased susceptibility to cephalothin at 3.33 mM IS, whereas at 10 and 30 mM IS the isolates remained intermediate resistant.

Inhibition zone diameter measurements showed significant reductions in inhibition by nalidixic acid for SGSC1512 isolates exposed to AGW for 12 and 18 hours at all IS and 6 hours at 3.33 mM. Similar reductions in the presence of chloramphenicol and tetracycline were observed after 12 hours at all IS and after 6 and 18 hours at 3.33 mM, respectively as compared to the control group (Table 4.1). The wild type *Salmonella typhimurium* strain ST5383 control group showed full susceptibility to all antibiotics except amoxicillin-clavulanic acid and nalidixic acid, for which only intermediate resistance was observed (Table 4.2). Exposure to AGW at all conditions increased the resistance of ST5383 against nalidixic acid except for 12 hours at 30 mM IS. ST5383's intermediate resistance to amoxicillin-clavulanic acid decreased to the susceptible level when it was exposed to AGW for 24 hours at all IS and after 18 hours at 10 mM IS. The only significant change in inhibition zone diameters were observed for nalidixic acid at 12 hours exposure at 3.33 mM IS and 18 hours exposure at 10 mM IS as reduction in diameters (Table 4.2).

The wild type, non-motile *Salmonella pullorum* strain SA1685 did not show any changes in antibiotic resistance and remained susceptible to all antibiotics tested for all experimental conditions as shown in Table 3. Although there were significant changes in inhibition zone diameters at a few tested AGW exposure condition, the susceptibility profile of SA1685 remained the same (Table 4.3).

Table 4.1. Measured diameter values (in mm) of antibiotic inhibition zones of SGSC1512 exposed to different IS (in mM) AGW for given time periods.

Antibiotic ^a	Control (mm)	6 Hours			12 Hours			18 Hours			24 Hours		
		3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM
AMK30 ^b	19.91±3.83(S) ^c	22.94±0.73(S)	22.00±0.95(S)	21.11±0.98(S)	22.68±0.77(S)	23.18±0.53(S)	23.29±0.52(S)	22.86±0.27(S)	23.15±0.46(S)	24.27±0.78(S)	22.42±0.71(S)	22.23±0.93(S)	22.73±0.98(S)
AMC30	20.94±6.67(S)	20.60±7.05(S)	22.16±7.59(S)	20.70±7.93(S)	21.40±6.76(S)	20.81±7.37(S)	20.13±6.54(S)	21.97±5.63(S)	21.35±6.15(S)	22.95±7.43(S)	20.79±6.40(S)	21.31±6.69(S)	20.06±6.04(S)
FOX30	23.17±1.11(S)	22.43±0.68(S)	23.58±0.61(S)	22.68±1.18(S)	23.83±1.08(S)	24.04±1.35(S)	22.71±1.22(S)	23.25±0.34(S)	23.05±1.14(S)	24.20±2.14(S)	22.97±1.22(S)	23.17±1.29(S)	23.76±1.82(S)
CRO30	29.15±5.55(S)	26.32±0.32(S)	28.14±2.45(S)	28.24±1.73(S)	29.13±1.61(S)	29.51±2.51(S)	27.88±0.72(S)	27.96±0.94(S)	29.21±0.51(S)	30.47±1.35(S)	29.03±1.28(S)	28.72±0.49(S)	30.08±2.45(S)
KF30	16.71±5.70(I)	18.79±8.03(S)	17.95±7.54(I)	17.92±7.11(I)	18.07±7.37(S)	20.55±8.78(S)	17.90±6.42(I)	18.56±6.36(S)	18.76±6.38(S)	18.12±6.90(S)	18.37±5.69(S)	18.32±6.57(S)	18.82±5.38(S)
C30	29.34±1.71(S)	25.92±3.40(S)	28.85±1.40(S)	27.29±0.98(S)	26.20±0.40(S)	26.03±0.99(S)	26.43±0.25(S)	26.98±1.02(S)	28.40±1.29(S)	28.08±1.03(S)	27.21±2.81(S)	26.88±2.70(S)	26.97±1.01(S)
CIP5	36.31±4.06(S)	38.15±1.45(S)	38.39±0.80(S)	38.87±2.14(S)	37.65±0.77(S)	39.36±1.87(S)	37.33±1.38(S)	38.42±1.29(S)	39.00±0.28(S)	38.58±0.28(S)	38.51±0.46(S)	39.43±1.40(S)	37.42±0.80(S)
NA30	18.20±0.76(I)	16.10±0.68(I)^d	16.58±0.68(I)	18.20±0.75(I)	16.32±0.65(I)	16.33±0.82(I)	16.32±0.66(I)	16.20±0.23(I)	15.87±0.73(I)	15.57±0.93(I)	17.20±1.83(I)	16.62±1.2(I)	16.24±0.97(I)
TE30	23.78±0.58(S)	22.75±0.09(S)	22.33±0.94(S)	22.82±1.72(S)	23.03±1.50(S)	24.04±1.05(S)	21.53±1.68(S)	22.73±0.25(S)	22.45±0.73(S)	22.88±0.86(S)	21.77±1.75(S)	24.01±2.83(S)	22.15±2.88(S)
SXT25	28.03±0.99(S)	27.57±0.40(S)	28.27±1.76(S)	26.29±0.96(S)	28.23±3.07(S)	28.35±2.29(S)	27.62±2.22(S)	26.97±2.20(S)	27.29±3.05(S)	27.63±2.68(S)	27.93±1.84(S)	25.95±1.12(S)	26.63±1.63(S)

^a Antibiotic abbreviations are as follows: Amikacin: (AMK); Amoxicillin-clavulanic acid: (AMC); Cefoxitin: (FOX); Ceftriaxone: (CRO); Cephalothin: KF 30; Chloramphenicol: (C); Ciprofloxacin: (CIP); Nalidixic Acid: (NA); Tetracycline: (TE); Trimethoprim-sulfamethoxazole: (SXT).

^b Numbers denote the concentration (in µg) of the given antibiotic impregnated on 6mm disks.

^c Letter in parantheses denote the degree of resistance as follows: Susceptible (S); Intermediate Susceptible (I); Resistant (R).

^d Numbers in bold shows the changes in the inhibition zone diameter is statistically significant compared to the control group.

Table 4.1. Measured diameter values (in mm) of antibiotic inhibition zones of ST5383 exposed to different IS (in mM) AGW for given time periods.

Antibiotic ^a	Control (mm)	6 Hours			12 Hours			18 Hours			24 Hours		
		3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM
AMK30 ^b	21.48±0.63(S) ^c	21.24±0.39(S)	21.04±0.19(S)	20.67±0.72(S)	20.53±0.98(S)	20.70±0.23(S)	20.93±0.40(S)	20.65±0.74(S)	22.13±1.82(S)	22.01±1.01(S)	23.25±2.30(S)	21.36±0.46(S)	20.94±0.70(S)
AMC30	17.39±4.06(I)	17.38±4.00(I)	17.53±4.23(I)	17.84±3.59(I)	16.72±3.58(I)	17.76±4.68(I)	16.85±3.96(I)	16.96±2.90(I)	18.53±4.13(S)	16.69±3.07(I)	18.16±2.54(S)	18.46±2.56(S)	19.52±3.50(S)
FOX30	22.47±0.86(S)	20.63±1.35(S)	21.28±0.28(S)	21.37±0.71(S)	21.56±1.30(S)	22.51±1.51(S)	22.71±2.43(S)	21.81±0.26(S)	20.85±1.36(S)	21.95±0.82(S)	23.07±2.08(S)	23.74±2.25(S)	21.72±1.03(S)
CRO30	25.88±2.19(S)	27.13±1.23(S)	26.40±2.39(S)	28.52±1.80(S)	27.03±1.44(S)	28.96±0.34(S)	27.26±0.67(S)	26.53±0.22(S)	27.46±0.75(S)	26.79±1.05(S)	27.83±0.62(S)	27.15±1.12(S)	28.14±0.88(S)
KF30	15.99±5.15(I)	16.46±4.75(I)	15.96±4.60(I)	15.47±4.16(I)	15.34±4.05(I)	15.57±3.87(I)	15.75±5.04(I)	15.71±3.65(I)	17.53±4.51(I)	16.85±4.30(I)	16.25±3.59(I)	17.26±4.56(I)	16.64±1.95(I)
C30	26.18±2.13(S)	25.17±3.47(S)	25.33±1.20(S)	25.82±3.19(S)	25.85±1.43(S)	25.61±0.91(S)	26.06±1.85(S)	25.77±1.79(S)	26.92±1.06(S)	26.67±0.43(S)	27.71±0.57(S)	26.54±0.57(S)	27.33±0.25(S)
CIP5	33.73±1.18(S)	34.38±0.63(S)	34.63±0.22(S)	32.71±0.75(S)	33.83±1.10(S)	35.19±1.38(S)	33.05±1.93(S)	33.00±1.28(S)	33.73±1.64(S)	33.75±0.89(S)	34.19±1.28(S)	34.20±1.02(S)	34.41±0.88(S)
NA30	19.69±0.33(S)	18.53±1.19(I)	18.71±0.72(I)	18.00±1.41(I)	17.74±0.81(I)^d	18.50±1.15(I)	21.54±4.54(S)	18.43±0.91(I)	18.17±0.49(I)	18.19±1.03(I)	18.74±0.90(I)	18.81±1.47(I)	18.93±1.98(I)
TE30	21.38±1.38(S)	21.61±0.69(S)	21.91±0.73(S)	20.81±1.01(S)	20.22±1.47(S)	19.87±0.78(S)	20.85±0.65(S)	20.59±0.75(S)	21.45±1.35(S)	20.64±0.09(S)	22.11±0.96(S)	21.67±0.94(S)	21.24±0.29(S)
SXT25	28.13±1.81(S)	26.88±3.64(S)	28.52±1.32(S)	27.51±3.16(S)	28.08±4.12(S)	28.81±1.50(S)	27.61±2.92(S)	28.02±3.57(S)	28.58±3.90(S)	28.92±2.16(S)	27.51±1.04(S)	28.79±1.77(S)	30.45±2.33(S)

^a Antibiotic abbreviations are as follows: Amikacin: (AMK); Amoxicillin-clavulanic acid: (AMC); Cefoxitin: (FOX); Ceftriaxone: (CRO); Cephalothin: KF 30; Chloramphenicol: (C); Ciprofloxacin: (CIP); Nalidixic Acid: (NA); Tetracycline: (TE); Trimethoprim-sulfamethoxazole: (SXT).

^b Numbers denote the concentration (in µg) of the given antibiotic impregnated on 6mm disks.

^c Letter in parantheses denote the degree of resistance as follows: Susceptible (S); Intermediate Susceptible (I); Resistant (R).

^d Numbers in bold shows the changes in the inhibition zone diameter is statistically significant compared to the control group.

Table 4.1. Measured diameter values (in mm) of antibiotic inhibition zones of SA1685 exposed to different IS (in mM) AGW for given time periods.

Antibiotic ^a	6 Hours				12 Hours			18 Hours			24 Hours		
	Control (mm)	3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM
AMK30 ^b	24.50±2.07(S) ^c	26.57±1.75(S)	25.75±0.81(S)	24.55±1.23(S)	25.09±1.02(S)	24.15±0.30(S)	23.82±1.12(S)	24.57±1.90(S)	25.06±0.48(S)	23.27±1.22(S)	25.42±2.19(S)	23.12±3.24(S)	22.81±2.71(S)
AMC30	30.73±3.18(S)	28.05±2.36(S)	28.03±0.81(S)	26.13±1.80(S)	28.69±1.05(S)	27.68±2.41(S)	26.77±2.37(S)	28.14±0.86(S)	25.75±1.91(S)	25.57±1.22(S)	28.19±4.11(S)	26.13±2.60(S)	24.72±1.33(S)
FOX30	23.91±1.77(S)	24.57±1.85(S)	24.72±1.39(S)	23.58±2.61(S)	25.72±0.22(S)	25.17±0.72(S)	24.19±0.40(S)	25.81±0.44(S)	26.35±1.21(S)	24.27±3.32(S)	26.07±2.67(S)	24.09±1.35(S)	24.99±0.50(S)
CRO30	28.30±3.42(S)	29.44±3.33(S)	28.65±2.53(S)	28.06±1.96(S)	31.04±1.86(S)	30.36±1.36(S)	28.85±1.12(S)	29.13±3.40(S)	29.99±2.61(S)	26.43±0.81(S)	30.11±1.53(S)	27.61±2.34(S)	26.20±2.01(S)
KF30	21.27±1.13(S)	23.32±1.54(S)	21.72±1.57(S)	21.35±2.36(S)	24.84±1.28(S)	22.65±0.24(S)	20.61±0.58(S)	23.84±1.17(S)	23.43±2.77(S)	21.08±2.00(S)	24.86±3.28(S)	22.62±3.43(S)	20.58±0.96(S)
C30	28.67±2.86(S)	28.56±0.54(S)	27.78±2.37(S)	25.17±1.49(S)	29.07±2.99(S)	28.24±0.35(S)	26.19±0.38(S)	28.39±0.79(S)	28.69±1.36(S)	28.76±1.97(S)	30.98±1.86(S)	28.32±2.97(S)	26.49±2.90(S)
CIP5	36.45±1.75(S)	34.91±1.87(S)	32.41±1.55(S)	31.47±2.17(S)	33.81±3.31(S)	33.00±3.80(S)	31.23±1.53(S)	36.01±2.75(S)	36.73±2.22(S)	28.61±4.04(S)	34.81±2.09(S)	35.66±4.00(S)	32.85±3.77(S)
NA30	22.91±0.65(S)	24.18±0.97(S)	21.85±1.05(S)	22.22±1.24(S)	23.71±1.57(S)	23.89±1.09(S)	24.04±0.90(S)	24.41±1.69(S)	23.56±1.69(S)	22.93±0.60(S)	25.48±4.15(S)	23.59±2.01(S)	23.99±0.69(S)
TE30	29.25±1.58(S)	27.56±4.10(S)	25.95±1.58(S)	23.87±0.23(S)	29.32±1.84(S)	26.15±0.46(S)	25.99±0.97(S)	28.97±2.11(S)	27.53±3.01(S)	25.69±4.14(S)	28.45±3.33(S)	26.86±4.26(S)	25.45±3.65(S)
SXT25	27.61±0.30(S)	29.30±0.77(S)^d	28.35±1.45(S)	28.16±1.61(S)	29.85±1.50(S)	29.72±2.86(S)	29.46±2.15(S)	29.08±3.30(S)	27.59±3.05(S)	27.71±1.10(S)	30.06±3.37(S)	29.17±3.19(S)	26.41±2.79(S)

^a Antibiotic abbreviations are as follows: Amikacin: (AMK); Amoxicillin-clavulanic acid: (AMC); Cefoxitin: (FOX); Ceftriaxone: (CRO); Cephalothin: KF 30; Chloramphenicol: (C); Ciprofloxacin: (CIP); Nalidixic Acid: (NA); Tetracycline: (TE); Trimethoprim-sulfamethoxazole: (SXT).

^b Numbers denote the concentration (in µg) of the given antibiotic impregnated on 6mm disks.

^c Letter in parantheses denote the degree of resistance as follows: Susceptible (S); Intermediate Susceptible (I); Resistant (R).

^d Numbers in bold shows the changes in the inhibition zone diameter is statistically significant compared to the control group.

Table 4.4. Susceptibility breakpoints, classification, and function of antibiotics tested in this study.

Susceptibility Breakpoints (mm)					
Antibiotic	Susceptible	Intermediate	Resistant	Class	Function
Amikacin (AMK30)	≥ 17	15-16	≤ 14	Aminoglycoside	Binds to the bacterial 30S ribosomal subunit, causes misreading of mRNA and inhibition of protein synthesis
Amoxicillin-clavulanic acid (AMC30)	≥ 18	14-17	≤ 13	β -lactam	β -lactamase inhibitor
Cefoxitin (FOX30)	≥ 18	15-17	≤ 14	β -lactam	Interferes with cell wall synthesis
Ceftriaxone (CRO30)	≥ 21	14-20	≤ 13	β -lactam	Inhibition cell wall synthesis
Cephalothin (KF30)	≥ 18	15-17	≤ 14	β -lactam	β -lactamase inhibitor
Chloramphenicol (C30)	≥ 18	13-17	≤ 12		Binds to the bacterial 50S ribosome
Ciprofloxacin (CIP5)	≥ 21	16-20	≤ 15	Quinolone	Inhibits DNA gyrase
Nalidixic acid (NA30)	≥ 19	14-18	≤ 13	Quinolone	Inhibits DNA replication & transcription
Tetracycline (TE)	≥ 19	15-18	≤ 14	Tetracycline	Inhibits 30S ribosome
Trimethoprim-sulfamethoxazole (SXT25)	≥ 16	11-15	≤ 10	Sulfonamide	Folate inhibitor

4.3.2 Epithelial Cell Invasion Assay

As presented in Table 4.5, the number of CFUs for control groups invading HEp2 cells ranged from approximately 300 to 420. After exposure to AGW for 6 and 12 hours at all IS tested, the number of bacteria invading the epithelial cells lines decreased significantly. The only exception was a single condition for ST5383, *i.e.*, 10 mM AGW after 6 hours exposure. The same decreasing trend was also observed after 18 hours of exposure to 3.33 mM AGW for all strains. Interestingly, longer exposure periods to AGW, *i.e.*, 24 hours, was associated with an increase in the number of bacteria invading the HEp2 cells at all IS, with the exception of 3.33 mM for SGSC1512 and ST5383 only. The invasiveness of SA1685 exposed to all IS for 24 hours increased significantly compared to the control group (Table 4.5).

4.3.3 Viability of *Salmonella* under Stress

Table 4.6 presents the percentage of viable cells of both the control and AGW-exposed *Salmonella enterica* spp. at 3.33, 10, and 30 mM IS. As can be seen in the table, the control groups' viability values were determined to be over 90%. Throughout the spectrum of exposure conditions, the lowest viability values were approximately 85%, 83%, and 86% for SGSC1512, ST5383, and SA1685 respectively.

Table 4.5. Number of *Salmonella* CFUs infecting HEp2 cells exposed to different IS (in mM) AGW for given time periods and compared to control group.

Strain	Control	6 Hours			12 Hours			18 Hours			24 Hours		
		3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM
SGSC1512	371±17	104±15^a	72±9	137±16	83±9	98±12	166±22	256±28	359±36	447±26	267±14	464±40	490±17
ST5383	420±13	200±31	326±84	297±13	158±29	189±20	159±14	203±16	447±37	553±35	217±30	521±23	564±35
SA1685	298±21	173±25	182±36	189±12	212±33	199±27	221±13	276±12	248±21	236±15	376±22	348±12	357±19

^a Numbers in bold indicates the changes in the CFU numbers are statistically significant compared to the control group.

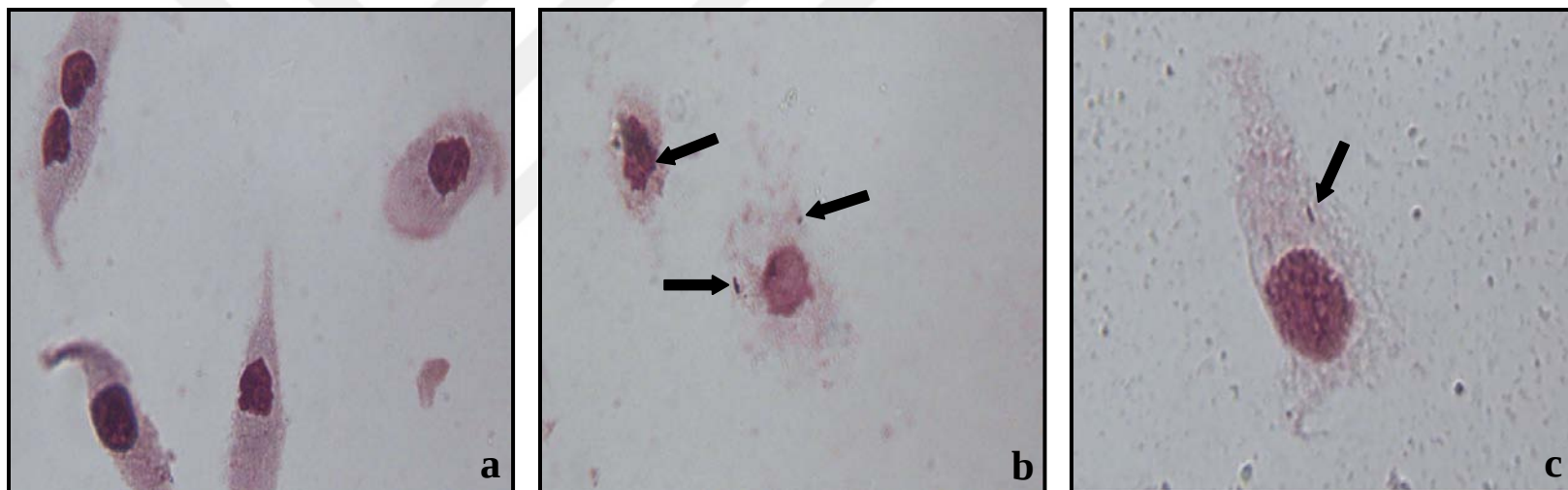


Figure 4.1. Representative images of HEp2 cells under light microscope (400×). Uninfected cells (a); HEp2 cells infected with SGSC1512 (b,c) pointed with arrow.

Table 4.6. Percent viability of *Salmonella* exposed to AGW with respect to time and IS.

Strain	Control	6 Hours			12 Hours			18 Hours			24 Hours		
		3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM	3.33 mM	10 mM	30 mM
SGSC1512	98.34±4.0	97.04±3.4	96.71±2.8	93.17±1.3	96.25±3.4	94.27±5.2	93.81±3.7	94.67±1.4	92.39±3.7	91.64±4.5	93.81±5.4	86.11±3.8	85.70±4.4
ST5383	89.08±4.9	85.39±7.5	89.02±7.5	85.2±8.59	84.25±3.2	91.89±2.9	87.62±3.9	83.26±2.9	89.25±3.7	88.90±2.1	83.42±4.4	87.21±2.8	89.75±1.4
SA1685	92.16±4.9	89.13±4.0	90.90±1.6	94.47±1.1	90.30±3.5	89.46±2.5	91.23±4.7	88.45±4.7	88.97±3.9	87.63±2.9	87.56±1.2	88.25±3.0	86.32±4.3

4.4 Discussion

In this study, the changes in antibiotic resistance and virulence of *Salmonella enterica* spp. exposed to environments simulating groundwater was investigated to determine whether the ion-rich matrix of groundwater may impose energy-driven mechanisms of antimicrobial resistance and invasiveness to target cell lines. The results have shown strain-dependent, efflux pump-mediated antibiotic resistance and increased virulence (at longer durations) by exposing *Salmonella enterica* spp. to AGW.

The first organism tested was a mutant *Salmonella typhimurium* strain (SGSC1512) with dysfunctional flagella, *i.e.*, a non-motile strain. During exposure to AGW, SGSC1512's resistance to cephalothin changed from intermediate resistance level to susceptible level for the most part (with the exception of 3.33 mM IS exposure after 6 hours) although the changes in inhibition zone diameters were not statistically significant. Yet, cephalothin is a member of the β -lactam class antibiotics, the trend was not observed for the other β -lactam antibiotics. The influence of efflux pumps on resistance to β -lactams class antibiotics is documented in literature (27, 31), however this was not confirmed in this study. Interestingly, the inhibition zone diameters of SGSC1512's control group decreased significantly when the bacterium was tested against nalidixic acid, at 12 hours and 18 hours of exposure to all IS conditions plus 3.33 mM exposure for 6 hours. This was an expected outcome since nalidixic acid is a quinolone class antibiotic, which is pumped out by gram negative bacteria using their active efflux pump. This is in agreement with previous studies showing the effects of efflux pumps mediating reduced quinolone susceptibility in *Salmonella* (44, 45); however, we did not observe

increased resistance in the case of ciprofloxacin, another quinolone antibiotic. Possible reason for this observation might be due to slightly different physical and chemical structures of the two drugs. The other antibiotic that decreased the inhibition zone diameters of control group of SGSC1512 was tetracycline for all tested conditions except 12 and 24 hours of exposure to 10 mM AGW. The decrease was statistically significant for 6 and 18 hours of exposure to 3.33 mM AGW although, the susceptibility threshold levels did not change. This was not expected since the tetracyclines can easily be transported to external cellular environment with presence of cations (21, 22, 40).

The second organism tested was a wild type, outbreak-causing *Salmonella typhimurium* strain (ST5383). Exposure to AGW resulted in increased resistance of ST5383's control group from susceptible level to intermediate resistance for all tested conditions with significant increases at 3.33 mM for 12 hours and at 10 mM for 18 hours against nalidixic acid. Similar to SGSC1512's resistance profile, no changes were observed against the other quinolone, ciprofloxacin, for the tested conditions. At longer exposure conditions, *i.e.*, 24 hours for all IS the ST5383 control group's intermediate level resistance against amoxicillin-clavulanic acid decreased to susceptible levels. The same decrease was also observed at 10 mM AGW for 18 hours of exposure. Decreases in the inhibition zone diameter values were not significant in the case of amoxicillin-clavulanic acid. This particular antibiotic is a member of β -lactams as are cefoxitin, ceftriaxone, and cephalothin. The inhibition zone diameters of ST5383 for these three β -lactams also increased at 24 hours exposure to all IS, suggesting a common trend of decreased resistance although the resistance profile threshold was not achieved as in the

case of amoxicillin-clavulanic acid. As mentioned earlier, *Salmonella enterica* spp. shows resistance to β -lactams by the AcrAB efflux system; however, the secretion and release of β -lactamase are more direct ways of mediating the β -lactams. Therefore, ST5383 exposed to AGW for all tested conditions did not cause an increase in resistance against β -lactam class antibiotics. On the contrary, long-term stress exposure might have impacted the synthesis of β -lactamase, which is observed as increased susceptibility.

The last organism tested was *Salmonella pullorum*, a different non-flagellated serovar (SA1685) that causes avian diseases in the poultry industry. The control group of SA1685 was observed to be susceptible to all of the antibiotics tested in this study. The exposure of this strain to AGW did not cause a change in the antibiotic resistance profile. Although significant decreases and increases were observed for a few test conditions (Table 4.3), the inhibition zone diameters were much higher than susceptible profile threshold values. Since SA1685 is a different serovar than SGSC1512 and ST5383, this trend might be due to the morphological features of the bacterium and its response to stress conditions. This is also evident when the inhibition zone diameters of the three serovars were compared. ST5383, the fully functional flagellated wild type *Salmonella typhimurium* outbreak strain has shown smaller diameters, whereas SGSC1512, the dysfunctional flagellated *Salmonella typhimurium* had slightly larger inhibition zone diameters. SA1685, the non-motile *Salmonella pullorum* strain showed the largest inhibition zone diameters.

Epithelial cell invasion assay results showed that exposure to AGW for 6 and 12 hours result in decreased invasiveness of *Salmonella enterica* spp. into HEp2 cell lines

for all tested IS conditions. In addition, the number of SA1685 that can invade epithelial cells also decreased at all IS for 18 hours of exposure. The number of bacteria that can invade HEp2 cells decreased for 3.33 mM and 10 mM AGW; and 3.33 mM for 18 hours of exposure in the cases of SGSC1512 and ST5383 respectively. However, at 24 hours of exposure to AGW increased the number of ST5383 and SGSC1512 that can invade the epithelial cells at 10, and 30 mM IS. In the case of SA1685, invasiveness increased for all tested IS for 24 hours of exposure. The decreases in the number of invading bacteria observed up to 18 hours of exposure might be related to the effect of stress induced on the bacteria. After 24 hours of exposure, they metabolically get accustomed to AGW medium; this in return increased the number of invading bacteria. This is in agreement with previous studies mentioned earlier, that showed increased invasion of human cell lines with increased calcium concentrations (34, 35).

4.5 Conclusions

Exposure of an outbreak-causing *Salmonella enterica* spp. to an environment that simulates groundwater results in environmental stress. As an ion-rich environment, the groundwater might affect the active efflux pumps of bacteria, which in turn change the resistance against certain antibiotic classes. This study has shown that, for short-term exposure to different concentrations of ions in artificial groundwater, changes in resistance to several antibiotics may be induced. Groundwater exposure may also result in changes in the invasiveness of *Salmonella enterica* spp. into human epithelial cell lines, more evident at 24 hours of exposure.

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Chapter 5

Effects of Long Term Exposure to Antibiotic Containing Groundwater in *Salmonella enterica* serovar Typhimurium: Changes in Antibiotic Resistance, *in vivo* and *in vitro* Pathogenicity

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Abstract

An outbreak causing strain of *Salmonella enterica* serovar Typhimurium was exposed to artificial groundwater with residual antibiotics for four weeks. Representative concentrations (0.05, 1, and 100 µg/L) of amoxicillin, tetracycline, and a mixture of antibiotics (1 µg/L) were supplemented to the groundwater flasks. Antibiotic susceptibility analysis and virulence response of stressed *Salmonella* were determined by using human epithelial cells (HEp2) and nematodes (*C. elegans*) weekly. Results have shown that *Salmonella typhimurium* can remain viable for long periods of exposure to antibiotic supplemented groundwater; however, may fail to cultivate as an indication of exposed cells entering a viable but nonculturable state. Interestingly, prolonged antibiotics exposure did not induce any changes in the antibiotic susceptibility profile of *S. typhimurium* strain used in this study. However, *S. typhimurium* exposed to 0.05 and 1 µg/L amoxicillin, and 1 µg/L tetracycline showed hyper-virulent profiles in both *in vitro* and *in vivo* virulence assays with the HEp2 and *C. elegans* respectively, which was particularly evident at 2 and 3 weeks of exposure.

5.1 Introduction

Salmonella enterica serovar Typhimurium is one of the most problematic food- and waterborne enteric pathogens in the world, with a high percentage of multiple antibiotic-resistant isolates (1). As a resilient pathogen, *Salmonella typhimurium* can cope well with a variety of stress conditions both in its target host and in natural environments (2). Once *Salmonella* gets excreted from a host and enters environmental systems, it experiences several stress conditions such as changes in temperature, osmolarity, and pH (3). One of the most common matrices that *Salmonella* contaminates is aquatic environments (4-8). Ultimately, it is critically important to understand how the stress conditions imposed in aquatic environments affect the virulence of *S. typhimurium* before the pathogen infects its targeted host again (9).

Pharmaceutical products are another major contaminant of the aquatic systems (10-14). A majority of the pharmaceutical products found in both surface and groundwater systems are antibiotics (15-18). Although the resistance mechanisms of *S. typhimurium* against antibiotics are a widely explored topic (19-23), there still exists a knowledge gap regarding bacterial response to antibiotics as an environmental stress and how exposure to low concentration level antibiotics induces stress hardening, and protection (3).

The goal of this study was to understand the effects of stress induced by groundwater environments and antibiotic presence on the virulence and antibiotic susceptibility of *S. typhimurium* for prolonged exposure periods. To fulfill this goal, artificial groundwater (AGW) was prepared and supplemented with residual antibiotics. Due to low levels of degradation (24-27) and prevalence in the environment (10, 12-14), amoxicillin and

tetracycline were selected as the model antibiotics. To represent a wide range of pristine and contaminated groundwater conditions, both low (14, 28) and high (29) concentrations of antibiotics were used (12, 13). In addition to amoxicillin and tetracycline, a cocktail of antibiotics was prepared by mixing nine common antibiotics in the AGW. Antibiotic susceptibility analysis of stressed cells, along with *in vitro* and *in vivo* virulence assays, were performed for *S. typhimurium* exposed to these combinations of antibiotics for up to 4 weeks. Results indicated that long term exposure to AGW supplemented with residual antibiotics may increase the virulence of *S. typhimurium* against human epithelial cells and nematodes. During the course of the study, bacteria remained mostly viable, however, failed to cultivate. No further antibiotic resistance was induced to susceptible control group due to exposure to the tested antibiotics.

5.2 Materials and Methods

5.2.1 Bacterial Cell Growth and Preparation

Salmonella enterica serovar Typhimurium strain ST5383 used in this study was obtained from Salmonella Genetic Stock Centre (SGSC) of University of Calgary, Alberta, Canada. *S. typhimurium* strain ST5383 is a wild type strain and originally isolated from an interprovincial outbreak that infected more than 1700 people (30). *Salmonella* cells used during the course of study was cultured in Luria-Bertani (LB) broth, (Fisher Scientific, Fair Lawn, NJ) at 37°C overnight, shaken continuously at 120 rpm. A refrigerated bench-top centrifuge (5804R; Eppendorf, Hamburg, Germany) equipped with fixed angle rotor (F-34-6-38; Eppendorf) was used to pellet the cells with an applied $3,700 \times g$ force

for 15 min at 4°C. Growth medium was decanted and the pellet was resuspended in 3 mM artificial groundwater (AGW) (described below). Concentration of cell stock solution was determined by using a cell counting hemocytometer (Bürker-Turk, Germany) under a light microscope (Fisher Scientific).

5.2.2 Application of Stress Conditions

Bacteria cultured and harvested as described above were exposed to several antibiotic-containing AGW for the duration of 1 to 4 weeks. AGW solutions used in cell preparation and other experiments were prepared with de-ionized water (DIW) (Millipore, Billerica, MA) and reagent-grade salts (Fisher Scientific) with a slight modification to a previously used recipe (31) by dissolving following: $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (36mg), $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ (25mg), KNO_3 (20mg), NaHCO_3 (36mg), $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (35mg), and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (60mg). The pH of solutions kept constant at 7.0 ± 0.2 . Ionic strength (IS) of solutions tested were 3 mM, a typical level for groundwater (32, 33).

The levels of antibiotics added to AGW were 0.05, 1, and 100 µg/L ampicillin (MP Biomedicals, Solon, OH), 0.05, 1, and 100 µg/L tetracycline (MP Biomedicals), and a cocktail of 1 µg/L of each the following antibiotics; amoxicillin (MP Biomedicals), tetracycline (EMD Chemicals, Darmstadt, Germany), ampicillin (EMD Chemicals), chloramphenicol (EMD Chemicals), kanamycin (EMD Chemicals), gentamicin (MP Biomedicals), streptomycin (MP Biomedicals), sulfamethoxazole (MP Biomedicals), and penicillin (Fisher Scientific).

Bacteria with 10^7 cells/mL concentration were exposed to the aforementioned stress conditions in 500 mL tissue culture flasks with phenolic caps (Corning, MA), placed on orbital shakers, and mildly shaken (70 rpm) for the desired time periods.

5.2.3 Viability and Cultivability

At the end of stress exposure to AGW, viability of the cells was determined by using the Live/Dead BacLight® kit (L-7012; Molecular Probes, Eugene, OR) according to the manufacturer's directions. Direct counting of the stained live and dead cells was done using an inverted microscope (IX70; Olympus, Japan) operated under red/green fluorescence filter set (Chroma Technology Corp., Brattleboro, VT). Stressed organisms were also tested for cultivability by spread plating serial dilutions of stressed cells on LB agar (Fisher Scientific) plates followed by overnight incubation at 37°C. Colony forming units (CFUs) were enumerated the following day.

5.2.4 Antibiotic Susceptibility Analysis

Antibiotic susceptibility tests were employed to assess the changes in *S. typhimurium*'s resistance to 11 antimicrobials commonly used by the U.S. National Antimicrobial Resistance Monitoring System (NARMS). Tests were performed in compliance with the Clinical and Laboratory Standards Institute/National Committee for Clinical Laboratory Standards (CLSI/NCCLS) (34) for the following antimicrobial agents (numbers in parentheses denote the amount of antimicrobials impregnated in 6 mm disks): amikacin (30 µg), amoxicillin-clavulanic acid (30 µg), cefoxitin (30 µg), ceftriaxone (30 µg), ceftiofur (30 µg), cephalothin (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg),

nalidixic acid (30 µg), tetracycline (30 µg), trimethoprim-sulfamethoxazole (25 µg). Antibiotic susceptibility analyses were performed in triplicate.

5.2.5 *In vitro* Invasion Assays

Human epithelial cell line HEp2 was obtained from American Type Culture Collection (CCL-23; ATCC, Manassas, VA) and was grown in Eagle's Minimum Essential Medium (ATCC) supplemented with 10% fetal bovine serum (ATCC), and 1% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO). Epithelial cells were incubated at 37°C under 5% CO₂.

Invasion of stressed *S. typhimurium* into epithelial cells was quantified with slight modifications to a protocol as described elsewhere (35). Briefly, a monolayer of HEp2 cells was grown until confluence in 24-well plates (Corning, Corning, NY), and was subsequently inoculated with 10⁵ *Salmonella* cells and incubated for 2 hours at 37°C to allow for internalization. Following the incubation, each well was washed three times with phosphate buffered saline (PBS) to remove unbound bacteria. Bacteria that were bound to the epithelial cells, but had not been internalized, were killed by applying fresh growth medium containing penicillin and gentamicin (5 and 100 µg/mL respectively) and incubated for 2 hours at 37°C. Following incubation, cells were washed with PBS, treated with trypsin-EDTA complex (ATCC), and lysed with 1 % Triton-X100 (Fisher). The lysates were spread onto LB agar plates and incubated for 18 hours at 37°C. The number CFUs were counted to quantify number of *S. typhimurium* that had successfully invaded the monolayer of epithelial cells. Invasion assays and CFU enumeration were performed in triplicate.

5.2.6 *Caenorhabditis elegans* Maintenance and *in vivo* Virulence Assays

C. elegans strain SS104 [*glp-4 (ts)*], a temperature sensitive mutant of nematode worms that produces progeny at 15°C but not at 25°C (36), was obtained from the *Caenorhabditis* Genetics Center (University of Minnesota, Saint Paul) and used for virulence assays. Worms were maintained by slight changes to standard procedures described elsewhere (37). Briefly, worms were grown on modified nematode growth medium (NGM) (US Biological, Swampscott, MA) [with 0.35% peptone instead of 0.25%, and supplemented with uracil (Fisher Scientific) (2 g/L final concentration)] plates at 15 °C, and fed with *E. coli* strain OP50 as previously described (38).

Virulence assays were performed based on similar studies described in literature (39-41). Fifteen synchronized worms in larval stage L4 were transferred to NGM plates seeded with 10 µl of stressed *S. typhimurium* mixed with nonpathogenic *E. coli* OP50 (1:100 ratio respectively), and maintained at 25°C. The number of viable worms was counted everyday and reported as the number of survivors until all fifteen worms were killed. Worms that were sessile and unresponsive to touch were considered to be dead. For each stress condition, virulence assays were performed in triplicate.

5.2.7 Statistical Analysis of Data

Changes in the diameters of antibiotic inhibition zones and the number of CFUs of *S. typhimurium* infecting HEp2 cells were statistically compared to the control groups using unpaired Student's t-test using Minitab® Version 14 (State College, PA). Differences between control and stressed *Salmonella* cells were considered to be significant at 95% confidence interval ($P < 0.05$).

5.3 Results and Discussion

5.3.1 Viability and Cultivability of *Salmonella typhimurium*

Changes in the percent viability and cultivability of *S. typhimurium* exposed to antibiotic-containing AGW were determined with respect to time and reported in Figures 5.1 and 5.2 respectively. As can be seen from Figure 5.1, the percent viability of the control group that was not exposed to antibiotics, i.e. week 0, ranged from 92% to 100%. At the end of week 1, the viabilities of the cells exposed to low-concentrations of antibiotics (94-96% viability for amoxicillin; 85-94% viability for tetracycline) were higher than those exposed to high-concentration (71% viability for amoxicillin; 67% viability for tetracycline). Percent viability of the cells exposed to the cocktail of antibiotics was 80%. As anticipated, the percentage of viable cells decreased gradually as cells were exposed to antibiotic-containing AGW for longer time periods (2 and 3 weeks) (Figure 5.1). By the end of week 4, the percent viabilities of cells exposed to amoxicillin were 38%, 41%, and 34% for concentrations of 0.05, 1, and 100 µg/L, respectively.

The percent viabilities of the cells exposed to tetracycline were 37% and 32% for concentrations of 0.05 and 1 µg/L respectively. At higher tetracycline concentration, i.e. 100 µg/L, the percent viability was much lower (14%). The lowest percent viability (12%) was observed in the flask containing the cocktail of antibiotics at the end of week 4.

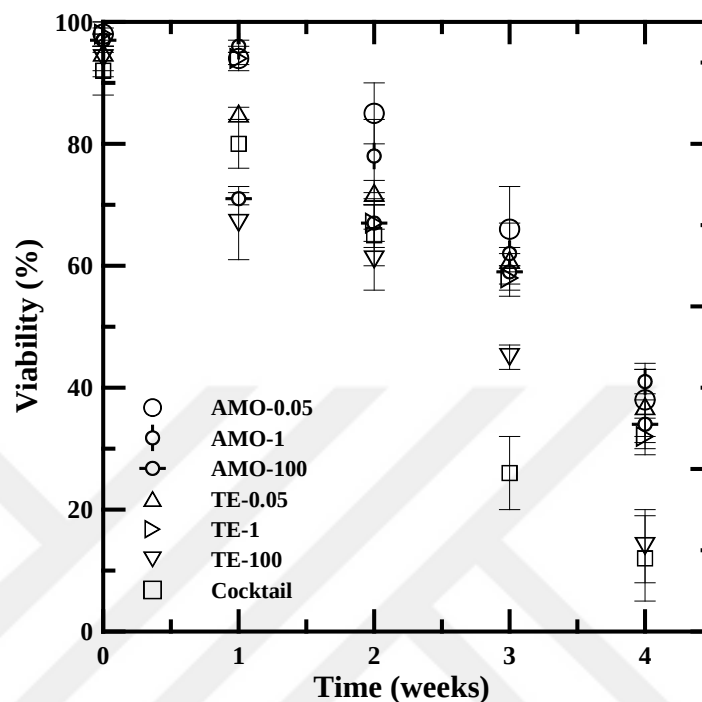


Figure 5.1. Changes in percent viability of *S. typhimurium* exposed to antibiotic-containing AGW with respect to time and compared to control group. The abbreviations in the legend refer to the tested antibiotics (AMO for amoxicillin, and TE for tetracycline). The numbers in the legend refer to the concentrations of tested antibiotics in $\mu\text{g/L}$. Error bars indicate the standard deviation of three replicates.

During the course of study the cultivability of *S. typhimurium*, cells were also enumerated and reported as percent cultivability (Figure 5.2). The initial (week 0) cultivability percentages were very close to the percentage of viable cells determined. However, significant differences were observed between percent viability and cultivability values from the end of week 1 through the end of week 4. The cells exposed to low concentration amoxicillin were 85% and 74% cultivable (for 0.05 and 1 $\mu\text{g/L}$ respectively); however, only 13% cultivability was observed for cells exposed to the high concentration of amoxicillin. In the case of tetracycline exposed cells, the cultivability was 52% and 70% for 0.05 and 1 $\mu\text{g/L}$, respectively. For high concentration of

tetracycline exposure (100 µg/L), none of the cells were capable of being cultivated by the end of week 1. This was also observed for the cells exposed to the cocktail of antibiotics (Figure 5.2). Similar to the trend observed in percent viability measurements, the cultivability of cells in the presence of amoxicillin decreased during the exposure period of week 2 and week 3. The cells exposed to high concentration of tetracycline and the cocktail of antibiotics failed to cultivate for weeks 2, 3 and 4. By the end of week 4, percent cultivability was pretty low for other conditions as well; 14% and 11% (for 0.05 and 1 µg/L amoxicillin exposure, respectively), 1% (for 1 µg/L amoxicillin exposure), as well as 6% and 2% (for 0.05 and 1 µg/L tetracyclin exposure, respectively). No bacterial growth was observed for 4 weeks of exposure to 100 µg/L tetracycline and cocktail of antibiotics (Figure 5.2).

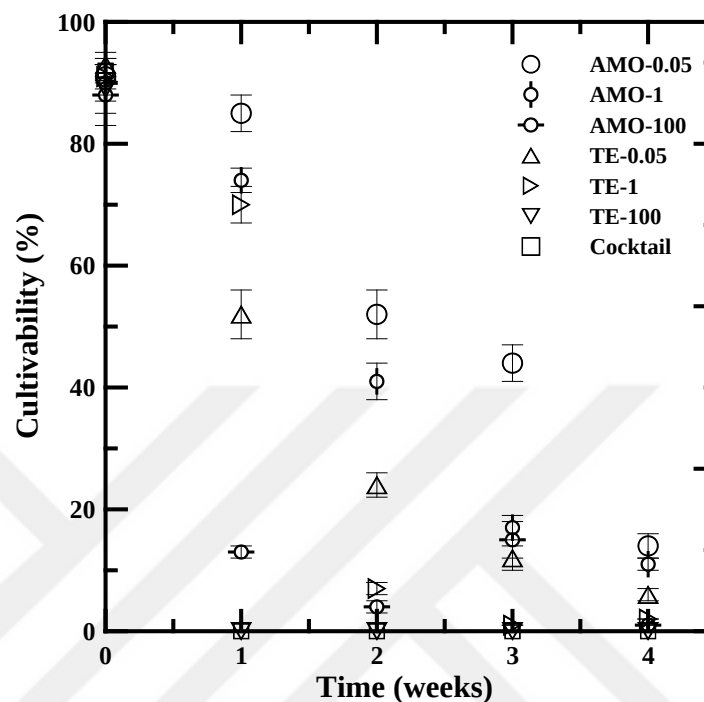


Figure 5.2. Changes in percent cultivability of *S. typhimurium* exposed to antibiotic-containing AGW with respect to time and compared to control group. The abbreviations in the legend refer to the tested antibiotics (AMO for amoxicillin, and TE for tetracycline). The numbers in the legend refer to the concentrations of tested antibiotics in $\mu\text{g/L}$. Error bars indicate the standard deviation of three replicates.

It is widely accepted that human pathogenic bacteria enter the viable but nonculturable (VBNC) state when exposed to harsh environmental conditions (45-47). However, it is a matter of continuous debate whether or not VBNC cells pose a risk to human and animal health (47-51). Therefore, both percent viability and cultivability values were determined (Figure 5.1 and 5.2, respectively). Results have shown that although the cells remain viable, they may not be cultivable at all (i.e. exposure to 100 $\mu\text{g/L}$ tetracycline and cocktail of antibiotics from week 1 to week 4) or cultivable at low levels [i.e. exposure to 100 $\mu\text{g/L}$ amoxicillin from week 1 to week 4 and low concentration of tetracycline (0.05 and 1 $\mu\text{g/L}$) from week 2 to week 4]. Overall, the

results showed that the majority of the cells exposed to antibiotics up to two weeks may remain structurally intact (52) (as determined by viability); however, fail to cultivate. As the exposure time to antibiotics is prolonged, both the percent viability and cultivability decreases to minimal levels (Figure 5.1 and 5.2, respectively).

5.3.2 Antibiotic Susceptibility Analysis

The full names and abbreviations of all antibiotics used for the susceptibility test are presented in Table 5.1, along with the susceptibility breakpoints indicating the thresholds of susceptible, intermediate resistance, and resistant levels (34). As further indication and confirmation of low levels of (exposure to 100 µg/L amoxicillin) or no cultivability (exposure to 100 µg/L tetracycline) when exposed to high concentrations of antibiotics, no bacteria was observed on the antibiotic susceptibility plates and the tests were failed to be performed. This was also the case for the cells exposed to the cocktail of antibiotics.

Table 5.1. Susceptibility breakpoints (34) of antibiotics tested in this study.

Antibiotic ^a	Susceptibility breakpoints (mm)		
	Susceptible	Intermediate resistance	Resistant
Amikacin (AMK)	≥17	15-16	≤14
Amoxicillin-clavulanic acid (AMC)	≥18	14-17	≤13
Cefoxitin (FOX)	≥18	15-17	≤14
Ceftriaxone (CRO)	≥21	14-20	≤13
Cephalothin (KF)	≥18	15-17	≤14
Chloramphenicol (C)	≥18	13-17	≤12
Ciprofloxacin (CIP)	≥21	16-20	≤15
Nalidixic acid (NA)	≥19	14-18	≤13
Tetracycline (TE)	≥19	15-18	≤14
Ceftiofur (XNL)	≥21	18-20	≤17
Trimethoprim-sulfamethoxazole (SXT)	≥16	11-15	≤10

^a Antibiotics abbreviations are given in parentheses.

Table 5.2 reports the measured diameters of the zones of inhibition for *S. typhimurium* exposed to AGW with amoxicillin (0.05 µg/L). Week 0 data denotes the susceptibility profile of the control group consisting of the cells that were not exposed to antibiotic stress (same control for all susceptibility tests). As can be seen in Table 5.2, the control group of *S. typhimurium* strain used in this study (ST5383) was susceptible to all 11 antibiotics tested. As cells were exposed to low concentration of amoxicillin (0.05 µg/L), subtle increases were observed in the size of the inhibition zone. These increases were statistically significant for only a few cases as marked in Table 5.2.

Table 5.2. Measured diameters (in mm) of antibiotic inhibition zones of *S. typhimurium* exposed to AGW with amoxicillin (0.05 µg/L) for given time periods compared to control group.

Antibiotics	Week 0	Week 1	Week 2	Week 3	Week 4
AMK	24.81 ± 2.79 (S)	25.20 ± 1.33 (S)	27.88 ± 2.62 (S)	26.72 ± 2.06 (S)	24.60 ± 2.12 (S)
AMC	25.38 ± 2.13 (S)	26.00 ± 2.11 (S)	29.16 ± 2.41 (S)	29.76 ± 2.58 (S)	31.52 ± 3.41 (S)
FOX	25.16 ± 2.32 (S)	26.96 ± 1.78 (S)	28.40 ± 1.99 (S)	28.12 ± 1.67 (S)	29.58 ± 2.51 (S)
CRO	33.20 ± 3.79 (S)	34.20 ± 2.43 (S)	32.86 ± 3.65 (S)	38.92 ± 3.21 (S)	36.18 ± 3.28 (S)
KF	23.72 ± 2.36 (S)	25.62 ± 1.65 (S)	27.12 ± 2.93 (S)	28.26 ± 2.47 (S)	26.38 ± 2.14 (S)
C	26.92 ± 2.42 (S)	26.96 ± 1.09 (S)	24.10 ± 2.08 (S)	27.00 ± 2.93 (S)	27.32 ± 2.65 (S)
CIP	38.18 ± 2.86 (S)	38.20 ± 3.01 (S)	37.46 ± 3.48 (S)	38.28 ± 3.46 (S)	35.92 ± 3.43 (S)
NA	19.88 ± 1.25 (S)	20.96 ± 2.07 (S)	22.28 ± 2.39 (S)	24.06 ± 1.78 (S)^a	24.78 ± 2.54 (S)
TE	23.21 ± 1.98 (S)	25.82 ± 1.17 (S)	26.66 ± 2.08 (S)	28.96 ± 2.15 (S)	26.08 ± 2.42 (S)
XNL	27.46 ± 2.12 (S)	25.46 ± 2.73 (S)	26.00 ± 2.42 (S)	27.96 ± 2.19 (S)	28.36 ± 2.74 (S)
SXT	34.00 ± 3.11 (S)	32.50 ± 3.74 (S)	35.56 ± 2.69 (S)	36.02 ± 3.17 (S)	35.88 ± 3.48 (S)

^a Numbers in bold denote the changes in the inhibition zone diameter are statistically significant compared to the control group.

Similarly, exposure to AGW with 1 µg/L amoxicillin did not change the susceptibility profile of the control group for any of the antibiotics tested. As can be seen in Table 5.3, the zone diameters were increased for the majority of the cells during the exposure period of week 1 to week 4.

Table 5.3. Measured diameters (in mm) of antibiotic inhibition zones of *S. typhimurium* exposed to AGW with amoxicillin (1 µg/L) for given time periods compared to control group.

Antibiotics	Week 0	Week 1	Week 2	Week 3	Week 4
AMK	24.81 ± 2.79 (S)	23.46 ± 1.72 (S)	24.68 ± 2.35 (S)	26.78 ± 2.11 (S)	26.08 ± 2.77 (S)
AMC	25.38 ± 2.13 (S)	26.38 ± 2.74 (S)	24.10 ± 2.66 (S)	30.32 ± 3.54 (S)	29.18 ± 2.52 (S)
FOX	25.16 ± 2.32 (S)	25.18 ± 1.25 (S)	27.72 ± 2.06 (S)	29.62 ± 1.74 (S)	29.54 ± 2.10 (S)
CRO	33.20 ± 3.79 (S)	32.64 ± 3.12 (S)	34.84 ± 3.58 (S)	37.12 ± 3.32 (S)	34.98 ± 3.53 (S)
KF	23.72 ± 2.36 (S)	25.82 ± 1.17 (S)	26.94 ± 2.16 (S)	26.48 ± 2.42 (S)	27.68 ± 2.66 (S)
C	26.92 ± 2.42 (S)	28.46 ± 2.81 (S)	25.94 ± 2.45 (S)	27.94 ± 2.55 (S)	28.58 ± 2.27 (S)
CIP	38.18 ± 2.86 (S)	36.84 ± 3.51 (S)	32.06 ± 3.21 (S)^a	39.30 ± 3.22 (S)	33.06 ± 3.19 (S)
NA	19.88 ± 1.25 (S)	20.18 ± 1.94 (S)	21.38 ± 1.77 (S)	24.06 ± 1.78 (S)	23.06 ± 2.11 (S)
TE	23.21 ± 1.98 (S)	26.56 ± 2.08 (S)	27.08 ± 2.74 (S)	25.96 ± 2.39 (S)	26.46 ± 2.67 (S)
XNL	27.46 ± 2.12 (S)	26.76 ± 2.73 (S)	25.38 ± 2.57 (S)	26.46 ± 2.87 (S)	26.50 ± 2.76 (S)
SXT	34.00 ± 3.11 (S)	33.90 ± 3.41 (S)	34.72 ± 3.03 (S)	36.76 ± 2.61 (S)	35.32 ± 3.20 (S)

^a Numbers in bold denote the changes in the inhibition zone diameter are statistically significant compared to the control group.

Antibiotic susceptibility of cells exposed to tetracycline containing AGW resulted in similar trends as the amoxicillin exposure. The inhibition zone diameters are given in Table 5.4 and Table 5.5 for tetracycline concentrations of 0.05 and 1 µg/L, respectively. The results indicate that cells exposed to tetracycline containing AGW sustained their susceptibility to the tested antibiotics for the duration of week 1 to week 4 for either concentration of tetracycline.

Table 5.4. Measured diameters (in mm) of antibiotic inhibition zones of *S. typhimurium* exposed to AGW with tetracycline (0.05 µg/L) for given time periods compared to control group.

Antibiotics	Week 0	Week 1	Week 2	Week 3	Week 4
AMK	24.81 ± 2.79 (S)	26.38 ± 2.14 (S)	26.46 ± 2.25 (S)	26.04 ± 1.76 (S)	27.94 ± 2.04 (S)
AMC	25.38 ± 2.13 (S)	24.62 ± 2.63 (S)	27.78 ± 2.41 (S)	30.22 ± 3.36 (S)	28.14 ± 1.68 (S)
FOX	25.16 ± 2.32 (S)	27.68 ± 2.17 (S)	29.62 ± 2.77 (S)	28.94 ± 2.18 (S)	28.84 ± 2.25 (S)
CRO	33.20 ± 3.79 (S)	36.40 ± 4.09 (S)	34.26 ± 2.69 (S)	35.98 ± 2.81 (S)	35.94 ± 3.09 (S)
KF	23.72 ± 2.36 (S)	23.70 ± 2.81 (S)	27.60 ± 2.47 (S)	26.20 ± 1.22 (S)	28.20 ± 1.71 (S)
C	26.92 ± 2.42 (S)	30.78 ± 3.44 (S)	28.08 ± 2.05 (S)	28.14 ± 1.92 (S)	27.94 ± 2.18 (S)
CIP	38.18 ± 2.86 (S)	38.66 ± 3.74 (S)	32.54 ± 3.73 (S)	35.64 ± 3.73 (S)	39.80 ± 2.62 (S)
NA	19.88 ± 1.25 (S)	20.72 ± 0.81 (S)	23.24 ± 2.84 (S)	24.66 ± 1.69 (S)	25.82 ± 2.59 (S)
TE	23.21 ± 1.98 (S)	25.94 ± 2.10 (S)	26.40 ± 2.45 (S)	25.24 ± 1.90 (S)	27.66 ± 2.50 (S)
XNL	27.46 ± 2.12 (S)	21.20 ± 2.94 (S)^a	25.02 ± 1.97 (S)	27.94 ± 2.14 (S)	27.24 ± 2.19 (S)
SXT	34.00 ± 3.11 (S)	34.74 ± 2.88 (S)	36.52 ± 2.88 (S)	35.94 ± 3.29 (S)	34.50 ± 3.71 (S)

^a Numbers in bold denote the changes in the inhibition zone diameter are statistically significant compared to the control group.

Table 5.5. Measured diameters (in mm) of antibiotic inhibition zones of *S. typhimurium* exposed to AGW with tetracycline (1 µg/L) for given time periods compared to control group.

Antibiotics	Week 0	Week 1	Week 2	Week 3	Week 4
AMK	24.81 ± 2.79 (S)	24.50 ± 1.09 (S)	27.04 ± 2.51 (S)	25.84 ± 2.71 (S)	25.56 ± 1.41 (S)
AMC	25.38 ± 2.13 (S)	27.74 ± 2.11 (S)	29.48 ± 2.84 (S)	31.08 ± 3.16 (S)	32.04 ± 3.46 (S)
FOX	25.16 ± 2.32 (S)	28.86 ± 2.49 (S)	30.20 ± 3.17 (S)	30.50 ± 3.73 (S)	32.16 ± 3.87 (S)
CRO	33.20 ± 3.79 (S)	34.44 ± 3.25 (S)	36.86 ± 3.22 (S)	38.56 ± 3.51 (S)	37.56 ± 3.22 (S)
KF	23.72 ± 2.36 (S)	25.64 ± 2.60 (S)	27.60 ± 2.47 (S)	28.54 ± 2.58 (S)	31.90 ± 3.51 (S)
C	26.92 ± 2.42 (S)	29.70 ± 2.52 (S)	27.94 ± 1.51 (S)	28.86 ± 2.09 (S)	28.60 ± 2.84 (S)
CIP	38.18 ± 2.86 (S)	39.40 ± 3.26 (S)	35.82 ± 3.29 (S)	36.36 ± 2.83 (S)	34.96 ± 3.16 (S)
NA	19.88 ± 1.25 (S)	22.26 ± 1.19 (S)	25.22 ± 2.87 (S)^a	24.40 ± 1.52 (S)	28.22 ± 2.63 (S)
TE	23.21 ± 1.98 (S)	25.24 ± 1.56 (S)	27.76 ± 2.63 (S)	29.18 ± 2.28 (S)	28.06 ± 2.85 (S)
XNL	27.46 ± 2.12 (S)	27.20 ± 2.44 (S)	29.06 ± 2.51 (S)	28.20 ± 1.87 (S)	28.18 ± 2.40 (S)
SXT	34.00 ± 3.11 (S)	34.64 ± 3.16 (S)	36.40 ± 3.08 (S)	35.98 ± 3.40 (S)	37.72 ± 3.51 (S)

^a Numbers in bold denote the changes in the inhibition zone diameter are statistically significant compared to the control group.

The antibiotic susceptibility tests could not be performed for the cells that failed to cultivate [tetracycline (100 µg/L), and cocktail of antibiotics]. This was also the case for cells exposed to AGW with high concentration of amoxicillin (100 µg/L) due to the low cultivability (Table 5.1). Antibiotic susceptibility tests were successfully performed for cells exposed to AGW with tetracycline (0.05 and 1 µg/L), even though they showed similar cultivability (especially after week 2) to cells exposed to AGW with high concentration of amoxicillin (100 µg/L), a. This may be due to the fact that different media was used for the cultivability (LB agar) and antibiotic susceptibility (Mueller-Hinton agar) tests resulting in different growth characteristics.

Results of the antibiotic susceptibility tests were quite unexpected (Tables 5.2-5.5). As bacteria are exposed to antibiotics in different habitats, it is inevitable that resistance is gained to those antibiotics (10, 23, 53-56). However, there was no increased amoxicillin resistance induced by exposure to this antibiotic condition during the prolonged duration of this study. On the contrary, the cells showed increased susceptibility (Tables 5.2, 5.3). This observation was also confirmed with tetracycline exposure; the susceptibility of cells exposed to 0.05 and 1 µg/L of tetracycline containing AGW showed increased susceptibility to tetracycline (Tables 5.4, 5.5). This may be explained by a couple of reasons. First, the concentration of the antibiotics tested might be too low (0.05 and 1 µg/L) to induce a resistance to the bacteria. This hypothesis could have been confirmed with the cells exposed to high concentration level antibiotics; however, the tests failed as mentioned earlier. Secondly, although both tetracycline (24-26) and amoxicillin (27) are quite resistant to degradation, they might have been

degraded in the AGW with time (10, 15, 57) and resulted in a lesser or insignificant level of activity (58).

5.3.3 *In vitro* Invasion Assays

The number of *S. typhimurium* that can successfully invade human epithelial cell cultures (HEp2) after the exposure to antibiotic-containing AGW was determined with *in vitro* invasion assays and is presented in Table 5.6 as CFUs. The control group that was not exposed to stress conditions (week 0) showed that 258 CFUs can invade HEp2 cells. For further periods of exposure, there was no clear trend with regards to HEp2 cell invasion as a function of antibiotic type and concentration, other than the fact the exposure to antibiotics resulted in a lower invasion that occurred for the control. At the end of week 1, the CFUs decreased significantly to 192 for cells exposed to tetracycline (0.05 µg/L), and 128 and 64 for cells exposed to amoxicillin (0.05 and 100 µg/L, respectively) ($P < 0.05$). However, cells exposed to amoxicillin and tetracycline (both 1 µg/L) resulted in the number of CFUs that could invaded HEp2 cells increased significantly to 448 and 384, respectively ($P < 0.05$). None of the cells exposed to the cocktail of antibiotics and high concentration of tetracycline (100 µg/L) were able to invade HEp2 cells throughout the study. By the end of week 2, the number of CFUs has increased significantly to 2667 and 1600 for cells exposed to 0.05 and 1 µg/L amoxicillin, respectively. The number of CFUs that can invade HEp2 cells was 107 for high concentration amoxicillin (100 µg/L) exposed cells, higher than week 1 results but still less than the control group. Among the cells exposed to 1 µg/L tetracycline, none of them were able to invade the epithelial cells starting from week 1 to the end of the study.

At the end of week 3, the number of CFUs from amoxicillin exposed cells (0.05 and 1 µg/L) was significantly higher than the control group; however, less than week 2 exposed cells. By the end of the study, the number of CFUs that can successfully invade the epithelial cells was less than those of the control group, except cells exposed to high concentration amoxicillin (100 µg/L).

Table 5.6. Number of *S. typhimurium* CFUs infecting HEp2 cells exposed to antibiotics containing AGW with respect to time and compared to control group.

Stress conditions	Week 0	Week 1	Week 2	Week 3	Week 4
Amoxicillin (0.05 µg/L)	258 ± 34	128 ± 18^a	2667 ± 269	720 ± 83	213 ± 45
Amoxicillin (1 µg/L)		448 ± 56	1600 ± 127	480 ± 57	107 ± 39
Amoxicillin (100 µg/L)		64 ± 12	107 ± 18	400 ± 38	320 ± 49
Tetracycline (0.05 µg/L)		192 ± 24	0	0	0
Tetracycline (1 µg/L)		384 ± 28	320 ± 34	320 ± 42	53 ± 17
Tetracycline (100 µg/L)		0	0	0	0
Cocktail (1 µg/L)		0	0	0	0

^a Numbers in bold denote the changes in the CFUs are statistically significant compared to the control group.

Usage of human epithelial cell lines to test the *in vitro* virulence of *Salmonella* is a quite common and effective way of analyzing the degree of pathogenicity (59-62). However, *in vitro* virulence assays may not fully represent the internal habitat of a living organism. Therefore, commonly studied soil nematode *C. elegans* (63) were utilized to test the *in vivo* virulence of *S. typhimurium* (39-41, 64-67) exposed to antibiotic-containing AGW in complement to *in vitro* assays.

In vitro virulence assays have shown that exposing *S. typhimurium* to antibiotic-containing AGW may increase the virulence of the bacteria as indicated by a greater number of cells entering the host epithelial cell lines (Table 5.6). This was more evident for cells exposed to low concentration amoxicillin during the periods of week 2 and week

3. The changes in CFU numbers involved in invasion was not pronounced for week 1 and week 4 under the low concentration amoxicillin condition. The cells exposed to high and low (after week 1) concentration tetracycline and the cocktail of antibiotics did not invoke virulence against HEp2 cells as described earlier.

5.3.4 *In vivo* Virulence Assays

To confirm the virulence trends of stressed *S. typhimurium* cells against HEp2 cells, *in vivo* virulence assays were performed with *C. elegans* and the results are presented in Figure 5.3. Worms that were fed with *E. coli* OP50 and *S. typhimurium* cells not exposed to stress conditions were referred to as the control. As can be seen in Figure 5.3, the control group died in 17 days. When *E. coli* OP50 was mixed with *S. typhimurium* cells exposed to amoxicillin (0.05, 1 and 100 µg/L) and tetracycline (1 µg/L) for the durations of week 1 to week 4, worms died in a shorter period of time than the control group. *S. typhimurium* exposed to tetracycline (0.05 and 100 µg/L) and the cocktail of antibiotics killed the worms in 16 and 17 days, respectively; very similar to the control group (data not shown). *S. typhimurium* cells exposed 1 µg/L tetracycline for one week were able to kill worms in 13 days (Figure 5.3). The cells exposed to tetracycline (1 µg/L) for two weeks or later resulted in very similar results to the control group as well (data not shown).

S. typhimurium cells exposed to low concentration amoxicillin containing AGW were the fastest killers of the worms as an indication of hyper-virulence (42-44). It took 10, 7, 9, and 11 days to kill all the worms after one, two, three, and four weeks of exposure to 0.05 µg/L amoxicillin respectively. For 1 µg/L amoxicillin exposed cells, worms were

dead in 11, 8, 10, and 12 days (week 1, 2, 3, and 4 exposure, respectively). Exposure to high concentration amoxicillin (100 µg/L) and tetracycline (1 µg/L) showed similar results in terms of the days required to kill all worms.

In vivo virulence assays were in agreement with the *in vivo* virulence assays; the worms were killed in a shorter amount of time with *S. typhimurium* exposed to low concentration amoxicillin for two and three weeks as compared to the control group (Figure 5.3b, c). Cells exposed to stress conditions for four weeks were still virulent to *C. elegans*; however, the days required to kill the whole population was longer than those exposed for one to three weeks (Figure 5.3).

The reason of increased virulence at exposure periods of two and three weeks may be the cells becoming accustomed to the exposed stress conditions during the period of first week and eventually adjusting their metabolism in AGW environment during the following weeks. This has been proven with K^+ , Ca^{2+} and Mg^{2+} ions (all present in AGW), known promoters of increased virulence of *S. typhimurium* (68-73). The decreased virulence observed in week 4 is possibly due to cells starting to die and lose their infectious characteristics as indicated by viability and cultivability data (Figures 5.1, 5.2). This is in agreement with other studies that has shown that *S. typhimurium* stressed with UV-C light and seawater may lose its cultivability and virulence, whereas remained structurally intact and viable (74).

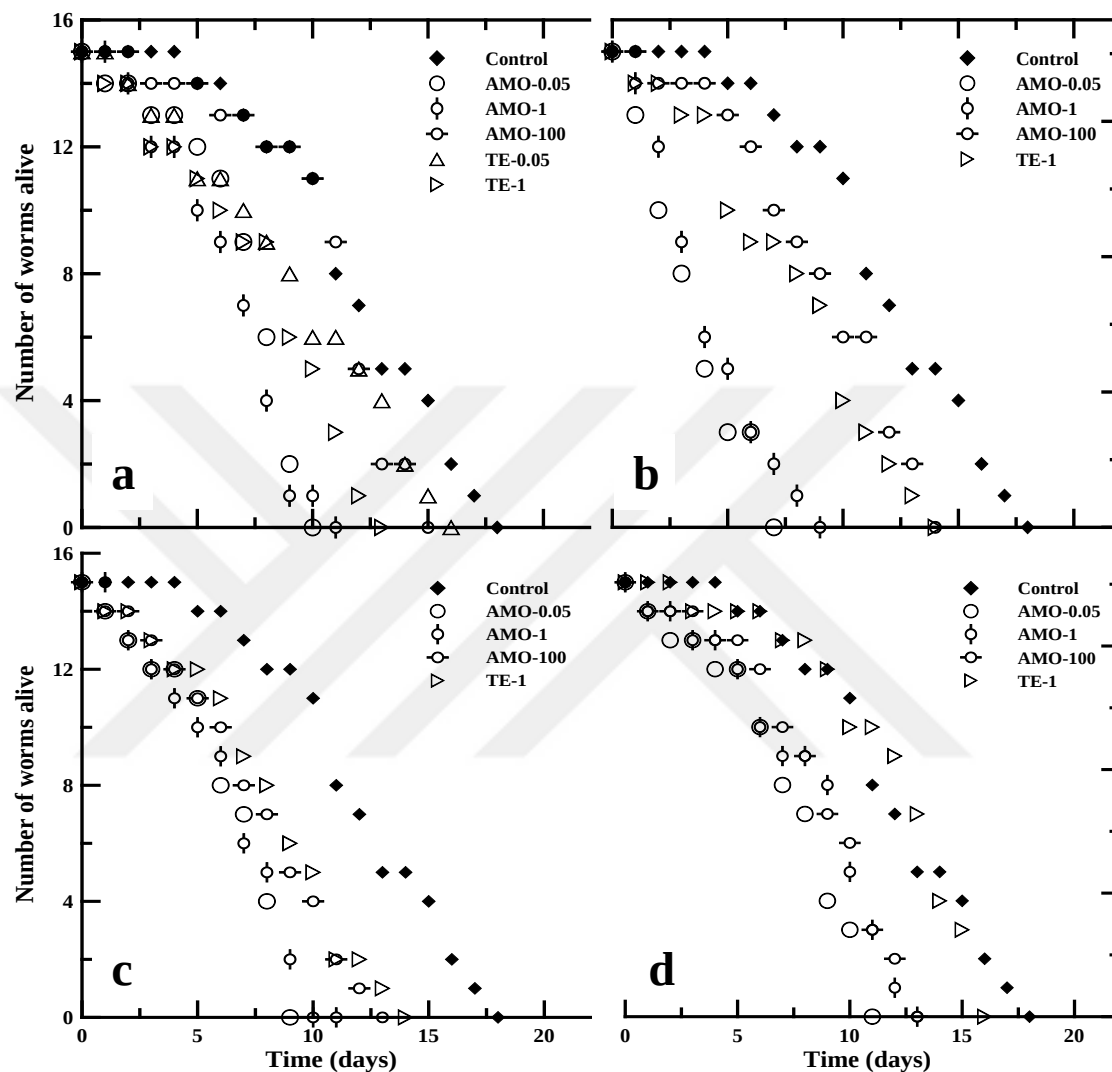


Figure 5.3. Number of *C. elegans* remaining alive when fed with *S. typhimurium* exposed antibiotic-containing AGW for one week (a), two weeks (b), three weeks (c), and four weeks (d). The abbreviations in the legend refer to the tested antibiotics (AMO for amoxicillin, and TE for tetracycline). The numbers in the legend refer to the concentrations of tested antibiotics in $\mu\text{g/L}$. All experiments were performed in triplicates, error bars are not shown for clarity purposes.

5.4 Conclusions

In this study, evidence was provided that long term exposure (up to approximately one month) to artificial groundwater (AGW) supplemented with several antibiotics may

induce viable but nonculturable (VBNC) state in *Salmonella typhimurium*. It has been also shown that *S. typhimurium* remains viable in AGW up to four weeks. During the course of the study, *S. typhimurium* exposed to low concentration amoxicillin containing AGW for two and three weeks showed hyper-virulence against human epithelial (HEp2) cells and nematodes (*C. elegans*) as shown by both *in vitro* and *in vivo* virulence assays, respectively. Enhanced virulence was also observed to be due to exposure to AGW with representative concentration of tetracycline. Exposure of *S. typhimurium* to amoxicillin and tetracycline containing AGW up to four weeks did not induce any resistance against these antibiotics among the bacteria. It has been concluded that, *S. typhimurium* in groundwater with trace antibiotics may remain viable and show increased virulence to either human and/or animal hosts.

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Chapter 6

Survival and Fitness of Random Generated *Salmonella typhimurium* Transposon Library under Long Term Environmental Stress: From *in vitro* to *in silico*

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Abstract

A random transposon library of *Salmonella enterica* serovar Typhimurium ATCC 14028 mutants were generated and exposed to several groundwater and seawater conditions to identify the genes required for fitness and survival under these conditions. The mutant library was monitored using a novel screening strategy and analyzed by hybridization to a high density genome tiling array. The results showed that the same set of genes is required for survival of most of the stress conditions tested. Those genes are generally involved in universal stress response regulation, carbon starvation and source depletion, osmotic shock, sugar uptake, and cell division. Additionally, several genes involved in the virulence of *S. typhimurium* were identified as required for survival. The number of genes required for survival in groundwater was slightly higher than that for seawater. The majority of mutants remained structurally intact following a four week exposure to the stress condition, but failed to cultivate.

6.1 Introduction

Random mutagenesis strategy (1, 2) can be combined with array-based methods (3-8) to analyze the expression of multiple genes of interest simultaneously. Specifically, random transposon mutagenesis (RTM) (9-11) can be utilized to determine the behaviour of genes in a pool of mutants following exposure to a particular environmental condition. The biggest disadvantage of RTM is that tens of thousands of random transposon insertion mutants are necessary to ensure that the mutations occur in every gene of interest, some of which are short in length (12). In the *S. Typhimurium* genome, there are 4,600 annotated open reading frames (ORFs) (13) and mathematical calculations estimate that at least 40,000 independent random transposon insertion mutants are required to knock-out the majority of those short genes (12).

In this study, a random transposon mutant library consisting of 90,000 independent mutants was generated and exposed to various stress conditions for four weeks to identify the genes of importance in terms of fitness and survival of *Salmonella typhimurium* in aquatic environments. The stress conditions that were used to challenge the *S. typhimurium* mutant library included artificial seawater (ASW), artificial groundwater (AGW) with natural organic matter, at high pH, at low temperature, and AGW under static conditions. Finally, a unique microarray was designed and utilized to quantify the changes in transposon representation in the library of surviving mutants at the end of each stress exposure period.

6.2 Materials and Methods

6.2.1 Bacterial Growth and Preparation

Salmonella enterica serovar Typhimurium strain ATCC14028s (ATCC, Manassas, VA) and its random generated transposon derivatives (described below) were used in this study. Strains were cultured in Luria-Bertani (LB) broth (Fisher Scientific, Fair Lawn, NJ), supplemented with 50 µg/mL kanamycin (Kan) and 15 µg/mL chloramphenicol (Cm), at 37°C overnight, shaken continuously at 120 rpm. A refrigerated bench-top centrifuge (5804R; Eppendorf, Hamburg, Germany) equipped with fixed angle rotor (F-34-6-38; Eppendorf) was used to pellet the cells with an applied $3,700 \times g$ force for 15 min at 4°C. Growth medium was decanted and the pellet was resuspended in 3 mM artificial groundwater (AGW) (described below). The rinsing procedure was performed twice to ensure complete removal of the growth media. Concentration of cell stock solution was determined using a cell counting hemocytometer (Bürker-Turk, Germany) under a light microscope (Fisher Scientific).

6.2.2 Construction of the Random Transposon Library

The random transposon library was constructed in the laboratory of Prof. Brian Ahmer at The Ohio State University. A chloramphenicol (Cm) resistant *S. typhimurium* 14028 derivative (referred to as JLD1214) was generated by targeted mutagenesis that introduced a Cm resistance cassette into the *Salmonella* genome at a neutral location just downstream of *pagC*. A random transposon library was then produced in JLD1214 using the EZ-Tn5™ <T7/KAN-2> Promoter Insertion Kit (Epicentre, Madison, WI).

Transposome complexes, i.e. transposase and transposon DNA complexes, were assembled *in vitro*, according to manufacturer's recommendations, and electroporated into electro-competent 14028s cells in logarithmic growth phase. Successful recombinants were recovered in selective broth, and counted on selective agar (LB w/ Kan and Cm). A total of 90,000 independent recombinants were recovered and pooled. Library complexity was confirmed by hybridization of labeled RNA generated from inserted T7 promoters onto a high resolution Nimblegen (NimbleGen Systems, Madison, WI) tiling array covering the entire *S. typhimurium* 14028 genome. Glycerol stocks of the library were aliquoted and stored frozen at -80°C until further use.

6.2.3 Application of Stress Conditions

The mutant library cultured and harvested as described above was exposed to several stress conditions for the duration of 4 weeks. The inoculation concentration of the library that introduced to 500 mL tissue culture flasks with 0.2 µm filtered caps (Corning, MA) was 10^7 cells/mL. Three biological replicates for each stress condition (cultured and harvested on different days) were placed on orbital shakers (OS-500, VWR, West Chester, PA), mildly shaken at 70 rpm and incubated at room temperature, unless otherwise noted, for four weeks. The solutions used in cell preparation and stress experiments were prepared with de-ionized water (DIW) (Millipore, Billerica, MA) and reagent-grade salts (Fisher Scientific).

The first stress condition (denoted as AGW-NOM) was designed with the background artificial groundwater (AGW), prepared according to slight modifications to a previously used recipe (14) by dissolving the followings in 1 L of DIW: $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (36mg),

CaSO₄·2H₂O (25mg), KNO₃ (20mg), NaHCO₃ (36mg), Ca(NO₃)₂·4H₂O (35mg), and MgSO₄·7H₂O (60mg). The pH of solution was constant at 7.0±0.2. The ionic strength (IS) of the solution was 3 mM, typical for groundwater (15, 16). Suwannee River natural organic matter (NOM) obtained from the International Humic Substances Society (St. Paul, MN) was added (final concentration of 10 mg/L) to the background AGW. The second stress condition (denoted as AGW-4C) was background AGW solution kept at 4°C following the inoculation of the mutant. The third stress condition (denoted as AGW-pH10) was background AGW with a modified pH of 10. The fourth stress condition (denoted as AGW-Static) was background AGW which was not shaken and kept static at room temperature during the course of the study. The last stress condition was artificial seawater (denoted as ASW), prepared by dissolving a mixture of sea salts (final concentration of 30 g/L) (Sigma-Aldrich, St. Louis, MO) in DIW, mildly shaken and kept at room temperature. The IS of ASW was ~700 mM.

6.2.4 Viability and Cultivability

At the end of four weeks of stress exposure, percent viabilities of the cells were determined by using the Live/Dead BacLight® kit (L-7012; Molecular Probes, Eugene, OR) according to the manufacturer's directions. Direct counting of the stained live and dead cells was done using an inverted microscope (IX70; Olympus, Japan) operated under red/green fluorescence filter set (Chroma Technology Corp., Brattleboro, VT). Stressed organisms were also tested for cultivability by spread plating serial dilutions of cells on LB agar (w/ Kan, Cm) (Fisher Scientific) plates followed by overnight incubation at 37°C. Colony forming units (CFUs) were enumerated the following day.

6.2.5 Re-growth of Stressed Bacteria and DNA Extraction

At the end of four weeks period, 25 mL of bacterial suspension was recovered from each stress condition flask and inoculated into 50 mL of growth medium (2× LB w/ Kan and Cm) and incubated at 37°C for 12 hours. Following, 40 mL of re-grown library strains were centrifuged at $3,700 \times g$ force for 15 min at 4°C (5804R; Eppendorf) equipped with fixed angle rotor (F-34-6-38; Eppendorf). Growth medium was decanted and the pellet was resuspended in ice-cold PBS. Resuspended cells were transferred to eppendorf tubes and centrifuged at $14,000 \times g$ force for 2 min at room temperature (5415D; Eppendorf) equipped with fixed angle rotor (F-45-24-11; Eppendorf) to remove PBS. Genomic DNA was extracted by using GenElute Mammalian Genomic DNA kit (Sigma), following the manufacturer's instructions. Extracted DNA samples' quantity was measured by a Nanospec (ND-1000, NanoDrop Technologies, Wilmington, DE). DNA integrity was checked with 1% agarose gel electrophoresis. DNA samples were kept frozen at -20°C until further use.

6.2.6 Labeling Protocol, Part I: DNA Sonication and Polyadenylation

The first part of the labeling protocol is as follows. 4 µg of genomic DNA was fragmented by sonication using five pulses of five seconds (Branson Sonifier 150, Branson Ultrasonics Corp., Danbury, CT). PolyA tails were added to fragmented genomic DNA using terminal transferase (TdT) as follows: 1.5 µg of DNA fragments were incubated for 30 min at 37°C in a total reaction volume of 50 µl containing 40 U TdT (New England BioLabs, Ipswich, MA), 0.25 mM CoCl₂, and 0.4 mM dATP.

Terminal transferase was subsequently inactivated at 70°C for 10 min and the tailed product was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA).

6.2.7 Labeling Protocol, Part II: PCR Amplification and Labeling of Fragments Adjacent the Deletion Location

Following the first part, nested PCR was used to amplify the polyA-tailed DNA fragments containing the insert P_{T7} and the flanking inserted region. In the first PCR reaction, 50 ng of purified polyA-tailed DNA was used as template for a PCR reaction using primer DOPR2 (5'-CAACGCAGACCGTTCGTGGCA), and a primer (CCT₂₄VN) designed to anneal to the polyA-tail (5'-CCTTTTTTTTTTTTTTTTTTTTTTTTVN). The reaction mixture consisted of 1× PCR buffer, 0.2 mM of dNTP, 1.5 mM MgCl₂, 0.5 U/μl Taq polymerase (Promega, Fitchburg, WI), and 0.2 μM of each forward and reverse primer in a total reaction volume of 25 μl. The PCR reaction was performed under the following conditions; initial denaturation at 94°C for 1 min followed by 30 cycles with denaturation at 94°C for 10 s, annealing at 50°C for 10 s, and extension at 72°C for 5 s. The last cycle was followed by a final extension for 3 min at 72°C. In the second amplification step, a nested PCR was performed using 1 μl amplified product from the initial PCR in a total volume of 50 μl. Internal primer KAN2FP1-B (5'-GTCCACCTACAACAAAGCTCTCATCAACC) and primer CCT₂₄VN were used under identical cycling conditions during the initial PCR reaction. PCR products were analyzed on 1% agarose gels. An aliquot of 8 μl of the nested PCR reaction product was used directly as template for a 20 μl *in vitro* transcription reaction using the AmpliScribe T7 transcription kit (Epicentre) following the manufacturer's protocol with minor

modifications. The RNA was labeled during the synthesis by including 2 μ l of 5 mM Cy5- or Cy3-UTP (GE Healthcare, Waukesha, WI) in the *in vitro* transcription reaction. Labeled RNA was treated with RNase-free DNase (Epicentre) and purified with the RNeasy Mini Kit (Qiagen).

6.2.8 Nimblegen High Density Array and Hybridization Protocol

High density oligo arrays were manufactured at Roche Nimblegen, Madison, WI. The arrays consisted of 386,010 *Salmonella*-specific oligos, designed to cover the entire genomes of *S. typhimurium* strains LT2 and 14028s on both strands in a tiling pattern; each oligo overlapping with the next one (centers are 24 bases apart on each strand, interlocking every 12 bases with oligos representing the opposite strand). The oligos were matched by T_m , with a minimum of 46 bases in length. About 700 oligo probes were represented in duplicate on the array, and about 900 *Salmonella enterica* serovar Enteritidis PT4-specific oligos were added as negative controls.

Hybridizations were performed according to the manufacturer's protocols (NimbleGen) with slight modifications. For each hybridization, 4 μ g of labeled RNA was mixed with the alignment oligo, NimbleGen hybridization components, and hybridization buffers. The arrays were hybridized at 42°C for 16 hours. Arrays were washed according to the manufacturer's protocol, and scanned using a GenePix 4000B laser scanner (Molecular Devices, Sunnyvale, CA) at 5 μ m resolution. The signal intensities were quantified using NimbleScan software v2.4 (NimbleGen). The data was normalized and analyzed using Webarray (17, 18) and WebarrayDB (19), with quantile

normalization. The moving median intensity of five adjacent probes on the same strand in the genome was calculated and plotted against the genome annotation.

6.3 Results and Discussion

6.3.1 Viability and Cultivability of *Salmonella typhimurium*

Changes in percent viability and cultivability of the *S. typhimurium* mutant library exposed to stress conditions were determined at the end of four weeks and are presented in Table 6.1. Percent viability and cultivability of cells exposed to AGW as the background survived better than cells exposed to ASW. As can be seen in Table 6.1, 84% cells exposed to AGW with NOM were viable at the end of the tested period. The cultivability of cells was much lower, i.e. 32%, in AGW with NOM. Viabilities of the mutants that were exposed to AGW at a high pH and AGW kept at 4°C were similar, 88% and 83%, respectively. Additionally, the cultivability of these mutants was determined as 42% and 34% in AGW at high pH and low temperature, respectively. In the case of AGW under a static condition (not shaken), the viability and cultivability were lower than the other AGW background conditions, and determined to be 69% and 28%, respectively. At the end of four weeks, the mutant library exposed to ASW showed high percentage viability (87%), and poor cultivability (3%).

One of the reasons that both the viability and cultivability were monitored over the course of study was to determine what percentage of cells would remain structurally intact (2) (as indicated by viability) and sustain the ability to reproduce (indicated by cultivability). Secondly, it was important to observe the extent of cells that remained

viable but nonculturable (VBNC) (6, 58, 79, 80, 83) among the mutant library since they may possible pose health risks (6, 18, 29, 59, 82). The results have shown that the majority of mutants exposed to AGW and ASW remained viable, independent of the nature of the peripheral stress condition. Under the same conditions, 30% to 40% of mutants were cultivable, except mutants exposed to ASW, concluding that they still have the capacity to revive and multiply in case of an infection to another target host following the stress event.

Table 6.1. Percent viability and cultivability of *S. typhimurium* exposed to stress conditions at the end of 4 weeks.

Stress condition ^a	Viability (%)	Cultivability (%)
AGW-NOM	84 ± 3	32 ± 3
AGW-4C	88 ± 6	42 ± 6
AGW-pH 10	83 ± 4	34 ± 4
AGW-Static	69 ± 19	28 ± 5
ASW	87 ± 4	3 ± 1

^a Abbreviations denote the following stress conditions: AGW-NOM: Artificial groundwater-natural organic matter; AGW-4C: Artificial groundwater kept at 4°C; AGW-pH10: Artificial groundwater with pH adjustment to 10; AGW-Static: Artificial groundwater kept at static conditions; ASW: Artificial seawater.

6.3.2 Survival and Fitness of *Salmonella typhimurium* mutants under Stress

Microarray based detection of genes required for fitness and survival is presented in Appendix A for each stress condition. Mutants which harvested a transposon in a gene required for survival at the specified stress condition died and returned a lower signal than in the input pool (control group, no exposure to stress). Signal intensities were detected and quantified to identify genes required for survival (referred to as down-regulated).

Certain genes that resulted in higher signal intensities after exposure than in the input pool (referred to as up-regulated) were also determined. Deletion of these genes probably provides an advantage to the mutant for either fitness or survival at the specified stress condition. The following sections provide detailed information on down-regulated genes for each stress condition. Due to the amount and complexity of the data obtained from the microarray analysis, only down-regulated genes were analyzed in detail. However, the genes that consistently resulted in an up-regulated signal in all stress conditions were determined and presented as well (Table 6.2 and 6.3).

6.3.3 Effects of Artificial Groundwater with Natural Organic Matter

Microarray analysis performed with the library of mutants exposed to AGW-NOM identified 302 genes required for survival (down) and 815 gene mutations of which enhance the bacterium's fitness (up). The full annotated list of down-regulated genes due to exposure to AGW-NOM is presented in Appendix A.

Some of the genes identified with lower signal after exposure to this condition are related to cell division (*sulA*, *ftsW*, *ftsO*, and *ftsA*) (30), suggesting that *S. typhimurium* exposed to AGW with NOM needs a fully functional cell multiplication apparatus to survive. In addition, the signal for the transcriptional regulator PhoB (31) was down, another major regulatory protein required for survival in AGW-NOM. A carbon starvation protein coding gene, *cstA*, similar to the carbon starvation protein of *E. coli* (32), was also needed. The *cst* genes are cyclic AMP (cAMP)-dependent carbon starvation response genes that are involved in nutrient scavenging (32, 33). Previous studies have confirmed that *cstA* shows increased activity when *E. coli* growth medium

was depleted of succinate, glycerol, or glucose or when *E. coli* grown in Luria broth (LB) enters the stationary phase (34). Clearly, *S. typhimurium* experiences similar deprivation under these stress conditions as well. Several universal stress protein coding genes were required in AGW-NOM as well. One of those, *ybdQ* (35, 36), is involved in a universal stress protein similar to the function of *uspA* (37). In addition, *yecG* (38), coding for a membrane associated stress protein produced under nutrient deprivation, osmotic shock and oxidative stress was also necessary (i.e, signal was down after exposure to the stress condition). Mutants in genes involved in the flagellar regulon of *S. typhimurium*, *flgB* (39) and *fliI*, were also underrepresented after stress suggesting that the flagella may play a role in survival. Finally, several genes involved in virulence of *S. typhimurium* such as *mviN*, *spaR* (40, 41), and *ssaK* (42) (type III secretion system) were also necessary during the exposure to NOM containing AGW for four weeks. This observation suggests that *Salmonella* virulence genes assist in survival mechanisms under stress.

6.3.4 Effects of Artificial Groundwater Kept at 4°C

Microarray analysis performed on the library of mutants exposed to AGW at 4°C identified 207 genes required for survival (down) and 448 genes mutations of which enhance the bacterium's fitness (up). The fully annotated list of required genes after exposure to AGW kept at 4°C for four weeks is presented in Appendix A.

One of the genes of major importance is *pspA* (43), involved in phage shock. This gene maintains the cell membrane potential under stress conditions and also functions as an important surface antigen, since it causes the activation of host immune response (44). Genes involved in flagella expression and synthesis, *flgD* (43, 45) and *fliZ* (46, 47), were

also beneficial in 4°C kept AGW as previously observed in AGW-NOM. In addition, the signal for the gene involved in the potassium transport (*kefB*) through the glutathione-regulated potassium-efflux system (48, 49) was down; suggesting that mutants lacking this gene were not able to survive in AGW, a potassium rich matrix.

6.3.5 Effects of Artificial Groundwater with High pH

Exposure of the library of mutants to AGW with pH10 resulted in 157 genes required for survival (down) and 278 mutations of which enhance the bacterium's fitness (up). The fully annotated list of genes required during exposure to AGW with pH10 for four weeks is presented in Appendix A.

Signals for the universal stress protein coding genes, *uspA* (37), *ybdQ* (35, 36), and the transcriptional regulator *phoB* (31) were down, as observed in mutants exposed to AGW-NOM. In addition, the *pspA* signal (43), was also down, similar to AGW-4°C.

The signal for the two-component major system regulator *uvrY* (along with *barA*) was down after exposure to AGW-pH10. This response regulator functions in efficient switching between glycolytic and gluconeogenic carbon sources and also regulates virulence gene expression (50, 51).

Several genes involved in the sugar uptake and transport over the cell membrane were found to be necessary for survival in AGW-pH10. Specifically, signals for *yneA* (codes for a putative sugar transport protein), *malM* (52) (maltose regulon periplasmic protein), and *slrB* (catalyzes the phosphorylation of incoming sugar substrates concomitant with their translocation across the cell membrane) (13) were down, suggesting sugar uptake to be an essential mechanism of cell survival under high pH conditions. Signal for the gene

coding for a protein involved in glycogen synthesis, *glgS* (53), was also down. Glycogen plays an important role in the glucose metabolism, and is generally kept as an energy reserve (54).

6.3.6 Effects of Artificial Groundwater Kept at Static Condition

Microarray analysis of the library of mutants exposed to AGW and kept statically during the exposure period of four weeks resulted in 151 genes required for survival (down) and 258 genes mutations of which enhance the bacterium's fitness (up). The fully annotated list of genes the signal of which went down after exposure to AGW kept in static conditions (not shaken) for four weeks is presented in Appendix A.

Some of the major genes that were observed as important in previously mentioned AGW stress conditions, such as *phoB*, *ybdQ*, *sula*, *pspA*, *glgS* and *malM*, were also found to be necessary for survival in static conditions.

Signals for genes involved in the synthesis of fimbriae, *fimW* (55) and *pefI* (56), were down. This observation may be attributed to the fact that cell-cell and cell-surface interactions would be favored under static conditions as a mechanism to cope with external stress. Indeed, signals for the genes involved in the conjugal transfer pilus (the term is used interchangeably with fimbria (57)) assembly proteins, *traW* and *trbC* (58), were also down.

6.3.7 Effects of Artificial Seawater

Microarray analysis of the mutants exposed to ASW showed that the number of regulated genes were lower than those exposed to AGW background stress conditions. Specifically, the numbers of regulated genes were as follows: 92 genes beneficial for

survival (down) and 192 genes mutations of which enhance the bacterium's fitness (up).. The fully annotated list of genes signals of which went down after exposure to ASW for four weeks is presented in Appendix A.

The majority of genes helping survival of the bacterium in ASW were common to those identified under AGW stress conditions, such as *phoB*, *sulA*, *pspA*, and *glgS*. This observation suggests that *S. typhimurium* requires similar genes and regulators involved in energy source utilization and universal stress responses to survive the high osmotic stress present in both AGW and ASW.

6.3.8 Genes with Consistent Increase or Decrease in Signal in all Stress Conditions

As mentioned earlier, due to the size and complexity of the microarray data, it was impossible to interpret and evaluate data from all stress conditions. Therefore, only the genes with consistently lower or higher signal after exposure to the stress conditions tested are listed in Tables 6.2 and Table 6.3, respectively.

The signal for an extensively studied major sigma factor (RpoS) in *Salmonella*, controlling the regulation of genes required for protection against external stresses (59-66), was observed to be down under all stress conditions, confirming validity of the whole experimental setup (Table 6.2). Previous studies also documented that RpoS also regulates the virulence of *Salmonella* and is required for infection (63, 67-71). This observation confirms the previously mentioned hypothesis that the mechanisms of virulence and survival are co-regulated as *Salmonella* experiences external stress.

Other significant genes observed to be involved in survival for all stress conditions were *phoB*, a transcriptional and two component response regulator; *fimW*, a putative

fimbrial protein coding gene; *sulA*, the gene responsible for the inhibition of cell division; *phoH*, the gene induced by phosphate starvation and a PhoB-dependent ATP-binding Pho regulon component; *pspA*, the phage shock protein involved in the maintenance of membrane potential under stress; *malM*, the gene coding for the maltose regulon, and finally *traW* and *traC*, the genes responsible for pilus assembly.

The gene involved in the transfer of vitamin B₁₂ from the external environment into the cytosol, *btuD* (72), was also observed to be required for survival, suggesting sequestering of B₁₂ to be an important stress coping mechanism in *S. typhimurium*. This was further confirmed with the observation that signals for another biopolymer transport gene (including vitamin B₁₂ uptake), *exbB* (73, 74), went down in all stress conditions.

Another important gene observed to be consistently involved in survival was *tqsA*, involved in the transport of a quorum sensing signal auto-inducer (75, 76). This observation suggests that quorum sensing is also an essential component of survival (77, 78) among *Salmonella* experiencing stress. This phenomenon was confirmed in previous studies described elsewhere (79, 80).

Table 6.2. Genes increasing survival of *S. typhimurium* exposed to aquatic stress conditions for 4 weeks (signals down).

Gene symbol	Gene	AGW-NOM	AGW-4C	AGW-pH 10	AGW-Static	ASW	Function ^a
STM0070	<i>caiD</i>	U ^b	- ^c	U- ^d	I- ^e	U-	carnitiny-CoA dehydratase; catalyzes the dehydration of L-carnitiny-CoA to crotonobetainyl-CoA
STM0136	<i>secA</i>	I-	I-	-	-	U	preprotein translocase subunit SecA; synonyms: azi, div; functions in protein export
STM0397	<i>phoB</i>	-	U-	-	-	-	transcriptional regulator PhoB; two component response regulator for the phosphate regulon; PhoR phosphorylates PhoB
STM0474	<i>ybaJ</i>	-	-	-	-	-	hypothetical protein
STM0486	<i>recR</i>	-	-	I-	-	I-	recombination protein RecR; involved in a recombinational process of DNA repair, independent of the recBC complex
STM0502	<i>ybbL</i>	-	-	-	-	-	putative ABC transporter ATP-binding protein YbbL; similar to <i>Escherichia coli</i> putative ATP-binding component of a transport system
STM0552	<i>fimW</i>	I-	-	I-	-	-	putative fimbrial protein; fimbriae W protein
STM0555		U	U	-	I-	-	pseudogene; frameshift relative to <i>Escherichia coli</i> IS3 putative transposase
STM0559	<i>rfbI</i>	-	-	-	-	-	putative glycosyl translocase
STM0605	<i>ybdN</i>	I-	-	-	-	I-	putative 3'-phosphoadenosine 5'-phosphosulfate sulfotransferase; PAPS reductase/FAD synthetase
STM0614	<i>ybdQ</i>	-	-	-	-	I-	putative universal stress protein; similar to UspA and related nucleotide-binding protein
STM0619	<i>citG</i>	-	I-	U-	I-	U	triphosphoribosyl-dephospho-CoA synthase; catalyzes the formation of 2'-(5"-triphosphoribosyl)-3'-dephospho-CoA from ATP and 3-dephospho-CoA
STM0627	<i>dcuC</i>	-	-	-	-	I-	C4-dicarboxylate transporter DcuC; responsible for the transport of C4-dicarboxylates during anaerobic growth
STM0681	<i>nagD</i>	I-	-	I-	I-	U-	UMP phosphatase; similar to <i>Escherichia coli</i> N-acetylglucosamine metabolism
STM0682	<i>nagC</i>	I-	-	-	-	-	N-acetylglucosamine operon transcriptional repressor; similar to <i>Escherichia coli</i> transcriptional repressor of nag; N-acetylglucosamine operon; NagC/XylR family
STM0722		-	-	I-	-	U-	Not annotated
STM0766	<i>dcoC</i>	-	-	-	-	-	oxaloacetate decarboxylase subunit gamma; catalyzes the decarboxylation of oxaloacetate to form pyruvate and carbon dioxide
STM0812	<i>ybhO</i>	-	-	U-	I-	U-	cardiolipin synthase 2; similar to <i>Escherichia coli</i> putative synthetase; CL synthase
STM0826	<i>ybiN</i>	-	-	-	-	I-	putative SAM-dependent methyltransferase
STM0870		-	-	-	-	I-	hypothetical protein; similar to <i>Escherichia coli</i> putative transport protein
STM0970	<i>pflA</i>	-	I-	I-	-	I-	pyruvate formate lyase-activating enzyme 1; activates pyruvate formate-lyase 1 under anaerobic conditions

Table 6.2 (Cont.). Genes increasing survival of *S. typhimurium* exposed to aquatic stress conditions for 4 weeks (signals down).

Gene symbol	Gene	AGW-NOM	AGW-4C	AGW-pH 10	AGW-Static	ASW	Function
STM1010		-	-	-	-	-	hypothetical protein
STM1021		-	-	-	-	-	hypothetical protein
STM1029		-	I-	I-	I-	I-	hypothetical protein; bacteriophage ES18 gp15 protein
STM1047		-	-	-	-	-	tail assembly protein
STM1071	<i>sulA</i>	-	-	-	-	-	SOS cell division inhibitor; suppressor of lon; inhibitor of cell division and FtsZ ring formation upon DNA damage/inhibition; HslVU and Lon involved in its turnover
STM1115	<i>scsC</i>	I-	I-	I-	-	U	suppression of copper sensitivity protein; suppressor for copper-sensitivity C; suppression of copper sensitivity
STM1126	<i>phoH</i>	-	I-	-	I-	U-	similar to <i>Escherichia coli</i> PhoB-dependent ATP-binding pho regulon component; may be helicase; induced by P starvation; contains a frameshift; similar to STM0669
STM1136	<i>ycdX</i>	-	-	-	-	-	putative hydrolase; similar to hydrolases of the PHP family
STM1213	<i>ycfQ</i>	-	-	-	-	-	putative transcriptional repressor; TetR/AcrR family
STM1282	<i>yeaK</i>	-	-	-	-	-	putative cytoplasmic protein
STMsR050	<i>STnc530</i>	-	-	-	-	-	Not annotated
STM1304	<i>astA</i>	U	-	I-	I-	U-	arginine succinyltransferase
STM1342	<i>btuD</i>	-	-	-	-	-	vitamin B12-transporter ATPase; ATP-binding protein that acts with the transmembrane protein BtuC and the solute binding protein BtuF to transport vitamin B12 into the cell
STM1374	<i>ynhA</i>	-	-	-	-	I-	cysteine desulfuration protein SufE; Acts with SufS to catalyze the formation of L-alanine from L-cysteine
STM1478	<i>ydgH</i>	U	-	-	-	I-	putative periplasmic protein
STM1481	<i>tqsA</i>	-	-	U-	I-	U-	putative transport protein; synonym: ydgG; transport of quorum-sensing signal; mutations in this gene alters the transport of the quorum-sensing signal autoinducer 2 (AI-2) which affects expression of a large number of genes
STM1484		-	-	-	I-	I-	putative protease
STM1575		-	U-	I-	-	I-	putative transcriptional regulator; TetR family
STM1690	<i>pspA</i>	-	-	-	-	-	phage shock protein PspA; involved in maintaining membrane potential under membrane stress conditions; also acts as a negative transcriptional regulator of the phage shock protein (psp) operon (pspABCDE) by regulating the transcriptional activator PspF
STM1730	<i>yciE</i>	I-	-	-	-	-	putative cytoplasmic protein

Table 6.2 (Cont.). Genes increasing survival of *S. typhimurium* exposed to aquatic stress conditions for 4 weeks (signals down).

Gene symbol	Gene	AGW-NOM	AGW-4C	AGW-pH 10	AGW-Static	ASW	Function
STM1869		-	-	-	-	I-	phage-tail assembly-like protein
STM1869A		I-	-	I-	I-	NT ^d	hypothetical protein
STM1883	<i>purT</i>	U	I-	I-	I-	U	phosphoribosylglycinamide formyltransferase 2
STM1902	<i>yecD</i>	-	-	-	-	U	hypothetical protein
STM2008		-	-	-	-	-	putative periplasmic protein
STM2076	<i>hisA</i>	-	-	-	-	I-	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino]imidazole-4-carboxamide isomerase
STM2255	<i>napC</i>	I-	-	-	-	-	cytochrome c-type protein NapC; with NapABDFGH functions as a nitrate reductase; NapC functions as an electron shuttle between NapAB and NapGH or quinone
STM2269	<i>yojN</i>	-	I-	-	-	I-	phosphotransfer intermediate protein in two-component regulatory system with RcsBC; similar to <i>Escherichia coli</i> putative 2-component sensor protein; regulatory protein
STM2360		I-	-	-	-	-	putative diaminopimelate decarboxylase; similar to <i>Escherichia coli</i> diaminopimelate decarboxylase
STM2475		-	-	-	-	NT	putative cytoplasmic protein
STM2590		-	-	-	-	I-	tail assembly protein I-like; similar to tail assembly protein I in phage lambda
STM2591		-	-	-	-	-	tail assembly protein K-like; similar to tail assembly protein K in phage lambda
STM2656	<i>rrfG</i>	-	-	-	-	-	5S ribosomal RNA
STM2706		-	I-	I-	-	-	phage tail-like protein; similar to tail fiber protein in phage P2
STM2787		-	NT	-	-	-	tricarboxylic transport
STM2798	<i>ygaP</i>	I-	U-	U-	I-	U-	putative rhodanese-like sulfurtransferase
STM2815	<i>emrB</i>	-	I-	I-	-	U	putative multidrug transport protein; similar to <i>Escherichia coli</i> multidrug resistance; probably membrane translocase; MFS family
STM2871	<i>prgK</i>	-	-	-	-	-	needle complex inner membrane lipoprotein; PrgK protein precursor; cell invasion protein; interacts with PrgH and forms oligomeric rings; type III secretion apparatus
STM2872	<i>prgJ</i>	-	-	-	-	-	needle complex minor subunit; PrgJ protein; invasion protein; may cap needle complex; type III secretion apparatus
STM2924	<i>rpoS</i>	-	-	-	-	I-	This sigma factor controls a regulon of genes required for protection against external stresses
STM3015	<i>ygeA</i>	-	-	-	-	I-	putative racemase; similar to <i>Escherichia coli</i> putative resistance proteins

Table 6.2 (Cont.). Genes increasing survival of *S. typhimurium* exposed to aquatic stress conditions for 4 weeks (signals down).

Gene symbol	Gene	AGW-NOM	AGW-4C	AGW-pH 10	AGW-Static	ASW	Function
STM3057	<i>ubiH</i>	U-	-	I-	I-	I-	2-octaprenyl-6-methoxyphenyl hydroxylase; Oxygenase that introduces the hydroxyl group at carbon four of 2-octaprenyl-6-methoxyphenol resulting in the formation of 2-octaprenyl-6-methoxy-1,4-benzoquinone
STMsR119	<i>rygC</i>	-	I-	-	-	U-	Not anoted
STM3084.S		-	-	-	-	-	putative regulatory protein; similar to <i>Escherichia coli</i> regulator for uxu operon; GntR family
STM3119		-	-	-	-	-	putative monoamine oxidase; similar to <i>Escherichia coli</i> putative aldehyde dehydrogenase
STM3159	<i>exbB</i>	-	-	I-	-	U-	biopolymer transport protein ExbB; membrane spanning protein in TonB-ExbB-ExbD complex; involved in the tonB-independent energy-dependent transport iron-siderophore complexes and vitamin B12 into the cell
STM3161	<i>metC</i>	-	-	I-	I-	-	cystathionine beta-lyase; catalyzes the formation of L-homocysteine from cystathionine
STM3353	<i>oadG</i>	-	-	-	-	-	oxaloacetate decarboxylase subunit gamma; catalyzes the decarboxylation of oxaloacetate to form pyruvate and carbon dioxide
STM3365	<i>yhcQ</i>	-	-	-	-	I-	p-hydroxybenzoic acid efflux subunit AaeA; with AaeB forms an efflux pump whose substrates are p-hydroxybenzoic acid, 6-hydroxy-2-naphthoic and 2-hydroxycinnamate
STM3547.Sc		U-	-	-	-	I-	putative transcriptional regulator; regulator of sugar metabolism
STM3624A		U	-	-	-	-	cystathionine gamma-synthase
STM3653		-	-	-	-	-	putative acetyltransferase
STM3654		-	-	-	-	-	pseudogene; in-frame stop following codon 23
STM3657		-	-	-	-	U	putative outer membrane lipoprotein
STM3681		-	-	-	-	-	putative transcriptional regulator; similar to <i>Escherichia coli</i> possible NagC-like transcriptional regulator
STMsR141	<i>STnc380</i>	-	-	-	-	-	Not annotated
STM3747	<i>yicE</i>	-	-	I-	I-	I-	putative purine/xanthine transport protein; similar to <i>Escherichia coli</i> putative transport protein; NCS2 family
STM3754		-	-	I-	-	-	putative cytoplasmic protein; similar to putative ATP binding protein SugR
STM3758	<i>fidL</i>	-	-	-	-	-	putative inner membrane protein; FidL
STM3793		-	-	U-	I-	U-	putative sugar kinase; similar to <i>Escherichia coli</i> ribokinase; ribokinase family
STM3863		-	-	U-	I-	U-	putative permease
STM3873.S	<i>gidB</i>	-	U-	-	-	-	glucose-inhibited division protein B; probable SAM-dependent methyltransferase

Table 6.2 (Cont.). Genes increasing survival of *S. typhimurium* exposed to aquatic stress conditions for 4 weeks (signals down).

Gene symbol	Gene	AGW-NOM	AGW-4C	AGW-pH 10	AGW-Static	ASW	Function
STM3914	<i>rhlB</i>	-	-	-	I-	-	ATP-dependent RNA helicase RhlB; enables ATP-dependent unwinding of double stranded RNA as a component of the RNA degradosome, a multi-enzyme complex important in RNA processing and messenger RNA degradation
STM4073	<i>ydeW</i>	U	I-	-	-	I-	putative transcriptional repressor; similar to <i>Escherichia coli</i> putative transcriptional regulator, sorC family
STM4184	<i>aceA</i>	-	-	I-	-	I-	isocitrate lyase; Catalyzes the first step in the glyoxalate cycle, which converts lipids to carbohydrates
STM4185	<i>aceK</i>	-	I-	-	I-	-	bifunctional isocitrate dehydrogenase kinase/phosphatase protein
STM4232	<i>malM</i>	U-	-	-	-	I-	maltose regulon periplasmic protein; maltose operon periplasmic protein precursor; protein of mal regulon
STM4279	<i>nrfC</i>	I-	-	-	-	U	putative formate-dependent nitrite reductase; similar to <i>Escherichia coli</i> formate-dependent nitrite reductase; FeS centers
STM4298	<i>melA</i>	I-	-	I-	I-	U-	alpha-galactosidase; alpha-galactosidase
STM4421		I-	-	I-	I-	U-	putative NAD-dependent aldehyde dehydrogenase; similar to <i>Escherichia coli</i> phenylacetaldehyde dehydrogenase
STM4468	<i>yjgK</i>	-	-	U-	-	U-	putative cytoplasmic protein
STM4503		I-	U-	-	-	-	putative inner membrane protein
STM4542	<i>yjjA</i>	I-	-	-	I-	U-	hypothetical protein; similar to <i>Escherichia coli</i> putative glycoprotein/receptor
STM4550	<i>fhuF</i>	-	-	-	-	I-	ferric iron reductase involved in ferric hydroximate transport; involved in reduction of ferric iron in cytoplasmic ferrioxamine B
STM4558	<i>rimI</i>	NT	-	-	-	-	ribosomal-protein-alanine N-acetyltransferase; alanine acetyltransferase that specifically acetylates ribosomal protein S18
PSLT013	<i>pefI</i>	-	-	-	-	-	putative outer membrane lipoprotein
PSLT091	<i>traW</i>	-	-	-	-	-	conjugal transfer pilus assembly protein TraW; conjugative transfer
PSLT094	<i>trbC</i>	-	-	-	-	-	conjugal transfer pilus assembly protein TrbC; conjugative transfer
PSLT094.1N		-	-	-	-	U-	hypothetical protein
STM4232	<i>malM</i>	U-	-	-	-	I-	maltose regulon periplasmic protein; maltose operon periplasmic protein precursor; protein of mal regulon
STM4279	<i>nrfC</i>	I-	-	-	-	U	putative formate-dependent nitrite reductase; similar to <i>Escherichia coli</i> formate-dependent nitrite reductase; FeS centers
STM4298	<i>melA</i>	I-	-	I-	I-	U-	alpha-galactosidase; alpha-galactosidase

Table 6.2 (Cont.). Genes increasing survival of *S. typhimurium* exposed to aquatic stress conditions for 4 weeks (signals down).

Gene symbol	Gene	AGW-NOM	AGW-4C	AGW-pH 10	AGW-Static	ASW	Function
STM4421		I-	-	I-	I-	U-	putative NAD-dependent aldehyde dehydrogenase; similar to <i>Escherichia coli</i> phenylacetaldehyde dehydrogenase

^aThe annotated functions of genes were cited from (13).

^b Unchanged.

^c Down.

^d Unchanged; possibly down. This response is referred to mild but consistent changes in one direction (down) that did not reach the minimum cut-offs for the number of oligos that showed a change. Thus, the data is insufficient to make a definitive call but the tendency is in a particular direction (down).

^e Intermediate down.

^f No transposon. This abbreviation is referred to conditions that no transposon reliably detected for this gene, possibly due to the short size of the gene making it harder to be reached by a transposon.

Table 6.3 lists the functions of genes that signal of which was consistently up in all stress conditions. As mentioned earlier, mutation of these genes would provide the bacteria an advantage for survival under specified stress condition.

Signals of genes involved in the synthesis of specific fimbriae [*sthA*, *sthB* (81), *ppdD* (82)] and pili [*traA* (83), *traE* (84), *stbE* (85)] were determined to be commonly up in all stress conditions. In addition to pili and fimbriae, some genes coding for the flagella, *fliA* (86, 87), *flgF* (88, 89), and *fliO* (90), were also found to negatively affect survival. This observation suggests that production of these bacterial surface appendages may require too much energy during the starvation conditions experienced by *Salmonella* under stress, and mutants that cannot make these structures anymore may outgrow their counterparts under conditions where these appendages are not required.

The major Mg^{2+} transport protein coding gene, *mgtC* (91-93), required for intra macrophage survival and growth in low calcium and controlled by PhoP/PhoQ (94-97) was also found to decrease survival rates. This was an unexpected outcome, considering the Mg^{2+} rich environment of AGW and ASW. Finally, signals for two major invasion proteins, SipA (98-101) and HilD (102-104), were consistently up. The fact that signals for some of the major virulence genes were down (required for survival) and some of them were up (decreasing fitness and survival), confirms the complexity of the interplay between virulence and survival in *Salmonella*.

Table 6.3. Genes decreasing survival of *S. typhimurium* exposed to aquatic stress conditions for 4 weeks (signals up).

Gene symbol	Gene	AGW-NOM	AGW-4C	AGW-pH 10	AGW-Static	ASW	Function ^a
STM0053		+ ^b	+	+	+	I+ ^c	putative transcriptional regulator; similar to <i>Escherichia coli</i> putative sensor-type protein; histidine kinase for citrate
STM0071	<i>caiC</i>	+	+	+	+	I+	putative crotonobetaine/carnitine-CoA ligase; similar to <i>Escherichia coli</i> probable crotonobetaine/carnitine-CoA ligase
STM0080		+	+	+	+	+	putative outer membrane lipoprotein
STM0113	<i>leuA</i>	U+ ^d	I+	U+	I+	I+	2-isopropylmalate synthase; catalyzes the formation of 2-isopropylmalate from acetyl-CoA and 2-oxoisovalerate in leucine biosynthesis
STM0144	<i>ppdD</i>	+	+	+	+	+	putative major pilin subunit; similar to <i>Escherichia coli</i> prepilin peptidase dependent protein
STM0328.s		+	+	+	+	I+	putative permease; similar to <i>Escherichia coli</i> putative transport protein
STM0336	<i>stbE</i>	+	+	+	I+	I+	putative fimbrial chaparone; similar to <i>Escherichia coli</i> periplasmic chaperone; required for type 1 fimbriae
STM0512	<i>sfbC</i>	I+	U+	+	I+	+	putative ABC transporter permease component; similar to <i>Escherichia coli</i> putative transport system permease protein; binding-protein-dependent
STM0539		+	+	I+	+	NT ^e	putative inner membrane protein
STM0618	<i>citT</i>	+	I+	U+	+	U+	citrate/succinate transport antiport protein; similar to <i>Escherichia coli</i> putative membrane protein; DASS family
STMsR025	<i>sroC</i>	+	+	+	+	I+	Not annotated
STM0874	<i>mdaA</i>	+	+	I+	I+	U+	nitroreductase A; NADPH-dependent; oxygen-insensitive; catalyzes the reduction of nitroaromatic compounds
STM0881	<i>ybjO</i>	+	+	+	+	+	putative inner membrane protein
STM0884	<i>ulaA</i>	+	U+	+	I+	U+	ascorbate-specific PTS system enzyme IIC; synonym: sgaT; membrane component; functions with enzymes IIB (sgaB; ulaB) and IIA (sgaA; ulaC) enzyme I and HPr for anaerobic utilization and uptake of L-ascorbate; sgaTBA are regulated by yifQ as well as Crp and Fnr
STM1003		+	+	I+	+	I+	putative transcriptional regulator; similar to <i>Escherichia coli</i> inner membrane transport protein; Lrp family
STM1019		+	+	+	+	+	hypothetical protein
STM1050		+	+	+	+	I+	tail fiber assembly like-protein; similar to tail fiber assembly protein
STM1051	<i>sseI</i>	+	I+	+	+	+	secreted effector protein; secreted effector I; secreted by SPI-2; colocalizes with host polymerizing actin cytoskeleton
STM1112	<i>cbpA</i>	+	+	U+	I+	U+	curved DNA-binding protein CbpA; functional analog of DnaJ; co-chaperone with DnaK, molecular chaperone in an adaptive response to environmental stress

Table 6.3 (Cont.). Genes decreasing survival of *S. typhimurium* exposed to aquatic stress conditions for 4 weeks (signals up).

Gene symbol	Gene	AGW-NOM	AGW-4C	AGW-pH 10	AGW-Static	ASW	Function
STM1128		+	+	+	+	I+	putative sodium/glucose cotransporter; similar to <i>Escherichia coli</i> putative cotransporter
STM1149	<i>mdoC</i>	+	+	+	I+	I+	glucans biosynthesis protein; required for the transfer of succinyl residues to the glucan backbone
STM1163	<i>pyrC</i>	+	+	I+	+	I+	dihydroorotase; catalyzes the formation of N-carbamoyl-L-aspartate from (S)-dihydroorotate in pyrimidine biosynthesis
STM1178	<i>flgF</i>	+	I+	+	+	+	flagellar basal body rod protein FlgF; FlgF, with FlgB and C, makes up the proximal portion of the flagellar basal body rod
STM1182	<i>flgJ</i>	U ^f	I+	I+	+	+	peptidoglycan hydrolase; Flagellum-specific muramidase which hydrolyzes the peptidoglycan layer to assemble the rod structure in the periplasmic space
STM1226	<i>potA</i>	+	I+	I+	I+	U+	putrescine/spermidine ABC transporter ATPase protein; functions together with PotBCD in ATP-dependent polyamine transport; PotA is the membrane-associated ATPase
STM1249	<i>pliC</i>	+	I+	U+	+	+	lysozyme inhibitor; PliC; periplasmic lysozyme inhibitor of c-type lysozyme
STM1269		+	U+	+	+	U+	chorismate mutase; catalyzes the interconversion of chorismate to prephenate
STM1359	<i>aroE</i>	+	I+	I+	+	I+	quininate/shikimate dehydrogenase; YdiB; quininate/shikimate dehydrogenase from <i>Escherichia coli</i> uses both NAD and NAD(P) to convert quininate and shikimate to 3-dehydroquininate and 3-dehydroshikimate
STM1398	<i>sseB</i>	+	+	+	+	I+	translocation machinery component; putative secreted protein
STM1404	<i>sseF</i>	+	+	U	I+	I+	secreted effector protein; SseF
STM1418	<i>ssaQ</i>	+	I+	I+	I+	I+	type III secretion system protein; secretion system apparatus protein SsaQ; type III secretion system apparatus protein
STM1435	<i>gloA</i>	I+	U+	I+	+	U+	glyoxalase I; Ni-dependent; catalyzes the formation of S-lactoylglutathione from methylglyoxal and glutathione
STM1540		+	+	+	+	I+	putative hydrolase
STM1571	<i>yddG</i>	+	+	+	+	+	hypothetical protein
STM1657		+	U+	+	+	I+	putative methyl-accepting chemotaxis protein; similar to <i>Escherichia coli</i> methyl-accepting chemotaxis protein I serine sensor receptor; similar to C-terminus of many MCPs
STM1704	<i>yciT</i>	+	+	I+	+	I+	putative regulatory protein; similar to <i>Escherichia coli</i> putative DeoR-type transcriptional regulator; DeoR family
STM1705	<i>osmB</i>	+	U	+	+	+	lipoprotein; osmotically inducible lipoprotein B; osmotically inducible
STM1814	<i>minC</i>	+	+	+	I+	+	septum formation inhibitor; blocks the formation of polar Z-ring septums

Table 6.3 (Cont.). Genes decreasing survival of *S. typhimurium* exposed to aquatic stress conditions for 4 weeks (signals up).

Gene symbol	Gene	AGW-NOM	AGW-4C	AGW-pH 10	AGW-Static	ASW	Function
STM1874		+	I+	+	I+	I+	putative inner membrane protein; similar to <i>Escherichia coli</i> putative resistance protein
STM1933		+I	U+	+	+	+	hypothetical protein
STM1956	<i>fliA</i>	+	I+	I+	+	I+	flagellar biosynthesis sigma factor; sigma factors are initiation factors that promote the attachment of RNA polymerase to specific initiation sites and are then released; this sigma factor directs late flagellar biosynthesis genes
STM1976	<i>fliM</i>	I+	U	I+	I+	+	flagellar motor switch protein FliM; with FliG and FliN makes up the switch complex which is involved in switching the direction of the flagella rotation
STM1978	<i>fliO</i>	+	I+	+	+	+	flagellar biosynthesis protein FliO; with FlhA, FlhB, FliP, FliQ and FliR is one of the membrane components of the flagellar export apparatus
STM2017	<i>cobS</i>	+	I+	+	+	+	cobalamin synthase; catalyzes the formation of adenosylcobalamin from Ado-cobinamide-GDP and alpha-ribazole
STM2079	<i>wzzB</i>	+	+	U+	+	U+	lipopolysaccharide O-antigen chain length regulator; chain length determinant protein; polysaccharide antigen chainregulator; regulator of length of O-antigen component of lipopolysaccharide chains
STM2097	<i>rfbB</i>	+	+	I+	+	I+	dTDP-glucose 4,6 dehydratase; NAD(P) binding; catalyzes the formation of dTDP-4-dehydro-6-deoxy-D-glucose from dTDP-glucose
STM2131	<i>baeR</i>	+	I+	U+	I+	I+	DNA-binding transcriptional regulator BaeR; response regulator in two-component regulatory system with BaeS; regulator of RNA synthesis, flagellar biosynthesis, chemotaxis and transport
STM2153	<i>yehE</i>	+	+	+	+	+	putative outer membrane protein
STM2196		I+	+	+	I+	I+	putative L-serine dehydratase; similar to <i>Escherichia coli</i> L-serine deaminase
STM2222	<i>rsuA</i>	U	+	I+	I+	I+	16S rRNA pseudouridylyl synthase A; catalyzes the synthesis pseudouridine from uracil-516 in 16S ribosomal RNA
STM2239		+	+	I+	+	I+	putative phage antiterminator; similar to antiterminator protein Q of phage P5
STM2275		+	+	+	+	I+	putative regulatory protein; GntR family
STM2358		+	+	+	+	NT	putative cytoplasmic protein
STM2375		U+	+	U	I+	I+	putative cytoplasmic protein
STM2429	<i>cysZ</i>	+	+	+	+	I+	putative sulfate transport protein CysZ; putative role in sulfur assimilation
STM2534		+	I+	I+	+	I+	putative cytoplasmic protein
STM2547		+	+	+	+	NT	putative hydrolase; similar to <i>Escherichia coli</i> putative enzyme
STM2573		+	+	+	I+	+	2-dehydropantoate 2-reductase; catalyzes the formation of 2-dehydropantoate from (R)-pantoate

Table 6.3 (Cont.). Genes decreasing survival of *S. typhimurium* exposed to aquatic stress conditions for 4 weeks (signals up).

Gene symbol	Gene	AGW-NOM	AGW-4C	AGW-pH 10	AGW-Static	ASW	Function
STM2575		+	+	I+	I+	+	putative transcriptional regulator; similar to <i>Escherichia coli</i> cyn operon positive regulator; LysR family
STM2671	<i>yfiR</i>	+	+	+	+	+	putative periplasmic protein
STM2672	<i>yfiN</i>	+	+	I+	+	I+	hypothetical protein
STM2780	<i>pipB2</i>	+	+	+	+	+	secreted effector protein; PipB2; similar to STM1088; contains pentapeptide repeats; secreted by SPI-2 type III secretion; localizes to <i>Salmonella</i> -containing vacuole and host cell membranes
STM2782	<i>mig-14</i>	+	+	+	I+	U	putative transcriptional activator
STM2832	<i>srlA</i>	+	+	+	+	+	glucitol/sorbitol-specific enzyme IIC component; similar to <i>Escherichia coli</i> PTS system; one of two glucitol/sorbitol-specific components; PTS family
STM2864	<i>sitD</i>	I+	I+	U+	+	+	putative permease; SitD; <i>Salmonella</i> iron transporter; Fur-regulated
STM2865	<i>avrA</i>	+	+	+	+	+	secreted effector protein; invasion protein; inhibits activation of key proinflammatory transcription factor NF-kappaB
STM2868	<i>orgC</i>	+	+	+	+	NT	putative cytoplasmic protein
STM2869	<i>orgB</i>	+	+	U+	I+	U+	needle complex export protein; oxygen-regulated invasion protein OrgB; putative flagellar biosynthesis/type III secretion apparatus
STM2874	<i>prgH</i>	+	I+	I+	+	I+	needle complex inner membrane protein; PrgH protein; invasion protein; interacts with PrgK; tetramerizes; with PrgK forms multimeric ring-shaped structures; type III secretion apparatus
STM2875	<i>hilD</i>	+	+	+	+	+	invasion protein regulatory protein; HilD; helix-turn-helix proteins; AraC family; activator for invasion genes; derepresses hilA expression
STM2882	<i>sipA</i>	+	+	+	I+	I+	secreted effector protein; <i>Salmonella</i> invasion protein SipA; binds actin and promotes polymerization; results in membrane ruffling and internalization due to cytoskeleton rearrangements
STM2893	<i>invI</i>	+	I+	U+	I+	+	needle complex assembly protein; surface presentation of antigens protein SpaM; secretory proteins; type III secretion apparatus
STM2901		+	+	+	+	+	putative cytoplasmic protein
STM2907	<i>pphB</i>	+	+	+	+	+	serine/threonine-specific protein phosphatase 2; similar to <i>Escherichia coli</i> protein phosphatase 2
STM3007	<i>ygdR</i>	+	+	+	+	+	putative peptide transport protein; POT family
STM3030		+	+	+	+	+	hypothetical protein

Table 6.3 (Cont.). Genes decreasing survival of *S. typhimurium* exposed to aquatic stress conditions for 4 weeks (signals up).

Gene symbol	Gene	AGW-NOM	AGW-4C	AGW-pH 10	AGW-Static	ASW	Function
STM3124		+	+	+	+	+	putative response regulator; similar to <i>Escherichia coli</i> putative regulator; contains CheY-like receiver domain and HTH DNA-binding domain
STM3126		+	+	I+	+	U+	putative amino acid transporter; similar to <i>Escherichia coli</i> proline permease transport protein
STM3132		+	I+	+	I+	I+	putative xylanase/chitin deacetylase
STM3148	<i>hybB</i>	I+	I+	I+	I+	U+	putative hydrogenase 2 b cytochrome subunit; HybB; cytochrome b subunit of the hydrogenase 2 enzyme, composed of HybA, B, C, and O subunits
STM3154		+	+	+	+	+	putative ATP-dependent RNA helicase-like protein
STM3189	<i>ygiD</i>	I+	+	U+	I+	+	hypothetical protein; seems to be involved in biofilm formation; in asymptomatic bacteriuria <i>E. coli</i> strains 83972 and VR50, the <i>ygiD</i> gene is upregulated during biofilm formation in urine
STM3194		U	I+	I+	I+	+	putative disulfide oxidoreductase
STM3250	<i>garD</i>	I+	I+	I+	I+	I+	galactarate dehydrogenase; similar to <i>Escherichia coli</i> putative hydrolase
STM3255		+	+	U+	+	U+	putative phosphotransferase system fructose-specific component IIB; similar to <i>Escherichia coli</i> PTS system; fructose-specific transport protein
STM3278		+	+	I+	+	NT	putative cytoplasmic protein
STM3298.S	<i>yhbY</i>	+	+	NT	+	+	RNA-binding protein YhbY; RNA binding protein found associated to pre-50S subunit of the ribosome; putative role in ribosome assembly; necessary for optimal growth but not cell viability
STM3340	<i>yhcK</i>	+	+	I+	I+	U+	transcriptional regulator NanR; Transcriptional repressor of the nan operon that encodes proteins involved in sialic acid utilization
STM3357		+	+	I+	+	+	putative regulatory protein; GntR family
STM3384	<i>yhdG</i>	+	+	I+	I+	U+	tRNA-dihydrouridine synthase B; putative TIM-barrel enzyme; hypothetical 35.9 kDa protein in <i>pmrA-fis</i> intergenic region
STM3405	<i>smf</i>	+	+	I+	+	U+	hypothetical protein; similar to <i>Escherichia coli</i> orf; fragment 1; putative protein involved in DNA uptake
STM3458	<i>yheR</i>	+	+	+	+	+	glutathione-regulated potassium-efflux system ancillary protein KefG; required for KefB activity
STM3494.S	<i>nudE</i>	+	I+	U+	I+	U+	ADP-ribose diphosphatase NudE; ADP-sugar pyrophosphatase; catalyzes the formation of D-ribose 5-phosphate from ADP-ribose; can also act on ADP-mannose and ADP-glucose
STM3527		U+	+	+	I+	+	hypothetical protein

Table 6.3 (Cont.). Genes decreasing survival of *S. typhimurium* exposed to aquatic stress conditions for 4 weeks (signals up).

Gene symbol	Gene	AGW-NOM	AGW-4C	AGW-pH 10	AGW-Static	ASW	Function
STM3528		+	+	+	+	+	putative periplasmic phosphate-binding protein
STM3549		+	+	+	+	+	putative inner membrane protein
STM3604		+	I+	I+	I+	U+	putative inner membrane protein
STM3608	<i>yhjD</i>	+	+	+	I+	I+	putative tRNA-processing ribonuclease
STM3614	<i>dctA</i>	+	+	+	+	+	C4-dicarboxylate transporter DctA; involved in the transport of C4-dicarboxylates across the membrane
STM3621	<i>yhjR</i>	+	+	NT	+	+	putative cytoplasmic protein
STM3641	<i>yhjY</i>	+	+	+	+	+	putative lipase; similar to <i>Escherichia coli</i> putative
STM3651		+	+	U+	I+	I+	putative acetyltransferase
STM3698		+	+	+	+	+	putative permease; similar to <i>Escherichia coli</i> putative transport protein
STM3707	<i>yibD</i>	+	+	+	+	+	glycosyl transferase; similar to <i>Escherichia coli</i> putative regulator
STM3729	<i>radC</i>	+	+	NT	+	NT	DNA repair protein RadC; Involved in DNA double-strand break repair and recombination. Promotes the annealing of complementary single-stranded DNA and by simulation of RAD51 recombinase
STM3736		+	U+	I+	I+	U+	putative transcriptional regulator; similar to <i>Escherichia coli</i> putative transcriptional regulator LysR-type; LysR family
STM3737		I+	+	+	I+	U+	putative Zn-dependent hydrolase; similar to glyoxylases
STM3738	<i>yigC</i>	+	+	I+	I+	I+	putative inner membrane protein
STM3751	<i>selC</i>	+	+	+	+	U	tRNA-Sec
STM3764	<i>mgtC</i>	+	+	+	+	+	Mg ²⁺ transport protein; Mg ²⁺ transport ATPase protein C; required for intramacrophage survival and growth in low calcium; cotranscribed with <i>mgtB</i> and transcriptionally controlled by PhoP/PhoQ system
STM3796	<i>ilvB</i>	U	U+	U+	+	U+	acetolactate synthase catalytic subunit; catalyzes the formation of 2-acetolactate from pyruvate
STM3821	<i>torD</i>	+	+	+	+	I+	chaperone protein TorD; TorD; involved in the biogenesis of torA; acts on torA before the insertion of the molybdenum cofactor and, as a result, probably favors a conformation of the apoenzyme that is competent for acquiring the cofactor
STM3830	<i>dgoR</i>	+	+	+	+	NT	galactonate operon transcriptional repressor; similar to <i>Escherichia coli</i> regulator protein for dgo operon; GntR family
STM3919	<i>wzzE</i>	+	U+	I+	+	I+	lipopolysaccharide biosynthesis protein WzzE; Enterobacterial Common Antigen (ECA) polysaccharide chain length modulation protein
STM3955	<i>rarD</i>	I+	+	U+	+	I+	chloramphenicol resistance
STM3981		+	+	I+	I+	U+	putative cytoplasmic protein

Table 6.3 (Cont.). Genes decreasing survival of *S. typhimurium* exposed to aquatic stress conditions for 4 weeks (signals up).

Gene symbol	Gene	AGW-NOM	AGW-4C	AGW-pH 10	AGW-Static	ASW	Function
STM4015		+	+	+	+	+	putative cytoplasmic protein
STM4027	rbn	I+	+	+	I+	I+	ribonuclease BN; RNase BN; required for 3' maturation of certain phage T4-encoded tRNAs; forms a dimer; specific for immature tRNA substrates containing incorrect residues within the universal CCA sequence; 3' to 5' exoribonuclease
STM4032.2N		+	+	+	+	+	hypothetical protein
STM4039		+	+	+	+	+	putative inner membrane lipoprotein
STM4045	rhaD	+	+	I+	+	+	rhamnulose-1-phosphate aldolase; similar to <i>Escherichia coli</i> rhamnulose-phosphate aldolase
STM4098		I+	U+	U+	I+	I+	putative arylsulfate sulfotransferase
STM4107	yijF	+	+	+	+	I+	putative periplasmic protein
STM4129	trmA	+	+	+	+	U+	tRNA (uracil-5-)-methyltransferase; catalyzes the formation of 5-methyl-uridine at position 54 in all tRNAs
STM4174	hydG	I+	I+	+	I+	U+	transcriptional regulatory protein ZraR; DNA-binding response regulator in two-component regulatory system with ZraS; response regulator/sigma54 interaction protein
STM4206		+	+	+	I+	+	putative phage glucose translocase
STM4212		+	+	I+	+	+	putative phage tail core protein
STM4250	yjbQ	+	+	+	I+	I+	putative cytoplasmic protein
STM4263	yjcB	+	+	+	+	+	putative inner membrane protein
STM4288	phnB	I+	+	U+	I+	NT	hypothetical protein
STM4295	adiY	+	+	+	+	+	transcriptional activator; similar to <i>Escherichia coli</i> putative AraC-type regulatory protein; AraC/XylS family; activates aidA
STM4312		+	+	+	+	+	hypothetical protein
STM4317		+	+	+	+	+	hypothetical protein; CopG family; putative helix-turn-helix protein
STM4374	yjfl	+	+	+	+	+	putative inner membrane protein
STM4374	yjfl	+	+	+	+	+	putative inner membrane protein
STM4384	sgaB	I+	I+	+	+	+	L-ascorbate-specific enzyme IIB component of PTS; sga subunit
STM4385	ptxA	+	I+	I+	+	+	L-ascorbate-specific enzyme IIA component of PTS; similar to <i>Escherichia coli</i> putative PTS system enzyme II A component; sga subunit
STM4412		+	+	+	+	+	putative permease; similar to <i>Escherichia coli</i> D-galactonate transport

Table 6.3 (Cont.). Genes decreasing survival of *S. typhimurium* exposed to aquatic stress conditions for 4 weeks (signals up).

Gene symbol	Gene	AGW-NOM	AGW-4C	AGW-pH 10	AGW-Static	ASW	Function
STM4413		+	+	+	+	+	putative metallo-dependent hydrolase; Catalyzes the reversible hydrolysis of the amide bond within dihydroorotate. This metabolic intermediate is required for the biosynthesis of pyrimidine nucleotides
STM4423		+	+	+	+	+	putative DNA-binding protein; similar to <i>Escherichia coli</i> putative AraC-type regulatory protein; contains putative DNA-binding domain
STM4451	<i>nrdG</i>	+	+	U+	I+	I+	anaerobic ribonucleotide reductase-activating protein; activates anaerobic ribonucleoside-triphosphate reductase under anaerobic conditions
STM4472	<i>ytgA</i>	+	+	+	I+	I+	putative inner membrane protein; hypothetical 17.8 kKa protein in <i>miaE</i> 3'region (ORF 17.8)
STM4500	<i>yjhP</i>	I+	I+	I+	I+	I+	putative SAM-dependent methyltransferase; similar to <i>Escherichia coli</i> putative methyltransferase
STM4546	<i>yjiP</i>	+	+	+	I+	U+	hypothetical protein; similar to <i>Escherichia coli</i> putative structural protein
STM4593	<i>sthB</i>	+	+	I+	I+	+	putative fimbrial usher protein; similar to <i>Escherichia coli</i> putative outer membrane protein; export function
STM4594	<i>sthA</i>	+	+	+	+	I+	putative fimbrial chaparone; similar to <i>Escherichia coli</i> periplasmic chaperone; required for type 1 fimbriae
PSLT011	<i>srgA</i>	+	I+	+	+	+	putative thiol-disulfide isomerase or thioredoxin; <i>sdiA</i> -regulated gene; orf7
PSLT014	<i>orf6</i>	+	+	+	+	+	putative outer membrane protein; orf5
PSLT019	<i>pefB</i>	+	+	+	+	NT	regulatory protein; plasmid-encoded fimbriae
PSLT077	<i>traA</i>	+	+	+	+	+	conjugal transfer pilin subunit TraA; conjugative transfer; pilin
PSLT079	<i>traE</i>	+	I+	+	+	+	conjugal transfer pilus assembly protein TraE; conjugative transfer

^aThe annotated functions of genes were cited from (13).

^bUp.

^cIntermediate up.

^dUnchanged; possibly up. This response is referred to mild but consistent changes in one direction (up) that did not reach the minimum cut-offs for the number of oligos that showed a change. Thus, the data is insufficient to make a definitive call but the tendency is in a particular direction (up).

^eNo transposon. This abbreviation is referred to conditions that no transposon reliably detected for this gene, possibly due to the short size of the gene making it harder to be reached by a transposon.

^fUnchanged.

6.4 Conclusions

In this study, the fitness and survival of a library of mutants was tested after exposure to several stress conditions with background artificial groundwater (including different pH, temperature, organic matter, and static conditions) and artificial seawater for the duration of four weeks. Microarray analysis was performed to identify the genes required for survival and genes that are decreasing fitness and survival for each tested condition. The results suggested that *S. typhimurium* depends on similar genes to survive under different aquatic stress conditions. The major classes of genes essential for survival were universal stress response regulators, carbon starvation genes, genes involved in source depletion, osmotic shock, sugar uptake, and cell division. In addition, surface appendages (fimbriae, pili, and flagella) coding genes were both identified as required and disadvantageous, suggesting that complex cell-cell and cell-surface interactions play fine-tuned roles in survival under stress. Finally, some genes identified as advantageous for survival are known to be involved in virulence of *S. typhimurium* – therefore, virulence and survival might be co-regulated under stress.

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Chapter 7

Summary and Conclusions

The overall goal of this doctoral work was to understand and determine the ultimate fate and transport of *Salmonella enterica* spp. in groundwater environments. To reach the goal, the deposition of *Salmonella* onto solid surfaces was examined over a range of solution chemistries. Packed bed column and radial stagnation point flow systems (RSPF) were utilized for the deposition and transport experiments. These experiments were complemented by a range of cellular characterization techniques which provided information to better interpret the interaction mechanisms and transport behavior of *Salmonella*. Batch experiments of several groundwater stress conditions were used to evaluate the response of *Salmonella* in terms of fitness, survival, and virulence.

In Chapter 2, the transport experiments utilizing *Salmonella pullorum* and *E. coli* O157:H7 was employed to determine the influence of solution chemistry, species type, and cell concentration on the transport behavior and deposition kinetics of *Salmonella* in a packed bed column. The major outcomes of this chapter were: i) Most of the cell surface characteristics that play role in transport mechanisms were different for *Salmonella pullorum* and *E. coli* O157:H7; and ii) *E. coli* O157:H7 is more adhesive than *Salmonella pullorum* in saturated systems concluding *Salmonella* can be transported further distances than *E. coli* O157:H7 under the tested conditions.

Chapter 3 highlights the role of flagella on the transport and adhesion of *Salmonella* in groundwater environments. Three *Salmonella enterica* serovars with different motility phenotypes were employed in packed bed column and RSPF systems over a range of environmentally relevant solution chemistries. The results presented in Chapter 3 provide direct evidence of the role of flagellar motion in the deposition dynamics of

Salmonella and concludes that flagellated strains can be deposited more than nonflagellated cells.

In Chapter 4, changes in antibiotic resistance and invasiveness of *Salmonella* upon exposure to groundwater conditions in a short period of time were investigated. Stressed organisms' antibiotic resistance profiles were monitored with respect to the functioning of energy-driven active efflux pumps in ion-rich groundwater environments. In addition, *in vitro* invasion assays were employed to test whether or not environmental stress conditions trigger the invasiveness of *Salmonella* into human epithelial cells. The results have shown that i) two of the *Salmonella* strains may enhance its resistance to several antibiotics mediated by active efflux pumps, concluding the resistance mechanisms might be strain dependent; and ii) the invasiveness of *Salmonella* into human epithelial cells increases upon 24-hrs exposure to groundwater, concluding ions present in aquatic environments might result in enhanced virulence in *Salmonella*.

The study described in Chapter 5 was motivated by the results observed and reported in Chapter 4, which showed the possibility of increased virulence in relatively longer periods of groundwater stress exposure. To further investigate how the antibiotic resistance and virulence of *Salmonella* is affected, the exposure period to groundwater environments was increased to four weeks and the bacterial cultures were supplemented with residual antibiotics. Due to the limitations of *in vitro* virulence assays, *C. elegans* nematodes were used to test the virulence of *Salmonella* in *in vivo*. Results have shown that long terms exposure to groundwater environments may result in hyper-virulent characteristics in *Salmonella* even under the viable but nonculturable state (VBNC). It

was also concluded that exposing *Salmonella* to certain antibiotics does not always induce resistance to these antibiotics, at least up to four weeks as confirmed with extensive antibiotic resistance analysis.

The final chapter describes a genotypic approach to the survival and fitness of *Salmonella* in groundwater environments, complementary to the phenotypic evaluations of pathogenicity investigated in Chapter 4 and 5. Specifically, a library of mutants composed of 90,000 independent recombinants was exposed to several groundwater and seawater conditions to identify the genes required for fitness and survival at a particular stress condition. The mutations covering the complete genome of *S. typhimurium* were monitored by microarray analysis to quantify the degree of signal loss or gain after exposure to the respective condition. Results indicated that *Salmonella* depends on similar genes for survival under different aquatic stress conditions, and these genes are involved in global stress response, carbon source utilization and depletion, reproduction, and osmotic shock. This unique work also suggested that mechanisms of virulence and survival may be connected in *Salmonella* experiencing external stress. However, the nature of this connection was not clearly analysed as different virulence components affected survival of the bacterium in these aquatic test environments in different ways.

The findings from this dissertation suggest the following critical insights regarding the transport section: First, the mechanisms of pathogen deposition in subsurface environments are dependent on not only physical and chemical but also biological factors. Secondly, these biological components are mostly cell surface characteristics including membrane polymeric substances, and surface appendages, such as flagella. Third,

motility plays a major role in the transport of *Salmonella* as motile cells can swim towards different pore structures and collector surface irregularities that are otherwise inaccessible to nonmotile cells.

Regarding the fate of cell pathogenicity, *Salmonella* can survive successfully in groundwater environments for extended periods of time and remain structurally intact, if not reproduce. Besides, and perhaps most importantly, the virulence of this important pathogen is enhanced under most of the relevant groundwater conditions that were simulated in this study.

This Ph.D. work has clearly demonstrated the comprehensive framework of the fate and transport of *Salmonella* in subsurface environments. These results have implications in the future design of groundwater wells (in terms of relative distances to point sources of bacterial pollution), water filtration facilities, riverbank filtration, as well as the development of gene targeting antimicrobials for *Salmonella*.

Appendix A

Survival and Fitness of Random Generated *Salmonella enterica* serovar Typhimurium Transposon Library under Long Term Environmental Stress: From *in vitro* to *in silico*

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A.1 Results

A.1.1 Genetic Screening and Survival of *Salmonella typhimurium* in AGW with Organic Matter

Table A.1. List of the genes with decreased signal in *S. typhimurium* exposed to AGW supplemented with NOM for 4 weeks.

Gene symbol	Gene	Gene function ^a
STM0046	<i>ileS</i>	isoleucyl-tRNA synthetase
STM0088	<i>apaH</i>	diadenosine tetraphosphatase; hydrolyzes P(1),P(4)-bis(5'-adenosyl) tetraphosphate to form 2 ADP
STM0127	<i>ftsW</i>	cell division protein FtsW; integral membrane protein involved in stabilizing FtsZ ring during cell division
STM0131	<i>ftsQ</i>	cell division protein FtsQ; involved in septum formation
STM0132	<i>ftsA</i>	cell division protein FtsA; ATP-binding involved in recruitment of FtsK to Z ring; essential cell division protein; colocalizes with FtsZ through direct interaction to the septal ring structure; structurally similar to eukaryotic actin; binds directly to the cell membrane
STM0139	<i>yacF</i>	putative cytoplasmic protein
STM0216	<i>rpsB</i>	30S ribosomal protein S2; one of the last subunits in the assembly of the 30S subunit; absence of S2 does not inhibit assembly but results in an inactive subunit
STM0253	<i>rrfH</i>	5S ribosomal RNA
STM0287		putative periplasmic protein
STM0297		putative transposase
STM0321	<i>proB</i>	gamma-glutamyl kinase; catalyzes the formation of glutamate 5-phosphate from glutamate in proline biosynthesis
STM0327		putative cytoplasmic protein
STM0334		putative cytoplasmic protein
STM0355		putative copper chaperone
STM0386	<i>proC</i>	pyrroline-5-carboxylate reductase; catalyzes the formation of L-proline from pyrroline-5-carboxylate
STM0393	<i>yajF</i>	fructokinase; catalyzes phosphorylation of fructose; cytosolic enzyme
STM0397	<i>phoB</i>	transcriptional regulator PhoB; two component response regulator for the phosphate regulon; PhoR phosphorylates PhoB
	<i>queA</i>	S-adenosylmethionine:tRNA ribosyltransferase-isomerase; Synthesizes oQ from preQ1 in a single S-adenosylmethionine-requiring step
STM0404		
STM0423	<i>ispA</i>	geranyltranstransferase; similar to <i>Escherichia coli</i> geranyltranstransferase; farnesylldiphosphate synthase (AAC73524.1)
	<i>apbA</i>	2-dehydropantoate 2-reductase; ketopantoate reductase; catalyzes the NADPH reduction of ketopantoate to pantoate; functions in pantothenate (vitamin B5) biosynthesis
STM0434		
STM0474	<i>ybaJ</i>	putative cytoplasmic protein
STM0486	<i>recR</i>	recombination protein RecR; involved in a recombinational process of DNA repair, independent of the recBC complex
	<i>ushA</i>	bifunctional UDP-sugar hydrolase/5'-nucleotidase periplasmic precursor; catalyzes the degradation of periplasmic UDP-glucose to uridine, glucose-1-phosphate and inorganic phosphate; specific for uridine nucleotides
STM0494		

Table A.1 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW supplemented with NOM for 4 weeks.

Gene symbol	Gene	Gene function
STM0498	<i>copA</i>	copper exporting ATPase; similar to <i>Escherichia coli</i> putative ATPase (AAC73586.1)
STM0502	<i>ybbL</i>	putative ABC transporter ATP-binding protein YbbL; similar to <i>Escherichia coli</i> putative ATP-binding component of a transport system (AAC73592.1)
STM0503	<i>ybbM</i>	putative transport protein; similar to <i>Escherichia coli</i> putative metal resistance protein (AAC73593.1); YbbM family
STM0559	<i>rfbI</i>	putative glycosyl translocase
STM0568	<i>pheP</i>	phenylalanine transporter; similar to <i>Escherichia coli</i> phenylalanine-specific transport system (AAC73677.1); APC family phosphopantetheinyltransferase component of enterobactin synthase multienzyme complex; similar to <i>Escherichia coli</i> enterochelin synthetase component D (AAC73684.1); phosphopantetheinyltransferase
STM0584	<i>entD</i>	hypothetical protein; possibly involved in aromatic compounds catabolism; PaaI
STM0599	<i>ybdB</i>	carbon starvation protein; similar to <i>Escherichia coli</i> carbon starvation protein (AAC73699.1)
STM0600	<i>cstA</i>	putative universal stress protein; similar to UspA and related nucleotide-binding protein
STM0614	<i>ybdQ</i>	triphosphoribosyl-dephospho-CoA synthase; catalyzes the formation of 2'-(5"-triphosphoribosyl)-3'-dephospho-CoA from ATP and 3-dephospho-CoA
STM0619	<i>citG</i>	C4-dicarboxylate transporter DcuC; responsible for the transport of C4-dicarboxylates during anaerobic growth
STM0627	<i>dcuC</i>	cell wall shape-determining protein; similar to <i>Escherichia coli</i> rod shape-determining membrane protein; sensitivity to radiation and drugs (AAC73735.1); cell elongation in exponential phase
STM0639	<i>mrdB</i>	SPOUT methyltransferase superfamily protein; enzyme from <i>Escherichia coli</i> has not been shown to exhibit methyltransferase activity; crystal structure shows homodimer
STM0641	<i>ybeA</i>	ribonucleoside hydrolase 1; Hydrolyzes with equal efficiency cytidine or uridine to ribose and cytosine or uracil, respectively;
STM0661	<i>rihA</i>	pyrimidine-specific glutamate/aspartate transporter; similar to <i>Escherichia coli</i> glutamate/aspartate transport system permease (AAC73754.1); ABC superfamily membrane protein
STM0663	<i>gltK</i>	methionine tRNA (duplicate of metT)
STM0675	<i>metU</i>	N-acetylglucosamine-6-phosphate deacetylase; catalyzes the formation of glucosamine 6-phosphate from N-acetylglucosamine 6-phosphate
STM0683	<i>nagA</i>	putative ABC transporter permease protein
STM0722		succinate dehydrogenase cytochrome b556 small membrane subunit; similar to <i>Escherichia coli</i> succinate dehydrogenase; hydrophobic subunit (AAC73816.1)
STM0733	<i>sdhD</i>	hydrophobic subunit (AAC73816.1)
STM0738	<i>sucC</i>	succinyl-CoA synthetase subunit beta; catalyzes the interconversion of succinyl-CoA and succinate
STM0766	<i>dcoC</i>	oxaloacetate decarboxylase subunit gamma; catalyzes the decarboxylation of oxaloacetate to form pyruvate and carbon dioxide
STM0790	<i>hutU</i>	pseudogene; frameshift relative to <i>Pseudomonas putida</i> urocanate hydratase; HUTU (SW:P25080)

Table A.1 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW supplemented with NOM for 4 weeks.

Gene symbol	Gene	Gene function
STM0795	<i>bioF</i>	8-amino-7-oxononanoate synthase; catalyzes the formation of 8-amino-7-oxononanoate from 6-carboxyhexanoyl-CoA and L-alanine
STM0798	<i>uvrB</i>	excinuclease ABC subunit B; The UvrABC repair system catalyzes the recognition and processing of DNA lesions. The beta-hairpin of the Uvr-B subunit is inserted between the strands, where it probes for the presence of a lesion
STM0812	<i>ybhO</i>	cardiolipin synthase 2; similar to <i>Escherichia coli</i> putative synthetase (AAC73876.1); CL synthase
STM0814	<i>ybhQ</i>	putative inner membrane protein
STM0822	<i>ybiB</i>	hypothetical protein; similar to <i>Escherichia coli</i> putative enzyme (AAC73887.1)
STM0826	<i>ybiN</i>	putative SAM-dependent methyltransferase
STM0835		manganese transport regulator MntR; Transcriptional regulator that represses the manganese transporter MntH when manganese is present
STM0847	<i>ybiK</i>	L-asparaginase; similar to <i>Escherichia coli</i> putative asparaginase (AAC73915.1)
STM0865	<i>ybjG</i>	undecaprenyl pyrophosphate phosphatase
STM0870		hypothetical protein; similar to <i>Escherichia coli</i> putative transport protein (AAC73934.1)
STM0951		putative cytoplasmic protein
STM0952		putative transcriptional regulator; similar to <i>Escherichia coli</i> putative transcriptional regulator LysR-type (AAC73313.1); LysR family
STM0960	<i>ftsK</i>	DNA translocase FtsK; DNA-binding membrane protein required for chromosome resolution and partitioning
STM0963	<i>serS</i>	seryl-tRNA synthetase; catalyzes a two-step reaction, first charging a serine molecule by linking its carboxyl group to the alpha-phosphate of ATP, followed by transfer of the aminoacyl-adenylate to its tRNA
STM0974	<i>focA</i>	formate transporter; similar to <i>Escherichia coli</i> probable formate transporter; formate channel 1 (AAC73990.1); FNT family
STM0994	<i>mukB</i>	cell division protein MukB; SMC (structural maintenance of chromosomes) family of proteins; involved in chromosome condensation and partitioning; forms a homodimer and the C-terminal is essential for DNA-binding activity while the purified N-terminal domain binds FtsZ; mutations result in cell division defects
STM1025		Gifsy-2 prophage
STM1029		Gifsy-2 prophage
STM1033		Clp protease-like protein; similar to <i>Escherichia coli</i> ATP-dependent proteolytic subunit of ClpA-ClpP serine protease; heat shock protein F21.5 (AAC73540.1)
STM1039		minor tail protein
STM1047		tail assembly protein
STM1048.1N		hypothetical protein
STM1056		MsgA-like protein

Table A.1 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW supplemented with NOM for 4 weeks.

Gene symbol	Gene	Gene function
STM1071	<i>sulA</i>	SOS cell division inhibitor; suppressor of lon (SW:SULA_SALTY); inhibitor of cell division and FtsZ ring formation upon DNA damage/inhibition; HslVU and Lon involved in its turnover
STM1108	<i>hpaA</i>	4-hydroxyphenylacetate catabolism; similar to <i>Escherichia coli</i> putative AraC-type regulatory protein (AAC74766.1) similar to <i>Escherichia coli</i> PhoB-dependent ATP-binding pho regulon component; may be helicase; induced by P starvation
STM1126	<i>phoH</i>	(AAC74105.1); contains a frameshift; similar to STM0669
STM1136	<i>ycdX</i>	putative hydrolase; similar to hydrolases of the PHP family
STM1137	<i>ycdY</i>	putative oxidoreductase component; similar to <i>Escherichia coli</i> putative oxidoreductase component (AAC74119.1)
STM1138	<i>ycdZ</i>	putative inner membrane protein; hypothetical protein in phoH-csgG intergenic region (SW:YCDZ_SALTY)
STM1151	<i>mdoH</i>	glucosyltransferase MdoH; necessary for biosynthesis of osmoregulated periplasmic glucans possibly involved in the transfer to the periplasmic space
STM1167	<i>rimJ</i>	ribosomal-protein-S5-alanine N-acetyltransferase; similar to <i>Escherichia coli</i> acetylase; acetylates N-terminal alanine of 30S
STM1170	<i>mviN</i>	ribosomal subunit protein S5 (AAC74150.1)
		putative virulence protein; virulence factor MviN (SW:MVIN_SALTY)
STM1174	<i>flgB</i>	flagellar basal body rod protein FlgB; with FlgF and C makes up the proximal portion of the flagellar basal body rod; Vibrio
STM1208	<i>thiK</i>	parahaemolyticus protein is associated with the polar flagella
STM1213	<i>ycfQ</i>	thiamine kinase; catalyzes the phosphorylation of thiamine to thiamine phosphate
STM1237	<i>ymfC</i>	putative transcriptional repressor; TetR/AcrR family
STM1250		putative ribosomal large subunit pseudouridine synthase
STM1271	<i>yeaR</i>	putative cytoplasmic protein
STM1285	<i>yeaG</i>	putative cytoplasmic protein
STM1288		putative serine protein kinase
STM1293	<i>pncA</i>	putative aldehyde reductase; similar to <i>Escherichia coli</i> putative aldehyde reductase (AAC74851.1)
		nicotinamidase/pyrazinamidase; catalyzes the formation of nicotinate from nicotinamide in NAD biosynthesis
STM1342	<i>btuD</i>	vitamin B12-transporter ATPase; ATP-binding protein that acts with the transmembrane protein BtuC and the solute binding protein
STM1374	<i>ynhA</i>	BtuF to transport vitamin B12 into the cell
STM1385	<i>ttrB</i>	cysteine desulfuration protein SufE; Acts with SufS to catalyze the formation of L-alanine from L-cysteine
STM1394	<i>ssaC</i>	tetrathionate reductase complex subunit B; tetrathionate reductase subunit B; TtrB (gi 4456871)
STM1411	<i>ssaK</i>	outer membrane secretin precursor; SpiA (gi 1498307); type III secretion system apparatus
		type III secretion system apparatus protein; secretion system apparatus protein SsaK (SW:SSAK_SALTY)

Table A.1 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW supplemented with NOM for 4 weeks.

Gene symbol	Gene	Gene function
STM1424	<i>valV</i>	tRNA-Val
STM1425	<i>ydhE</i>	multidrug efflux protein; NorM; MdtK; functions as a Na(+)/drug antiporter; inactivation in <i>Vibrio cholerae</i> results in susceptibility to fluoroquinolones
STM1451	<i>gst</i>	glutathionine S-transferase; similar to <i>Escherichia coli</i> glutathionine S-transferase (AAC74707.1)
STM1452	<i>tppB</i>	putative tripeptide transporter permease; synonym: ydgR; mutations in this gene confer resistance to the toxic peptide alafosfalin in <i>Salmonella typhimurium</i> ; member of proton-dependent oligopeptide transport (POT) system family; in <i>Escherichia coli</i> this gene is regulated by OmpR although not via osmoregulation
STM1455	<i>ydgP</i>	electron transport complex protein RnfG; part of membrane-bound complex thought to be involved in electron transport to nitrogen
STM1457		electron transport complex protein RnfC; part of membrane-bound complex thought to be involved in electron transport to nitrogen
STM1459		Na(+)-translocating NADH-quinone reductase subunit E; inner membrane protein
STM1471	<i>rstB</i>	sensor protein RstB; similar to <i>Escherichia coli</i> sensor histidine protein kinase (RstA regulator) (AAC74681.1); in two-component regulatory system with RstA
STM1481	<i>tqsA</i>	putative transport protein; synonym: ydgG; transport of quorum-sensing signal; mutations in this gene alters the transport of the quorum-sensing signal autoinducer 2 (AI-2) which affects expression of a large number of genes
STM1484		putative protease
STM1494		putative transport system permease component; similar to <i>Escherichia coli</i> putative transport system permease protein (AAC75189.1); ABC-type transport systems
STM1506	<i>rspB</i>	putative dehydrogenase; similar to <i>Escherichia coli</i> starvation-sensing protein (AAC74652.1)
STM1521	<i>marC</i>	multiple drug resistance protein MarC; protein involved in resistance to different drugs (tetracycline, chloramphenicol, beta-lactams, and quinolones); part of the multiple antibiotic resistance (<i>mar</i>) locus, which is composed by the genes <i>marC</i> and <i>marRAB</i> ;
STM1531		unknown function
STM1539		putative hydrogenase; similar to <i>Escherichia coli</i> may modulate levels of hydrogenase-2 (AAC76027.1)
STM1551		putative hydrogenase-1 small subunit; similar to <i>Escherichia coli</i> hydrogenase-1 small subunit (AAC74057.1)
STM1573.Sc		putative cytoplasmic protein
STM1575		putative cytoplasmic protein
STM1612		putative transcriptional regulator; TetR family
STM1625	<i>ydcl</i>	putative cellulase protein; similar to <i>Escherichia coli</i> putative lyase/synthase (AAC77261.1)
		putative transcriptional regulator; similar to <i>Escherichia coli</i> putative transcriptional regulator LysR-type (AAC74504.1); LysR family

Table A.1 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW supplemented with NOM for 4 weeks.

Gene symbol	Gene	Gene function
STM1626	<i>trg</i>	methyl-accepting chemotaxis protein III; similar to <i>Escherichia coli</i> methyl-accepting chemotaxis protein III; ribose and galactose sensor receptor (AAC74503.1)
STM1650		putative reverse transcriptase
STM1665		putative cytoplasmic protein
STM1684	<i>ycjF</i>	hypothetical protein
STM1690	<i>pspA</i>	phage shock protein PspA; involved in maintaining membrane potential under membrane stress conditions; also acts as a negative transcriptional regulator of the phage shock protein (psp) operon (pspABCDE) by regulating the transcriptional activator PspF
STM1694	<i>sapC</i>	peptide transport protein; peptide transport system permease protein SapC (SW:SAPC_SALTY); ABC superfamily membrane protein
STM1709	<i>yciS</i>	putative inner membrane protein
STM1716	<i>sohB</i>	putative periplasmic protease; SohB; periplasmic protein; member of the peptidase S49 family
STM1742	<i>oppF</i>	oligopeptide transport protein; oligopeptide transport ATP-binding protein; OppF (SW:OPPF_SALTY); ABC superfamily ATP-binding protein
STM1773	<i>ychA</i>	putative transcriptional regulator
STM1791		putative hydrogenase-1 protein; similar to <i>Escherichia coli</i> nickel incorporation into hydrogenase-1 proteins (AAC74062.1)
STM1798	<i>ycgR</i>	putative inner membrane protein
STM1809		putative cytoplasmic protein
STM1820	<i>yeaZ</i>	putative molecular chaperone
STM1827.S		putative diguanylate cyclase/phosphodiesterase
STM1828	<i>yoaE</i>	putative inner membrane protein; similar to <i>Escherichia coli</i> putative transport protein (AAC74886.1)
STM1844	<i>htpX</i>	heat shock protein HtpX; putative metalloprotease
STM1859		putative cytoplasmic protein
STM1868A		lytic enzyme
STM1870		RecE-like protein; exoVIII; similar to <i>Escherichia coli</i> exonuclease VIII; ds DNA exonuclease; 5'-3' specific (AAC74432.1)
STMsR072	<i>ryeB</i>	
STM1879	<i>ptrB</i>	protease 2; PtrB; oligopeptidase that cleaves peptide bonds following arginine and lysine residues
STM1881	<i>yebF</i>	hypothetical protein; secreted protein; unknown function

Table A.1 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW supplemented with NOM for 4 weeks.

Gene symbol	Gene	Gene function
STM1902	<i>yecD</i>	putative isochorismatase
STM1927	<i>yecG</i>	universal stress protein UspC; ppGpp-dependent; membrane-associated, stress protein produced under conditions of nutrient deprivation, osmotic shock and oxidative stress
STM1938	<i>yecA</i>	hypothetical protein; similar to the C-terminal domain of SecA
STM1972	<i>flil</i>	flagellum-specific ATP synthase; involved in type III protein export during flagellum assembly
STM2069	<i>yeeY</i>	putative transcriptional regulator; similar to <i>Escherichia coli</i> putative transcriptional regulator LysR-type (AAC75076.1); LysR family
STM2076	<i>hisA</i>	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase; catalyzes the formation of 5-(5-phospho-1-deoxyribulos-1-ylamino)methylideneamino-1-(5-phosphoribosyl)imidazole-4-carboxamide from 1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide
STM2120	<i>asmA</i>	putative assembly protein; YegA; inner membrane protein involved in the assembly of outer membrane proteins (OMPs); asmA-null mutants show low lipopolysaccharide (LPS) levels, suggesting a role in LPS biogenesis and/or in restricting outer membrane fluidity, resulting on altered assembly of OMPs
STMsr082	<i>cyaR</i>	
STM2139		putative inner membrane protein
STM2206	<i>fruF</i>	bifunctional fructose-specific PTS IIA/HPr protein; phosphoenolpyruvate (PEP)-dependent, sugar transporting phosphotransferase system; catalyzes the phosphorylation of incoming sugar substrates concomitant with their translocation across the cell membrane; IIA is phosphorylated by phospho-HP which then transfers the phosphoryl group to the IIB component
STM2228	<i>yejM</i>	putative hydrolase; hypothetical protein in rplY-proL intergenic region (SW:YEJM_SALTY); alkaline phosphatase superfamily
STM2237		putative inner membrane protein
STM2269	<i>yojN</i>	phosphotransfer intermediate protein in two-component regulatory system with RcsBC; similar to <i>Escherichia coli</i> putative 2-component sensor protein (AAC75276.1); regulatory protein
STM2295	<i>yfaO</i>	putative NTP pyrophosphohydrolase; similar to oxidative damage repair enzymes
STM2310	<i>menF</i>	menaquinone-specific isochorismate synthase; synthesizes isochorismate acid from chorismate
STM2337	<i>ackA</i>	acetate kinase; AckA utilizes acetate and can acetylate CheY which increases signal strength during flagellar rotation; utilizes magnesium and ATP; also involved in conversion of acetate to acetyl-CoA
STM2344		putative phosphotransferase system enzyme II A component; similar to <i>Escherichia coli</i> putative PTS system enzyme II A component (AAC77152.1)
STM2355	<i>argT</i>	lysine/arginine/ornithine transport protein; lysine-arginine-ornithine-binding periplasmic protein precursor; LAO-binding protein (SW:ARGT_SALTY); ABC superfamily binding protein

Table A.1 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW supplemented with NOM for 4 weeks.

Gene symbol	Gene	Gene function
STM2383	<i>mepA</i>	penicillin-insensitive murein endopeptidase; D-alanyl-D-alanine endopeptidase; functions in hydrolyzing cell wall peptidoglycan; similar to LAS metallopeptidases; forms a dimer in periplasm
STM2385	<i>yfcB</i>	N5-glutamine S-adenosyl-L-methionine-dependent methyltransferase; involved in methylation of ribosomal protein L3
STM2434		putative cytoplasmic protein
STM2445	<i>ucpA</i>	short chain dehydrogenase; similar to <i>Escherichia coli</i> putative oxidoreductase (AAC75479.1)
STM2447		putative outer membrane lipoprotein
STM2475		putative cytoplasmic protein
STM2481	<i>acrD</i>	aminoglycoside/multidrug efflux system; similar to <i>Escherichia coli</i> protein that results in sensitivity to acriflavine; integral membrane protein; possible efflux pump (AAC75523.1); RND family
STM2492		putative glycerate kinase
STM2543	<i>nifS</i>	cysteine desulfurase; catalyzes the removal of sulfur from cysteine to form alanine
STM2582	<i>lepB</i>	signal peptidase I; catalyzes the cleavage of the amino-terminal leader peptide from secretory proteins
STM2590		tail assembly protein I-like; similar to tail assembly protein I in phage lambda
STM2591		tail assembly protein K-like; similar to tail assembly protein K in phage lambda
STM2665	<i>yfiA</i>	translation inhibitor protein RaiA; associated with 30S ribosomal subunit; interferes with translation elongation
STM2684	<i>recN</i>	recombination and repair protein; similar to <i>Escherichia coli</i> protein used in recombination and DNA repair (AAC75665.1)
STM2706		phage tail-like protein; similar to tail fiber protein in phage P2
STM2787		tricarboxylic transport
STM2794	<i>ygaE</i>	DNA-binding transcriptional regulator CsiR; regulator of gab gene expression
STM2806	<i>nrdI</i>	ribonucleotide reductase stimulatory protein; in <i>Salmonella</i> NrdI has a stimulatory effect on the ribonucleotide reductase activity of NrdH with NrdEF
STM2815	<i>emrB</i>	putative multidrug transport protein; similar to <i>Escherichia coli</i> multidrug resistance; probably membrane translocase (AAC75733.1); MFS family
STM2819	<i>yqaA</i>	putative inner membrane protein
STM2831	<i>mltB</i>	murein hydrolase B; membrane-bound lytic murein transglycosylase B; catalyzes the cleavage of the glycosidic bonds between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan
STM2834	<i>slrB</i>	glucitol/sorbitol-specific PTS system component IIA; phosphoenolpyruvate-dependent sugar phosphotransferase system; catalyzes the phosphorylation of incoming sugar substrates concomitant with their translocation across the cell membrane; IIB is phosphorylated by IIA and then transfers the phosphoryl group to the sugar; IIC forms the translocation channel
STM2872	<i>prgJ</i>	needle complex minor subunit; PrgJ protein (SW:PRGJ_SALTY); invasion protein; may cap needle complex; type III secretion apparatus

Table A.1 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW supplemented with NOM for 4 weeks.

Gene symbol	Gene	Gene function
STM2888	<i>spaR</i>	needle complex export protein; surface presentation of antigens protein SpaR (SW:SPAR_SALTY); inner membrane secretory protein; type III secretion apparatus
STM2912		putative transcriptional regulator; similar to <i>Escherichia coli</i> putative transcriptional regulator LysR-type (AAC73313.1); LysR family
STM2924	<i>rpoS</i>	RNA polymerase sigma factor RpoS; sigma factors are initiation factors that promote the attachment of RNA polymerase to specific initiation sites and are then released; this sigma factor controls a regulon of genes required for protection against external stresses
STM2925	<i>nlpD</i>	lipoprotein NlpD; outer membrane lipoprotein involved in stationary-phase cell survival; similar to LppB virulence determinant from <i>Haemophilus somnus</i>
STM2977	<i>fucK</i>	L-fuculokinase; similar to <i>Escherichia coli</i> L-fuculokinase (AAC75845.1)
STM2980	<i>ygdE</i>	putative RNA 2'-O-ribose methyltransferase
STM3001	<i>thyA</i>	thymidylate synthase; ThyA; catalyzes formation of dTMP and 7,8-dihydrofolate from 5,10-methylenetetrahydrofolate and dUMP; involved in deoxyribonucleotide biosynthesis; there are 2 copies in some Bacilli, one of which appears to be phage-derived
STM3050	<i>yqfB</i>	hypothetical protein
STM3084.S	<i>rygC</i>	
STM3156		putative regulatory protein; similar to <i>Escherichia coli</i> regulator for <i>uxu</i> operon (AAC77280.1); GntR family
STM3159	<i>exbB</i>	putative cytoplasmic protein
STM3161	<i>metC</i>	biopolymer transport protein ExbB; membrane spanning protein in TonB-ExbB-ExbD complex; involved in the tonB-independent energy-dependent transport iron-siderophore complexes and vitamin B12 into the cell
STM3177	<i>ygiX</i>	cystathionine beta-lyase; catalyzes the formation of L-homocysteine from cystathionine
STM3181	<i>parE</i>	DNA-binding transcriptional regulator QseB; response regulator in two-component regulatory system with QseC; regulates FlhCD which is the master regulator for flagella and motility genes
		DNA topoisomerase IV subunit B; decatenates newly replicated chromosomal DNA and relaxes positive and negative DNA supercoiling
STM3204	<i>cca</i>	multifunctional tRNA nucleotidyl transferase/2'3'-cyclic phosphodiesterase/2'nucleotidase/phosphatase; catalyzes the addition and repair of the essential 3'-terminal CCA sequence in tRNAs without using a nucleic acid template; phosphohydrolase activities include hydrolysis of pyrophosphate, 5'-nucleoside tri- and diphosphates, NADP, and 2'-AMP with the production of Pi, metal-dependent phosphodiesterase activity for 2',3'-cAMP, 2',3'-cGMP, and 2',3'-cCMP, and hydrolysis 2',3'-cyclic substrates with the formation of 2'-nucleotides and 3'-nucleotides; these phosphohydrolase activities are probably involved in the repair of the tRNA 3'-
STM3225	<i>ygjU</i>	CCA terminus degraded by intracellular RNases
		serine/threonine transporter SstT; involved in the import of serine and threonine coupled with the import of sodium

Table A.1 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW supplemented with NOM for 4 weeks.

Gene symbol	Gene	Gene function
STM3246	<i>rnpB</i>	misc_RNA; regulatory RNA
STM3249	<i>garL</i>	alpha-dehydro-beta-deoxy-D-glucarate aldolase
STM3269	<i>yhbO</i>	putative intracellular proteinase
STM3296	<i>hflB</i>	ATP-dependent metalloprotease; synonym: ftsH; inner membrane metalloprotease; may be involved in degradation of aberrant cytoplasmic and membrane proteins
STM3319	<i>yhbG</i>	putative ABC transporter ATP-binding protein YhbG; similar to <i>Escherichia coli</i> putative ATP-binding component of a transport system (AAC76233.1); putative ABC superfamily ATP-binding protein
STM3338	<i>nanT</i>	putative sialic acid transporter; similar to <i>Escherichia coli</i> sialic acid transporter (AAC76256.1); MFS family
STM3353	<i>oadG</i>	oxaloacetate decarboxylase subunit gamma; catalyzes the decarboxylation of oxaloacetate to form pyruvate and carbon dioxide
STM3372	<i>mreD</i>	rod shape-determining protein MreD; part of cell wall structural complex MreBCD; transmembrane component
STM3426	<i>rpsH</i>	30S ribosomal protein S8; binds directly to 16S rRNA central domain where it helps coordinate assembly of the platform of the 30S subunit
STM3429	<i>rplX</i>	50S ribosomal protein L24; assembly initiator protein; binds to 5' end of 23S rRNA and nucleates assembly of the 50S; surrounds polypeptide exit tunnel
STM3436	<i>rpsS</i>	30S ribosomal protein S19; protein S19 forms a complex with S13 that binds strongly to the 16S ribosomal RNA
STM3497	<i>yrfH</i>	ribosome-associated heat shock protein Hsp15; predicted small RNA-binding protein
STM3575	<i>yhhN</i>	putative inner membrane protein; similar to <i>Escherichia coli</i> putative enzyme (AAC76493.1)
STM3584	<i>nikR</i>	nickel responsive regulator; Inhibits transcription at high concentrations of nickel
STM3617		endo-1,4-D-glucanase; catalyzes the hydrolysis of 1,4-beta-D-glucosidic linkages in cellulose, lichenin and cereal beta-D-glucans
STM3653		putative acetyltransferase
STM3654		pseudogene; in-frame stop following codon 23
STM3657		putative outer membrane lipoprotein
STM3681		putative transcriptional regulator; similar to <i>Escherichia coli</i> possible NagC-like transcriptional regulator (AAC73497.1)
STM3726	<i>mutM</i>	formamidopyrimidine-DNA glycosylase; Involved in base excision repair of DNA damaged by oxidation or by mutagenic agents. Acts as DNA glycosylase that recognizes and removes damaged bases
STM3742	<i>spoT</i>	bifunctional (p)ppGpp synthetase II/ guanosine-3',5'-bis pyrophosphate 3'-pyrophosphohydrolase; similar to <i>Escherichia coli</i> (p)ppGpp synthetase II; also guanosine-3',5'-bis pyrophosphate 3'-pyrophosphohydrolase (AAC76674.1)
STM3747	<i>yicE</i>	putative purine/xanthine transport protein; similar to <i>Escherichia coli</i> putative transport protein (AAC76678.1); NCS2 family
STM3758	<i>fidL</i>	putative inner membrane protein; FidL (gi4324611)

Table A.1 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW supplemented with NOM for 4 weeks.

Gene symbol	Gene	Gene function
STM3786	<i>yicN</i>	putative inner membrane protein
STM3863		putative permease
STM3865	<i>atpD</i>	F ₀ F ₁ ATP synthase subunit beta; Produces ATP from ADP in the presence of a proton gradient across the membrane. The beta chain is a regulatory subunit
STM3886	<i>rbsR</i>	transcriptional repressor RbsR; DNA-binding transcriptional repressor of ribose metabolism
STM3914	<i>rhlB</i>	ATP-dependent RNA helicase RhlB; enables ATP-dependent unwinding of double stranded RNA as a component of the RNA degradosome, a multi-enzyme complex important in RNA processing and messenger RNA degradation
STM3937	<i>hemD</i>	uroporphyrinogen-III synthase; catalyzes the formation of uroporphyrinogen-III from hydroxymethylbilane; functions in tetrapyrrole and heme biosynthesis
STM3978	<i>yigC</i>	3-octaprenyl-4-hydroxybenzoate decarboxylase; catalyzes the decarboxylation of 3-octaprenyl-4-hydroxy benzoate to 2-octaprenylphenol
STM4004	<i>hemN</i>	coproporphyrinogen III oxidase; catalyzes the oxygen-independent formation of protoporphyrinogen-IX from coproporphyrinogen-III
STM4055	<i>sodA</i>	superoxide dismutase; SodA; manganese binding; only present under aerobic conditions; destroys free radicals
STM4069		putative periplasmic protein
STM4096	<i>rpmE</i>	50S ribosomal protein L31; RpmE; there appears to be two types of ribosomal proteins L31 in bacterial genomes; some contain a CxxC motif while others do not; Bacillus subtilis has both types; the proteins in this cluster have the CXXC motif; RpmE is found in exponentially growing Bacilli while YtiA was found after exponential growth; expression of ytiA is controlled by a zinc-specific transcriptional repressor; RpmE contains one zinc ion and a CxxC motif is responsible for this binding; forms an RNP particle along with proteins L5, L18, and L25 and 5S rRNA; found crosslinked to L2 and L25 and EF-G; may be near the peptidyltransferase site of the 50S ribosome
STM4155		putative inner membrane protein
STM4156		putative cytoplasmic protein
STM4160	<i>thiG</i>	thiazole synthase; functions in thiamine (vitamin B1) biosynthesis; in Bacillus subtilis this enzyme catalyzes the formation of thiazole from dehydroxyglycine and 1-deoxy-D-xylulose-5-phosphate and ThiS-thiocarboxylate
STM4172	<i>zraP</i>	zinc resistance protein
STM4184	<i>aceA</i>	isocitrate lyase; Catalyzes the first step in the glyoxalate cycle, which converts lipids to carbohydrates
STM4185	<i>aceK</i>	bifunctional isocitrate dehydrogenase kinase/phosphatase protein; catalyzes the phosphorylation/dephosphorylation of the enzyme isocitrate dehydrogenase on a specific serine which regulates activity; unphosphorylated IDH is fully active when cells are grown on glucose while the enzyme becomes phosphorylated and inactive in the presence of acetate or ethanol
STM4210		putative methyl-accepting chemotaxis protein
STM4307		putative anaerobic dimethylsulfoxide reductase subunit C; similar to <i>Escherichia coli</i> putative DMSO reductase anchor subunit (AAC74662.1)

Table A.1 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW supplemented with NOM for 4 weeks.

Gene symbol	Gene	Gene function
STM4365	<i>yjeT</i>	putative inner membrane protein
STM4379	<i>yjfO</i>	putative lipoprotein
STM4416	<i>mpl</i>	UDP-N-acetylmuramate/L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase; similar to <i>Escherichia coli</i> putative ligase (AAC77190.1)
STM4468	<i>yjgK</i>	putative cytoplasmic protein
STM4476.S	<i>holC</i>	DNA polymerase III subunit chi; binds to single-strand binding (SSB) protein and acts as a bridge between the DnaX clamp loader complex and the SSB
STM4534		putative transcriptional regulator; similar to <i>Escherichia coli</i> psp operon transcriptional activator (AAC74385.1); NtrC family; contains ATPase domain
STM4550	<i>fhuF</i>	ferric iron reductase involved in ferric hydroximate transport; involved in reduction of ferric iron in cytoplasmic ferrioxamine B
STM4564	<i>yjjV</i>	putative deoxyribonuclease YjjV
PSLT002		putative phospholipase D
PSLT013	<i>pefI</i>	regulator; plasmid-encoded fimbriae; orf6
PSLT030		putative cytoplasmic protein
PSLT032		putative diguanylate cyclase/phosphodiesterase
PSLT091	<i>traW</i>	conjugal transfer pilus assembly protein TraW; conjugative transfer
PSLT094.1N		hypothetical protein
PSLT096	<i>trbE</i>	conjugal transfer protein TrbE

^aThe annotated functions of genes were cited from (13).

A.1.2. Genetic Screening and Survival of *Salmonella typhimurium* in AGW Kept at 4°C

Table A.2. List of the genes with decreased signal in *S. typhimurium* exposed to AGW kept at 4°C for 4 weeks.

Gene symbol	Gene	Gene function ^a
STM0070	<i>caiD</i>	carnitiny-CoA dehydratase; catalyzes the dehydration of L-carnitiny-CoA to crotonobetainyl-CoA
STM0127	<i>ftsW</i>	cell division protein FtsW; integral membrane protein involved in stabilizing FtsZ ring during cell division
STM0139	<i>yacF</i>	putative cytoplasmic protein
STM0160	<i>yacL</i>	putative cytoplasmic protein
STM0216	<i>rpsB</i>	30S ribosomal protein S2; one of the last subunits in the assembly of the 30S subunit; absence of S2 does not inhibit assembly but results in an inactive subunit
STM0326		pseudogene; frameshift; putative HSP70 class molecular chaperone
STM0404	<i>queA</i>	S-adenosylmethionine:tRNA ribosyltransferase-isomerase; Synthesizes oQ from preQ1 in a single S-adenosylmethionine-requiring step
STM0486	<i>recR</i>	recombination protein RecR; involved in a recombinational process of DNA repair, independent of the recBC complex
STM0494	<i>ushA</i>	bifunctional UDP-sugar hydrolase/5'-nucleotidase periplasmic precursor; catalyzes the degradation of periplasmic UDP-glucose to uridine, glucose-1-phosphate and inorganic phosphate; specific for uridine nucleotides
STM0502	<i>ybbL</i>	putative ABC transporter ATP-binding protein YbbL; similar to <i>Escherichia coli</i> putative ATP-binding component of a transport system (AAC73592.1)
STM0503	<i>ybbM</i>	putative transport protein; similar to <i>Escherichia coli</i> putative metal resistance protein (AAC73593.1); YbbM family
STM0559	<i>rfbI</i>	putative glycosyl translocase
STM0565		putative periplasmic protein
STM0568	<i>pheP</i>	phenylalanine transporter; similar to <i>Escherichia coli</i> phenylalanine-specific transport system (AAC73677.1); APC family
STM0579	<i>ybdF</i>	putative cytoplasmic protein
STM0599	<i>ybdB</i>	hypothetical protein; possibly involved in aromatic compounds catabolism; PaaI
STM0605	<i>ybdN</i>	putative 3'-phosphoadenosine 5'-phosphosulfate sulfotransferase; PAPS reductase/FAD synthetase
STM0614	<i>ybdQ</i>	putative universal stress protein; similar to UspA and related nucleotide-binding protein
STM0627	<i>dcuC</i>	C4-dicarboxylate transporter DcuC; responsible for the transport of C4-dicarboxylates during anaerobic growth
STM0632	<i>tatE</i>	twin arginine translocase protein E; TatE; similar to TatA and found in some proteobacteria; part of system that translocates proteins with a conserved twin arginine motif across the inner membrane; capable of translocating folded substrates typically those with bound cofactors; similar to a protein import system in thylakoid membranes
STM0681	<i>nagD</i>	UMP phosphatase; similar to <i>Escherichia coli</i> N-acetylglucosamine metabolism (AAC73769.1)
STM0682	<i>nagC</i>	N-acetylglucosamine operon transcriptional repressor; similar to <i>Escherichia coli</i> transcriptional repressor of nag; N-acetylglucosamine operon (AAC73770.1); NagC/XylR family
STM0722		putative ABC transporter permease protein
STM0766	<i>dcoC</i>	oxaloacetate decarboxylase subunit gamma; catalyzes the decarboxylation of oxaloacetate to form pyruvate and CO ₂

Table A.2 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW kept at 4°C for 4 weeks.

Gene symbol	Gene	Gene function
STM0798	<i>uvrB</i>	excinuclease ABC subunit B; The UvrABC repair system catalyzes the recognition and processing of DNA lesions. The beta-hairpin of the Uvr-B subunit is inserted between the strands, where it probes for the presence of a lesion
STM0804	<i>moaC</i>	molybdenum cofactor biosynthesis protein C; MoaC; along with MoaA is involved in conversion of a guanosine derivative into molybdopterin precursor Z; involved in molybdenum cofactor biosynthesis
STM0812	<i>ybhO</i>	cardiolipin synthase 2; similar to <i>Escherichia coli</i> putative synthetase (AAC73876.1); CL synthase
STM0822	<i>ybiB</i>	hypothetical protein; similar to <i>Escherichia coli</i> putative enzyme (AAC73887.1)
STM0826	<i>ybiN</i>	putative SAM-dependent methyltransferase
STM0870		hypothetical protein; similar to <i>Escherichia coli</i> putative transport protein (AAC73934.1)
STM0969	<i>ycaM</i>	putative amino-acid transporter; similar to <i>Escherichia coli</i> putative transport (AAC73985.1); APC family
STM0974	<i>focA</i>	formate transporter; similar to <i>Escherichia coli</i> probable formate transporter; formate channel 1 (AAC73990.1); FNT family
STMsR037	<i>isrB-1</i>	
STM1010		Gifsy-2 prophage
STM1021		Gifsy-2 prophage
STM1047		tail assembly protein
STM1071	<i>sulA</i>	SOS cell division inhibitor; suppressor of lon (SW:SULA_SALTY); inhibitor of cell division and FtsZ ring formation upon DNA damage/inhibition; HslVU and Lon involved in its turnover
STMsR039	<i>STnc50</i>	
STM1136	<i>ycdX</i>	putative hydrolase; similar to hydrolases of the PHP family
STM1170	<i>mviN</i>	putative virulence protein; virulence factor MviN (SW:MVIN_SALTY)
STM1213	<i>ycfQ</i>	putative transcriptional repressor; TetR/AcrR family
STM1265		putative response regulator; contains CheY-like receiver domain and HTH DNA-binding domain
STM1282	<i>yeaK</i>	putative cytoplasmic protein
STMsR050	<i>STnc53</i>	
STM1298	<i>topB</i>	DNA topoisomerase III; decatenates replicating daughter chromosomes
STM1304	<i>astA</i>	arginine succinyltransferase
STM1342	<i>btuD</i>	vitamin B12-transporter ATPase; ATP-binding protein that acts with the transmembrane protein BtuC and the solute binding protein BtuF to transport vitamin B12 into the cell
STM1374	<i>ynhA</i>	cysteine desulfuration protein SufE; Acts with SufS to catalyze the formation of L-alanine from L-cysteine
STM1385	<i>ttrB</i>	tetrathionate reductase complex subunit B; tetrathionate reductase subunit B; TtrB (gi 4456871)
STM1444	<i>slyA</i>	transcriptional regulator SlyA; Transcription regulator that can both activate or repress expression

Table A.2 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW kept at 4°C for 4 weeks.

Gene symbol	Gene	Gene function
STM1451	<i>gst</i>	glutathionine S-transferase; similar to <i>Escherichia coli</i> glutathionine S-transferase (AAC74707.1)
STM1478	<i>ydgH</i>	putative periplasmic protein
STM1481	<i>tqsA</i>	putative transport protein; synonym: ydgG; transport of quorum-sensing signal; mutations in this gene alters the transport of the quorum-sensing signal autoinducer 2 (AI-2) which affects expression of a large number of genes
STM1484		putative protease
STM1541		putative regulatory protein; similar to <i>Escherichia coli</i> regulator for <i>uxu</i> operon (AAC77280.1); GntR family
STM1658	<i>ydaL</i>	hypothetical protein; contains putative Smr domain
STM1660.S	<i>fnr</i>	fumarate/nitrate reduction transcriptional regulator; Global transcription factor that controls the expression of over 100 target genes in response to anoxia
STM1690	<i>pspA</i>	phage shock protein PspA; involved in maintaining membrane potential under membrane stress conditions; also acts as a negative transcriptional regulator of the phage shock protein (<i>psp</i>) operon(<i>pspABCDE</i>) by regulating the transcriptional activator PspF
STM1716	<i>sohB</i>	putative periplasmic protease; SohB; periplasmic protein; member of the peptidase S49 family
STM1730	<i>yciE</i>	putative cytoplasmic protein
STM1844	<i>htpX</i>	heat shock protein HtpX; putative metalloprotease
STM1858		putative cytoplasmic protein
STM1859		putative cytoplasmic protein
STM1866		pseudogene; in-frame stop following codon 23
STM1868A		lytic enzyme
STM1869		phage-tail assembly-like protein
STM1869A		hypothetical protein
STM1902	<i>yecD</i>	hypothetical protein
STM1925	<i>flhD</i>	transcriptional activator FlhD; with FlhC is involved in the activation of class 2 flagellar genes and is involved in the regulation of a number of other genetic systems
STM1955	<i>fliZ</i>	protein FliZ; expression activator of the class 2 type of flagellar operons, essential to achieve maximal cell motility; activator of type 1 fimbrial gene expression; cell density-responsive regulator; FliZ in <i>Salmonella typhimurium</i> induces HilA, an activator of invasion
STM1963	<i>amyA</i>	genes necessary to achieve full virulence
STM2008		cytoplasmic alpha-amylase; converts 1,4-alpha-D-glucans to maltodextrin
STM2076	<i>hisA</i>	putative periplasmic protein
STM2083	<i>rfbK</i>	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase; catalyzes the formation of 5-(5-phospho-1-deoxyribulos-1-ylamino)methylideneamino-l-(5-phosphoribosyl)imidazole-4-carboxamide from 1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide
		phosphomannomutase; phosphomannomutase (SW:RFBK_SALTY); LPS side chain defect

Table A.2 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW kept at 4°C for 4 weeks.

Gene symbol	Gene	Gene function
STM2114	<i>wcaB</i>	putative colanic acid biosynthesis acetyltransferase WcaB; acetyltransferase believed to catalyze the addition of the acetyl group that is attached through an O linkage to the first fucosyl residue of the colanic acid repetitive unit (E unit); <i>wcaB</i> is induced in sessile bacteria and by osmotic shock, and repressed when grown in rich medium
STM2228	<i>yejM</i>	putative hydrolase; hypothetical protein in <i>rplY</i> - <i>proL</i> intergenic region (SW:YEJM_SALTY); alkaline phosphatase superfamily
STM2254	<i>ccmA</i>	cytochrome c biogenesis protein CcmA; ATP-binding protein; required for proper cytochrome c maturation
STM2255	<i>napC</i>	cytochrome c-type protein NapC; with NapABDFGH functions as a nitrate reductase; NapC functions as an electron shuttle between NapAB and NapGH or quinone
STM2344		putative phosphotransferase system enzyme II A component; similar to <i>Escherichia coli</i> putative PTS system enzyme II A component (AAC77152.1)
STM2360		putative diaminopimelate decarboxylase; similar to <i>Escherichia coli</i> diaminopimelate decarboxylase (AAC75877.1)
STM2389	<i>fadI</i>	3-ketoacyl-CoA thiolase; FadI; fatty acid oxidation complex component beta; functions in a heterotetramer with FadJ; similar to FadA2B2 complex; functions in beta-oxidation of fatty acids during anaerobic growth
STM2426		putative cytoplasmic protein
STM2475		putative cytoplasmic protein
STM2590		tail assembly protein I-like; similar to tail assembly protein I in phage lambda
STM2591		tail assembly protein K-like; similar to tail assembly protein K in phage lambda
STM2597		major tail-like protein; similar to major tail protein
STM2611.S		endopeptidase-like protein; similar to <i>Escherichia coli</i> bacteriophage lambda endopeptidase (AAC73657.1)
STMsr094	<i>isrI</i>	
STM2656	<i>rrfG</i>	5S ribosomal RNA
STM2795	<i>ygaU</i>	hypothetical protein; putative LysM domain
STM2871	<i>prgK</i>	needle complex inner membrane lipoprotein; PrgK protein precursor (SW:PRGK_SALTY); cell invasion protein; interacts with PrgH and forms oligomeric rings; type III secretion apparatus
STM2872	<i>prgJ</i>	needle complex minor subunit; PrgJ protein (SW:PRGJ_SALTY); invasion protein; may cap needle complex; type III secretion apparatus
STM2891	<i>spaO</i>	surface presentation of antigens protein SpaO; involved in a secretory pathway responsible for the surface presentation of determinants needed for the entry of <i>Salmonella</i> species into mammalian cells
STM2906		fragment of putative transposase
STM2925	<i>nlpD</i>	lipoprotein NlpD; outer membrane lipoprotein involved in stationary-phase cell survival; similar to LppB virulence determinant from <i>Haemophilus somnus</i>
STM2939	<i>ygcH</i>	putative cytoplasmic protein
STM3015	<i>ygeA</i>	putative racemase; similar to <i>Escherichia coli</i> putative resistance proteins (AAC75879.1)

Table A.2 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW kept at 4°C for 4 weeks.

Gene symbol	Gene	Gene function
STMsR115	<i>STnc28</i> 0	2-octaprenyl-6-methoxyphenyl hydroxylase; Oxygenase that introduces the hydroxyl group at carbon four of 2-octaprenyl-6-methoxyphenol resulting in the formation of 2-octaprenyl-6-methoxy-1,4-benzoquinone
STM3057	<i>ubiH</i>	putative regulatory protein; similar to <i>Escherichia coli</i> regulator for <i>uxu</i> operon (AAC77280.1); GntR family
STM3084.S	<i>yggJ</i>	hypothetical protein
STM3094	<i>nupG</i>	nucleoside transport; similar to <i>Escherichia coli</i> transport of nucleosides, permease protein (AAC76001.1); MFS family
STM3113		putative monoamine oxidase; similar to <i>Escherichia coli</i> putative aldehyde dehydrogenase (AAC74469.1)
STM3119		biopolymer transport protein ExbB; membrane spanning protein in TonB-ExbB-ExbD complex; involved in the tonB-independent energy-dependent transport iron-siderophore complexes and vitamin B12 into the cell
STM3159	<i>exbB</i>	cystathionine beta-lyase; catalyzes the formation of L-homocysteine from cystathionine
STM3161	<i>metC</i>	repressor protein for FtsI; SufI protein precursor (SW:SUF1_SALTY); putative periplasmic protein
STM3172	<i>sufI</i>	DNA-binding transcriptional regulator QseB; response regulator in two-component regulatory system with QseC; regulates FlhCD
STM3177	<i>ygiX</i>	which is the master regulator for flagella and motility genes
		transcription elongation factor GreA; necessary for efficient RNA polymerase transcription elongation past template-encoded arresting sites; arresting sites in DNA have the property of trapping a certain fraction of elongating RNA polymerases that pass through, resulting in locked ternary complexes. Cleavage of the nascent transcript by cleavage factors such as GreA or GreB allows the resumption of elongation from the new 3'terminus
STM3299	<i>greA</i>	oxaloacetate decarboxylase subunit gamma; catalyzes the decarboxylation of oxaloacetate to form pyruvate and carbon dioxide
STM3353	<i>oadG</i>	tartrate dehydratase subunit alpha; Involved in the tartrate degradation pathway
STM3355		p-hydroxybenzoic acid efflux subunit AaeA; with AaeB forms an efflux pump whose substrates are p-hydroxybenzoic acid, 6-hydroxy-2-naphthoic and 2-hydroxycinnamate
STM3365	<i>yhcQ</i>	rod shape-determining protein MreD; part of cell wall structural complex MreBCD; transmembrane component
STM3372	<i>mreD</i>	putative cytoplasmic protein
STM3456	<i>yheV</i>	glutathione-regulated potassium-efflux system protein KefB; involved in potassium efflux
STM3457	<i>kefB</i>	putative inner membrane protein; similar to <i>Escherichia coli</i> putative transport (AAC76427.1)
STM3499	<i>yhgE</i>	putative transcriptional regulator; similar to <i>Escherichia coli</i> putative regulator (AAC75308.1)
STM3533		putative transcriptional regulator; ORF408 (gi 4456874); regulator of sugar metabolism
STM3547.Sc		cystathionine gamma-synthase
STM3624A		putative acetyltransferase
STM3653		pseudogene; in-frame stop following codon 23
STM3654		putative outer membrane lipoprotein
STM3657		putative transcriptional regulator; similar to <i>Escherichia coli</i> possible NagC-like transcriptional regulator (AAC73497.1)
STM3681		

Table A.2 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW kept at 4°C for 4 weeks.

Gene symbol	Gene	Gene function
STM3688		putative cytoplasmic protein
	<i>STnc38</i>	
STMsR141	<i>0</i>	
STM3713	<i>rfaL</i>	O-antigen ligase; O-antigen ligase (SW:RFAL_SALTY) putative hexose transferase; lipopolysaccharide 1,2-N-acetylglucosaminetransferase (SW:RFAK_SALTY); lipopolysaccharide core
STM3714	<i>rfaK</i>	biosynthetic protein
STM3754		putative cytoplasmic protein; similar to putative ATP binding protein SugR (gi 4324607)
STM3758	<i>fidL</i>	putative inner membrane protein; FidL (gi 4324611)
STM3767		putative cytoplasmic protein
STM3779		putative phosphotransferase system HPr protein; similar to <i>Escherichia coli</i> PTS system protein HPr (AAC75468.1)
STM3786	<i>yicN</i>	putative inner membrane protein
STM3793		putative sugar kinase; similar to <i>Escherichia coli</i> ribokinase (AAC76775.1); ribokinase family
STM3891	<i>rrlC</i>	23S ribosomal RNA
STM3897	<i>yifA</i>	transcriptional regulator HdfR; Negatively regulates the transcription of the flagellar master operon flhDC by binding to the upstream region of the operon
STM3914	<i>rhlB</i>	ATP-dependent RNA helicase RhlB; enables ATP-dependent unwinding of double stranded RNA as a component of the RNA degradosome, a multi-enzyme complex important in RNA processing and messenger RNA degradation
STM3937	<i>hemD</i>	uroporphyrinogen-III synthase; catalyzes the formation of uroporphyrinogen-III from hydroxymethylbilane; functions in tetrapyrrole and heme biosynthesis
STM4055	<i>sodA</i>	superoxide dismutase; SodA; manganese binding; only present under aerobic conditions; destroys free radicals
STM4069		putative periplasmic protein
STM4155		putative inner membrane protein
STM4156		putative cytoplasmic protein
STM4170	<i>hupA</i>	transcriptional regulator HU subunit alpha; histone-like DNA-binding protein
STM4184	<i>aceA</i>	isocitrate lyase; Catalyzes the first step in the glyoxalate cycle, which converts lipids to carbohydrates
STM4232	<i>malM</i>	maltose regulon periplasmic protein; maltose operon periplasmic protein precursor (SW:MALM_SALTY); protein of mal regulon
STM4279	<i>nrfC</i>	putative formate-dependent nitrite reductase; similar to <i>Escherichia coli</i> formate-dependent nitrite reductase; FeS centers (AAC77042.1)
STM4307		putative anaerobic dimethylsulfoxide reductase subunit C; similar to <i>Escherichia coli</i> putative DMSO reductase anchor subunit (AAC74662.1)
STM4379	<i>yjfO</i>	putative lipoprotein
STM4382	<i>yjfR</i>	putative L-ascorbate 6-phosphate lactonase; contains beta-lactamase fold

Table A.2 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW kept at 4°C for 4 weeks.

Gene symbol	Gene	Gene function
STM4421		putative NAD-dependent aldehyde dehydrogenase; similar to <i>Escherichia coli</i> phenylacetaldehyde dehydrogenase (AAC74467.1)
STM4468	<i>yjgK</i>	putative cytoplasmic protein
STM4542	<i>yjjA</i>	hypothetical protein; similar to <i>Escherichia coli</i> putative glycoprotein/receptor (AAC77316.1)
STM4550	<i>fhuF</i>	ferric iron reductase involved in ferric hydroximate transport; involved in reduction of ferric iron in cytoplasmic ferrioxamine B
STM4558	<i>rimI</i>	ribosomal-protein-alanine N-acetyltransferase; alanine acetyltransferase that specifically acetylates ribosomal protein S18
PSLT013	<i>pefI</i>	regulator; plasmid-encoded fimbriae; orf6
PSLT030		putative cytoplasmic protein
PSLT091	<i>traW</i>	conjugal transfer pilus assembly protein TraW; conjugative transfer
PSLT094	<i>trbC</i>	conjugal transfer pilus assembly protein TrbC; conjugative transfer
PSLT094.1N		hypothetical protein
PSLT096	<i>trbE</i>	conjugal transfer protein TrbE

^aThe annotated functions of genes were cited from (13).

A.1.3. Genetic Screening and Survival of *Salmonella typhimurium* in AGW with High pH

Table A.3. List of the genes with decreased signal in *S. typhimurium* exposed to AGW with pH10 for 4 weeks.

Gene symbol	Gene	Gene function ^a
STM0136	<i>secA</i>	preprotein translocase subunit SecA; synonyms: azi, div; functions in protein export; can interact with acidic membrane phospholipids and the SecYEG protein complex; binds to preproteins; binds to ATP and undergoes a conformational change to promote membrane insertion of SecA/bound preprotein; ATP hydrolysis appears to drive release of the preprotein from SecA and deinsertion of SecA from the membrane; additional proteins SecD/F/YajC aid SecA recycling; exists in an equilibrium between monomers and dimers; may possibly form higher order oligomers; proteins in this cluster correspond SecA1; SecA2 is not essential and seems to play a role in secretion of a subset of proteins
STM0139	<i>yacF</i>	putative cytoplasmic protein
STM0253	<i>rrfH</i>	5S ribosomal RNA
STM0287		putative periplasmic protein
STM0397	<i>phoB</i>	transcriptional regulator PhoB; two component response regulator for the phosphate regulon; PhoR phosphorylates PhoB
STM0467	<i>ffs</i>	misc_RNA; RNA component of signal recognition particle; processed at 5' end by RNaseP; 4.5S ribosomal RNA
STM0474	<i>ybaJ</i>	putative cytoplasmic protein
STM0502	<i>ybbL</i>	putative ABC transporter ATP-binding protein YbbL; similar to <i>Escherichia coli</i> putative ATP-binding component of a transport system (AAC73592.1)
STM0555		pseudogene; frameshift relative to <i>Escherichia coli</i> IS3 putative transposase (GB:AAC75150.1)
STM0559	<i>rfbI</i>	putative glycosyl translocase
STM0605	<i>ybdN</i>	putative 3'-phosphoadenosine 5'-phosphosulfate sulfotransferase; PAPS reductase/FAD synthetase
STM0614	<i>ybdQ</i>	putative universal stress protein; similar to UspA and related nucleotide-binding protein
STM0627	<i>dcuC</i>	C4-dicarboxylate transporter DcuC; responsible for the transport of C4-dicarboxylates during anaerobic growth
STM0682	<i>nagC</i>	N-acetylglucosamine operon transcriptional repressor; similar to <i>Escherichia coli</i> transcriptional repressor of nag; N-acetylglucosamine operon (AAC73770.1); NagC/XylR family
STM0766	<i>dcoC</i>	oxaloacetate decarboxylase subunit gamma; catalyzes the decarboxylation of oxaloacetate to form pyruvate and carbon dioxide
STM0826	<i>ybiN</i>	putative SAM-dependent methyltransferase
STM0870		hypothetical protein; similar to <i>Escherichia coli</i> putative transport protein (AAC73934.1)
STM0972		SopD-like protein
STM1010		Gifsy-2 prophage
STM1010.1n		hypothetical protein
STM1021		Gifsy-2 prophage
STM1025		Gifsy-2 prophage
STM1047		tail assembly protein
STM1056		MsgA-like protein

Table A.3 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW with pH10 for 4 weeks.

Gene symbol	Gene	Gene function
STM1071	<i>sulA</i>	SOS cell division inhibitor; suppressor of lon (SW:SULA_SALTY); inhibitor of cell division and FtsZ ring formation upon DNA damage/inhibition; HslVU and Lon involved in its turnover
STM1136	<i>ycdX</i>	putative hydrolase; similar to hydrolases of the PHP family
STM1213	<i>ycfQ</i>	putative transcriptional repressor; TetR/AcrR family
STM1282	<i>yeaK</i>	putative cytoplasmic protein
STMsR050	<i>STnc53</i> <i>0</i>	
STM1342	<i>btuD</i>	vitamin B12-transporter ATPase; ATP-binding protein that acts with the transmembrane protein BtuC and the solute binding protein BtuF to transport vitamin B12 into the cell
STM1374	<i>ynhA</i>	cysteine desufuration protein SufE; Acts with SufS to catalyze the formation of L-alanine from L-cysteine
STM1478	<i>ydgH</i>	putative periplasmic protein
STM1484		putative protease
STM1690	<i>pspA</i>	phage shock protein PspA; involved in maintaining membrane potential under membrane stress conditions; also acts as a negative transcriptional regulator of the phage shock protein (psp) operon (pspABCDE) by regulating the transcriptional activator PspF
STM1828	<i>yoaE</i>	putative inner membrane protein; similar to <i>Escherichia coli</i> putative transport protein (AAC74886.1)
STM1838	<i>yobF</i>	putative cytoplasmic protein
STM1859		putative cytoplasmic protein
STM1868A		lytic enzyme
STM1902	<i>yecD</i>	hypothetical protein
STM1925	<i>flhD</i>	transcriptional activator FlhD; with FlhC is involved in the activation of class 2 flagellar genes and is involved in the regulation of a number of other genetic systems
STM1947	<i>uvrY</i>	response regulator; synonym: sirA; in <i>Escherichia coli</i> the protein UvrY is part of a two-component system along with BarA that is needed for efficient switching between glycolytic and gluconeogenic carbon sources possibly by regulating the Csr system; in <i>Salmonella</i> SirA and BarA regulate virulence gene expression also via the Csr system
STM2008		putative periplasmic protein
STM2076	<i>hisA</i>	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase; catalyzes the formation of 5-(5-phospho-1-deoxyribulos-1-ylamino)methylideneamino-1-(5-phosphoribosyl)imidazole-4-carboxamide from 1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide
STM2220	<i>yejG</i>	hypothetical protein
STM2255	<i>napC</i>	cytochrome c-type protein NapC; with NapABDFGH functions as a nitrate reductase; NapC functions as an electron shuttle between NapAB and NapGH or quinone
STM2269	<i>yojN</i>	phosphotransfer intermediate protein in two-component regulatory system with RcsBC; similar to <i>Escherichia coli</i> putative 2-component sensor protein (AAC75276.1); regulatory protein

Table A.3 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW with pH10 for 4 weeks.

Gene symbol	Gene	Gene function
STM2341		putative transketolase; similar to <i>Escherichia coli</i> transketolase 1 isozyme (AAC75972.1)
STM2344		putative phosphotransferase system enzyme II A component; similar to <i>Escherichia coli</i> putative PTS system enzyme II A component (AAC77152.1)
STM2360		putative diaminopimelate decarboxylase; similar to <i>Escherichia coli</i> diaminopimelate decarboxylase (AAC75877.1)
STM2363	<i>cvpA</i>	colicin V production protein; membrane protein required for colicin V production
STM2448	<i>yfeZ</i>	putative inner membrane protein
STM2452		putative inner membrane protein; hypothetical protein in eutR-hemF intergenic region; ORF33 (SW:YPPFK_SALTY)
STM2475		putative cytoplasmic protein
STM2590		tail assembly protein I-like; similar to tail assembly protein I in phage lambda
STM2591		tail assembly protein K-like; similar to tail assembly protein K in phage lambda
STM2611.S		endopeptidase-like protein; similar to <i>Escherichia coli</i> bacteriophage lambda endopeptidase (AAC73657.1)
STM2639	<i>rseA</i>	anti-RNA polymerase sigma factor SigE; similar to <i>Escherichia coli</i> anti sigma-E/sigma 24 factor; negative regulatory protein (AAC75625.1)
STM2656	<i>rrfG</i>	5S ribosomal RNA
STM2787		tricarboxylic transport
STM2795	<i>ygaU</i>	hypothetical protein; putative LysM domain
STM2834	<i>slrB</i>	glucitol/sorbitol-specific PTS system component IIA; phosphoenolpyruvate-dependent sugar phosphotransferase system; catalyzes the phosphorylation of incoming sugar substrates concomitant with their translocation across the cell membrane; IIB is phosphorylated by IIA and then transfers the phosphoryl group to the sugar; IIC forms the translocation channel
STM2871	<i>prgK</i>	needle complex inner membrane lipoprotein; PrgK protein precursor (SW:PRGK_SALTY); cell invasion protein; interacts with PrgH and forms oligomeric rings; type III secretion apparatus
STM2872	<i>prgJ</i>	needle complex minor subunit; PrgJ protein (SW:PRGJ_SALTY); invasion protein; may cap needle complex; type III secretion apparatus
STM2873	<i>prgI</i>	needle complex major subunit; PrgI protein (SW:PRGI_SALTY); invasion protein; type III secretion apparatus
STM2924	<i>rpoS</i>	RNA polymerase sigma factor RpoS; sigma factors are initiation factors that promote the attachment of RNA polymerase to specific initiation sites and are then released; this sigma factor controls a regulon of genes required for protection against external stresses
STM3015	<i>ygeA</i>	putative racemase; similar to <i>Escherichia coli</i> putative resistance proteins (AAC75879.1)
STM3019	<i>rygC</i>	
STM3084.S		putative regulatory protein; similar to <i>Escherichia coli</i> regulator for <i>uxu</i> operon (AAC77280.1); GntR family

Table A.3 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW with pH10 for 4 weeks.

Gene symbol	Gene	Gene function
STM3111	<i>yggX</i>	hypothetical protein
STM3119		putative monoamine oxidase; similar to <i>Escherichia coli</i> putative aldehyde dehydrogenase (AAC74469.1)
STM3197	<i>glgS</i>	glycogen synthesis protein GlgS; Involved in glycogen synthesis. May be involved in glycogen priming transcription elongation factor GreA; necessary for efficient RNA polymerase transcription elongation past template-encoded arresting sites; arresting sites in DNA have the property of trapping a certain fraction of elongating RNA polymerases that pass through, resulting in locked ternary complexes. Cleavage of the nascent transcript by cleavage factors such as GreA or GreB allows the resumption of elongation from the new 3'terminus
STM3299	<i>greA</i>	
STMsr129	<i>T18</i>	
STM3353	<i>oadG</i>	oxaloacetate decarboxylase subunit gamma; catalyzes the decarboxylation of oxaloacetate to form pyruvate and carbon dioxide
STM3365	<i>yhcQ</i>	p-hydroxybenzoic acid efflux subunit AaeA; with AaeB forms an efflux pump whose substrates are p-hydroxybenzoic acid, 6-hydroxy-2-naphthoic and 2-hydroxycinnamate
STM3392	<i>yhdV</i>	putative outer membrane lipoprotein
STM3534	<i>glgP</i>	glycogen phosphorylase; similar to <i>Escherichia coli</i> glycogen phosphorylase (AAC76453.1)
STM3547.Sc		putative transcriptional regulator; ORF408 (gi 4456874); regulator of sugar metabolism
STM3591	<i>uspA</i>	universal stress protein A; similar to <i>Escherichia coli</i> universal stress protein; broad regulatory function (AAC76520.1)
STM3617		endo-1,4-D-glucanase; catalyzes the hydrolysis of 1,4-beta-D-glucosidic linkages in cellulose, lichenin and cereal beta-D-glucans
STM3624A		cystathionine gamma-synthase
STM3653		putative acetyltransferase
STM3654		pseudogene; in-frame stop following codon 23
STM3657		putative outer membrane lipoprotein
STM3681		putative transcriptional regulator; similar to <i>Escherichia coli</i> possible NagC-like transcriptional regulator (AAC73497.1)
STM3687	<i>mtlR</i>	mannitol repressor protein; Acts as a repressor of the mtlAD operon
STMsr141	<i>STnc38</i>	
STM3758	<i>0</i>	
	<i>fidL</i>	putative inner membrane protein; FidL (gi 4324611)
		bifunctional N-acetylglucosamine-1-phosphate uridyltransferase/glucosamine-1-phosphate acetyltransferase; forms a homotrimer; catalyzes the acetylation of glucosamine-1-phosphate and uridylation of N-acetylglucosamine-1-phosphate to produce UDP-
STM3862	<i>glmU</i>	GlcNAc; function in cell wall synthesis
STM3873.S	<i>gidB</i>	glucose-inhibited division protein B; probable SAM-dependent methyltransferase
STM3886	<i>rbsR</i>	transcriptional repressor RbsR; DNA-binding transcriptional repressor of ribose metabolism

Table A.3 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW with pH10 for 4 weeks.

Gene symbol	Gene	Gene function
STM3897	<i>yifA</i>	transcriptional regulator HdfR; Negatively regulates the transcription of the flagellar master operon flhDC by binding to the upstream region of the operon
STM3914	<i>rhlB</i>	ATP-dependent RNA helicase RhlB; enables ATP-dependent unwinding of double stranded RNA as a component of the RNA degradosome, a multi-enzyme complex important in RNA processing and messenger RNA degradation
STM3937	<i>hemD</i>	uroporphyrinogen-III synthase; catalyzes the formation of uroporphyrinogen-III from hydroxymethylbilane; functions in tetrapyrrole and heme biosynthesis
STM4073	<i>ydeW</i>	putative transcriptional repressor; similar to <i>Escherichia coli</i> putative transcriptional regulator, sorC family (AAC74585.1)
STM4077	<i>yneA</i>	putative sugar transport protein; similar to <i>Escherichia coli</i> putative LacI-type transcriptional regulator (AAC74589.1); putative ABC superfamily periplasmic protein
STM4155		putative inner membrane protein
STM4156		putative cytoplasmic protein
STMsR160	<i>HB236/530</i>	bifunctional isocitrate dehydrogenase kinase/phosphatase protein; catalyzes the phosphorylation/dephosphorylation of the enzyme isocitrate dehydrogenase on a specific serine which regulates activity; unphosphorylated IDH is fully active when cells are grown on glucose while the enzyme becomes phosphorylated and inactive in the presence of acetate or ethanol
STM4185	<i>aceK</i>	maltose regulon periplasmic protein; maltose operon periplasmic protein precursor (SW:MALM_SALTY); protein of mal regulon
STM4232	<i>malM</i>	putative formate-dependent nitrite reductase; similar to <i>Escherichia coli</i> formate-dependent nitrite reductase; FeS centers (AAC77042.1)
STM4279	<i>nrfC</i>	putative lipoprotein
STM4379	<i>yjfO</i>	putative inner membrane protein
STM4503		hypothetical protein; similar to <i>Escherichia coli</i> putative glycoprotein/receptor (AAC77316.1)
STM4542	<i>yjjA</i>	putative cytoplasmic protein
STM4549	<i>fhuF</i>	ferric iron reductase involved in ferric hydroximate transport; involved in reduction of ferric iron in cytoplasmic ferrioxamine B
STM4550	<i>rimI</i>	ribosomal-protein-alanine N-acetyltransferase; alanine acetyltransferase that specifically acetylates ribosomal protein S18
STM4558	<i>yjjV</i>	putative deoxyribonuclease YjjV
STM4564	<i>pefI</i>	regulator; plasmid-encoded fimbriae; orf6
PSLT013		putative inner membrane protein; similar to DNA polymerase III theta subunit
PSLT064	<i>traW</i>	conjugal transfer pilus assembly protein TraW; conjugative transfer
PSLT091	<i>trbC</i>	conjugal transfer pilus assembly protein TrbC; conjugative transfer
PSLT094		hypothetical protein
PSLT094.1N		

Table A.3 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW with pH10 for 4 weeks.

Gene symbol	Gene	Gene function
PSLT096	<i>trbE</i>	conjugal transfer protein TrbE

^aThe annotated functions of genes were cited from (13).

A.1.4. Genetic Screening and Survival of *Salmonella typhimurium* in AGW Kept at Static Conditions

Table A.4. List of the genes with decreased signal in *S. typhimurium* exposed to AGW kept at static condition for 4 weeks.

Gene symbol	Gene	Gene function ^a
STM0136	<i>secA</i>	preprotein translocase subunit SecA; synonyms: azi, div; functions in protein export; can interact with acidic membrane phospholipids and the SecYEG protein complex; binds to preproteins; binds to ATP and undergoes a conformational change to promote membrane insertion of SecA/bound preprotein; ATP hydrolysis appears to drive release of the preprotein from SecA and deinsertion of SecA from the membrane; additional proteins SecD/F/YajC aid SecA recycling; exists in an equilibrium between monomers and dimers; may possibly form higher order oligomers; proteins in this cluster correspond SecA1; SecA2 is not essential and seems to play a role in secretion of a subset of proteins
STM0287		putative periplasmic protein
STM0362		putative cytoplasmic protein
STM0397	<i>phoB</i>	transcriptional regulator PhoB; two component response regulator for the phosphate regulon; PhoR phosphorylates PhoB
STM0474	<i>ybaJ</i>	putative cytoplasmic protein
STM0486	<i>recR</i>	recombination protein RecR; involved in a recombinational process of DNA repair, independent of the recBC complex
STM0502	<i>ybbL</i>	putative ABC transporter ATP-binding protein YbbL; similar to <i>Escherichia coli</i> putative ATP-binding component of a transport system (AAC73592.1)
STM0503	<i>ybbM</i>	putative transport protein; similar to <i>Escherichia coli</i> putative metal resistance protein (AAC73593.1); YbbM family
STM0552	<i>fimW</i>	putative fimbrial protein; fimbriae W protein (SW:FIMW_SALTY)
STM0559	<i>rfbI</i>	putative glycosyl translocase
STM0605	<i>ybdN</i>	putative 3'-phosphoadenosine 5'-phosphosulfate sulfotransferase; PAPS reductase/FAD synthetase
STM0624	<i>STnc70</i>	
STM0614	<i>ybdQ</i>	putative universal stress protein; similar to UspA and related nucleotide-binding protein
STM0627	<i>dcuC</i>	C4-dicarboxylate transporter DcuC; responsible for the transport of C4-dicarboxylates during anaerobic growth
STM0682	<i>nagC</i>	N-acetylglucosamine operon transcriptional repressor; similar to <i>Escherichia coli</i> transcriptional repressor of nag; N-acetylglucosamine operon (AAC73770.1); NagC/XylR family
STM0696	<i>ybfF</i>	putative enzyme
STM0722		putative ABC transporter permease protein
STM0766	<i>dcoC</i>	oxaloacetate decarboxylase subunit gamma; catalyzes the decarboxylation of oxaloacetate to form pyruvate and carbon dioxide
STM0826	<i>ybiN</i>	putative SAM-dependent methyltransferase
STM0870		hypothetical protein; similar to <i>Escherichia coli</i> putative transport protein (AAC73934.1)
STM0970	<i>pflA</i>	pyruvate formate lyase-activating enzyme 1; activates pyruvate formate-lyase 1 under anaerobic conditions
STM1010		Gifsy-2 prophage
STM1021		Gifsy-2 prophage

Table A.4 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW kept at static condition for 4 weeks.

Gene symbol	Gene	Gene function
STM1047		tail assembly protein
STM1071	<i>sulA</i>	SOS cell division inhibitor; suppressor of lon (SW:SULA_SALTY); inhibitor of cell division and FtsZ ring formation upon DNA damage/inhibition; HslVU and Lon involved in its turnover
STM1115	<i>scsC</i>	suppression of copper sensitivity protein; suppressor for copper-sensitivity C (gi 2327005); suppression of copper sensitivity
STMsR039	<i>STnc50</i>	
STM1136	<i>ycdX</i>	putative hydrolase; similar to hydrolases of the PHP family
STM1213	<i>ycfQ</i>	putative transcriptional repressor; TetR/AcrR family
STM1282	<i>yeaK</i>	putative cytoplasmic protein
STMsR050	<i>STnc53</i>	
STM1315	<i>celD</i>	DNA-binding transcriptional regulator ChbR; represses the celABCDF-ydjC operon involved in carbon uptake
STM1342	<i>btuD</i>	vitamin B12-transporter ATPase; ATP-binding protein that acts with the transmembrane protein BtuC and the solute binding protein BtuF to transport vitamin B12 into the cell
STM1374	<i>ynhA</i>	cysteine desulfuration protein SufE; Acts with SufS to catalyze the formation of L-alanine from L-cysteine
STM1444	<i>slyA</i>	transcriptional regulator SlyA; Transcription regulator that can both activate or repress expression
STM1478	<i>ydgH</i>	putative periplasmic protein
STM1575		putative transcriptional regulator; TetR family
STM1673		putative outer membrane lipoprotein
STM1690	<i>pspA</i>	phage shock protein PspA; involved in maintaining membrane potential under membrane stress conditions; also acts as a negative transcriptional regulator of the phage shock protein (psp) operon(pspABCDE) by regulating the transcriptional activator PspF
STM1697		hypothetical protein; putative diguanylate cyclase/phosphodiesterase domain 2
STM1730	<i>yciE</i>	putative cytoplasmic protein
STM1828	<i>yoeA</i>	putative inner membrane protein; similar to <i>Escherichia coli</i> putative transport protein (AAC74886.1)
STM1837	<i>cspC</i>	cold shock-like protein CspC; cold shock-like protein CspC (SW:CSPC_SALTY); multicopy suppresses mukB mutants; putative regulator
STM1838	<i>yobF</i>	putative cytoplasmic protein
STM1859		putative cytoplasmic protein
STM1866		pseudogene; in-frame stop following codon 23
STM1868A		lytic enzyme
STM1869		phage-tail assembly-like protein

Table A.4 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW kept at static condition for 4 weeks.

Gene symbol	Gene	Gene function
STM2008		putative periplasmic protein
STM2076	<i>hisA</i>	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase; catalyzes the formation of 5-(5-phospho-1-deoxyribulos-1-ylamino)methylideneamino-1-(5-phosphoribosyl)imidazole-4-carboxamide from 1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide
STM2255	<i>napC</i>	cytochrome c-type protein NapC; with NapABDFGH functions as a nitrate reductase; NapC functions as an electron shuttle between NapAB and NapGH or quinone
STM2269	<i>yojN</i>	phosphotransfer intermediate protein in two-component regulatory system with RcsBC; similar to <i>Escherichia coli</i> putative 2-component sensor protein (AAC75276.1); regulatory protein
STM2295	<i>yfaO</i>	putative NTP pyrophosphohydrolase; similar to oxidative damage repair enzymes
STM2360		putative diaminopimelate decarboxylase; similar to <i>Escherichia coli</i> diaminopimelate decarboxylase (AAC75877.1)
STM2426		putative cytoplasmic protein
STM2448	<i>yfeZ</i>	putative inner membrane protein
STM2475		putative cytoplasmic protein
STM2590		tail assembly protein I-like; similar to tail assembly protein I in phage lambda
STM2591		tail assembly protein K-like; similar to tail assembly protein K in phage lambda
STM2656	<i>rrfG</i>	5S ribosomal RNA
STM2706		phage tail-like protein; similar to tail fiber protein in phage P2
STM2787		tricarboxylic transport
STM2815	<i>emrB</i>	putative multidrug transport protein; similar to <i>Escherichia coli</i> multidrug resistance; probably membrane translocase (AAC75733.1); MFS family
STM2871	<i>prgK</i>	needle complex inner membrane lipoprotein; PrgK protein precursor (SW:PRGK_SALTY); cell invasion protein; interacts with PrgH and forms oligomeric rings; type III secretion apparatus
STM2872	<i>prgJ</i>	needle complex minor subunit; PrgJ protein (SW:PRGJ_SALTY); invasion protein; may cap needle complex; type III secretion apparatus
STM2873	<i>prgI</i>	needle complex major subunit; PrgI protein (SW:PRGI_SALTY); invasion protein; type III secretion apparatus
STM2924	<i>rpoS</i>	RNA polymerase sigma factor RpoS; sigma factors are initiation factors that promote the attachment of RNA polymerase to specific initiation sites and are then released; this sigma factor controls a regulon of genes required for protection against external stresses
STM3015	<i>ygeA</i>	putative racemase; similar to <i>Escherichia coli</i> putative resistance proteins (AAC75879.1)
STM3084.S	<i>rygC</i>	
STM3094	<i>yggJ</i>	putative regulatory protein; similar to <i>Escherichia coli</i> regulator for uxu operon (AAC77280.1); GntR family
STM3111	<i>yggX</i>	hypothetical protein

Table A.4 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW kept at static condition for 4 weeks.

Gene symbol	Gene	Gene function
STM3159	<i>exbB</i>	biopolymer transport protein ExbB; membrane spanning protein in TonB-ExbB-ExbD complex; involved in the tonB-independent energy-dependent transport iron-siderophore complexes and vitamin B12 into the cell
STM3197	<i>glgS</i>	glycogen synthesis protein GlgS; Involved in glycogen synthesis. May be involved in glycogen priming
STM3281	<i>nlpI</i>	lipoprotein NlpI; lipoprotein that appears to be involved in cell division; interacts with the periplasmic protease Prc and may be activated by protease processing
STMsR129	<i>T18</i>	
STM3353	<i>oadG</i>	oxaloacetate decarboxylase subunit gamma; catalyzes the decarboxylation of oxaloacetate to form pyruvate and carbon dioxide
STM3365	<i>yhcQ</i>	p-hydroxybenzoic acid efflux subunit AaeA; with AaeB forms an efflux pump whose substrates are p-hydroxybenzoic acid, 6-hydroxy-2-naphthoic and 2-hydroxycinnamate
STM3456	<i>yheV</i>	putative cytoplasmic protein
STM3533		putative transcriptional regulator; similar to <i>Escherichia coli</i> putative regulator (AAC75308.1)
STM3547.Sc		putative transcriptional regulator; ORF408 (gi 4456874); regulator of sugar metabolism
STM3624A		cystathionine gamma-synthase
STM3653		putative acetyltransferase
STM3654		pseudogene; in-frame stop following codon 23
STM3657		putative outer membrane lipoprotein
STM3681		putative transcriptional regulator; similar to <i>Escherichia coli</i> possible NagC-like transcriptional regulator (AAC73497.1)
STM3688		putative cytoplasmic protein
STMsR141	<i>STnc38</i>	
STM3754	<i>0</i>	
STM3758	<i>fidL</i>	putative cytoplasmic protein; similar to putative ATP binding protein SugR (gi 4324607)
STM3873.S	<i>gidB</i>	putative inner membrane protein; FidL (gi 4324611)
STM3886	<i>rbsR</i>	glucose-inhibited division protein B; probable SAM-dependent methyltransferase
		transcriptional repressor RbsR; DNA-binding transcriptional repressor of ribose metabolism
STM3897	<i>yifA</i>	transcriptional regulator HdfR; Negatively regulates the transcription of the flagellar master operon flhDC by binding to the upstream region of the operon
STM3946	<i>yifL</i>	putative outer membrane lipoprotein
STM4073	<i>ydeW</i>	putative transcriptional repressor; similar to <i>Escherichia coli</i> putative transcriptional regulator, sorC family (AAC74585.1)
STM4155		putative inner membrane protein
STM4170	<i>hupA</i>	transcriptional regulator HU subunit alpha; histone-like DNA-binding protein
STM4184	<i>aceA</i>	isocitrate lyase; Catalyzes the first step in the glyoxalate cycle, which converts lipids to carbohydrates

Table A.4 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to AGW kept at static condition for 4 weeks.

Gene symbol	Gene	Gene function
STM4232	<i>malm</i>	maltose regulon periplasmic protein; maltose operon periplasmic protein precursor (SW:MALM_SALTY); protein of mal regulon
STM4279	<i>nrfC</i>	putative formate-dependent nitrite reductase; similar to <i>Escherichia coli</i> formate-dependent nitrite reductase; FeS centers (AAC77042.1)
STM4382	<i>yjfR</i>	putative L-ascorbate 6-phosphate lactonase; contains beta-lactamase fold
STM4468	<i>yjgK</i>	putative cytoplasmic protein
STM4503		putative inner membrane protein
STM4550	<i>fhuF</i>	ferric iron reductase involved in ferric hydroximate transport; involved in reduction of ferric iron in cytoplasmic ferrioxamine B
STM4558	<i>rimI</i>	ribosomal-protein-alanine N-acetyltransferase; alanine acetyltransferase that specifically acetylates ribosomal protein S18
PSLT013	<i>pefI</i>	regulator; plasmid-encoded fimbriae; orf6
PSLT064		putative inner membrane protein; similar to DNA polymerase III theta subunit
PSLT091	<i>traW</i>	conjugal transfer pilus assembly protein TraW; conjugative transfer
PSLT094	<i>trbC</i>	conjugal transfer pilus assembly protein TrbC; conjugative transfer
PSLT094.1N		conjugative transfer: assembly

^aThe annotated functions of genes were cited from (13).

A.1.5. Genetic Screening and Survival of *Salmonella typhimurium* in ASW

Table A.5. List of the genes with decreased signal in *S. typhimurium* exposed to ASW for 4 weeks.

Gene symbol	Gene	Gene function ^a
STM0098		putative secreted protein
STM0160	<i>yacL</i>	putative cytoplasmic protein
STM0246	<i>yaeE</i>	DL-methionine transporter permease subunit; part of the MetNIQ methionine uptake system
STM0253	<i>rrfH</i>	5S ribosomal RNA
STM0287		putative periplasmic protein
STM0383	<i>yaiB</i>	putative cytoplasmic protein
STM0397	<i>phoB</i>	transcriptional regulator PhoB; two component response regulator for the phosphate regulon; PhoR phosphorylates PhoB
		hemolysin expression-modulating protein; with Hns involved in transcriptional regulation of hemolysin; non-specific DNA-binding
STM0473	<i>hha</i>	protein which affects the production of multiple proteins
STM0474	<i>ybaJ</i>	hypothetical protein
		putative ABC transporter ATP-binding protein YbbL; similar to <i>Escherichia coli</i> putative ATP-binding component of a transport
STM0502	<i>ybbL</i>	system (AAC73592.1)
STM0552	<i>fimW</i>	putative fimbrial protein; fimbriae W protein (SW:FIMW_SALTY)
STM0555		pseudogene; frameshift relative to <i>Escherichia coli</i> IS3 putative transposase (GB:AAC75150.1)
STM0766	<i>dcoC</i>	oxaloacetate decarboxylase subunit gamma; catalyzes the decarboxylation of oxaloacetate to form pyruvate and carbon dioxide
STM0969	<i>ycaM</i>	putative amino-acid transporter; similar to <i>Escherichia coli</i> putative transport (AAC73985.1); APC family
STMsr037	<i>isrB-1</i>	
STM1021		Gifsy-2 prophage
STM1047		tail assembly protein
		SOS cell division inhibitor; suppressor of lon (SW:SULA_SALTY); inhibitor of cell division and FtsZ ring formation upon DNA
STM1071	<i>sulA</i>	damage/inhibition; HslVU and Lon involved in its turnover
STM1136	<i>ycdX</i>	putative hydrolase; similar to hydrolases of the PHP family
STM1213	<i>ycfQ</i>	putative transcriptional repressor; TetR/AcrR family
STM1282	<i>yeaK</i>	putative cytoplasmic protein
	<i>STnc53</i>	
STMsr050	<i>0</i>	
		vitamin B12-transporter ATPase; ATP-binding protein that acts with the transmembrane protein BtuC and the solute binding protein
STM1342	<i>btuD</i>	BtuF to transport vitamin B12 into the cell
STM1541		putative regulatory protein; similar to <i>Escherichia coli</i> regulator for <i>uxu</i> operon (AAC77280.1); GntR family

Table A.5 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to ASW for 4 weeks.

Gene symbol	Gene	Gene function
STM1690	<i>pspA</i>	phage shock protein PspA; involved in maintaining membrane potential under membrane stress conditions; also acts as a negative transcriptional regulator of the phage shock protein (psp) operon(<i>pspABCDE</i>) by regulating the transcriptional activator PspF
STM1730	<i>yciE</i>	putative cytoplasmic protein
STM1838	<i>yobF</i>	putative cytoplasmic protein
STM1859		putative cytoplasmic protein
STM2008		putative periplasmic protein
STM2064	<i>phsB</i>	thiosulfate reductase electron transport protein; PhsB (SW:PHSB_SALTY); hydrogen sulfide production; iron-sulfur subunit; electron transfer
STM2082	<i>rfbP</i>	undecaprenol-phosphate galactosephosphotransferase/O-antigen transferase; undecaprenyl-phosphate galactosephosphotransferase (SW:RFBP_SALTY); mutation results in LPS side chain defect
STM2255	<i>napC</i>	cytochrome c-type protein NapC; with NapABDFGH functions as a nitrate reductase; NapC functions as an electron shuttle between NapAB and NapGH or quinone
STM2360		putative diaminopimelate decarboxylase; similar to <i>Escherichia coli</i> diaminopimelate decarboxylase (AAC75877.1)
STM2448	<i>yfeZ</i>	putative inner membrane protein
STM2591		tail assembly protein K-like; similar to tail assembly protein K in phage lambda
STM2656	<i>rrfG</i>	5S ribosomal RNA
STM2706		phage tail-like protein; similar to tail fiber protein in phage P2
STM2749		putative cytoplasmic protein; similar to <i>Escherichia coli</i> regulatory factor of maltose metabolism; similar to Ner repressor protein of phage Mu (AAC76220.1)
STM2834	<i>slrB</i>	glucitol/sorbitol-specific PTS system component IIA; phosphoenolpyruvate-dependent sugar phosphotransferase system; catalyzes the phosphorylation of incoming sugar substrates concomitant with their translocation across the cell membrane; IIB is phosphorylated by IIA and then transfers the phosphoryl group to the sugar; IIC forms the translocation channel
STM2872	<i>prgJ</i>	needle complex minor subunit; PrgJ protein (SW:PRGJ_SALTY); invasion protein; may cap needle complex; type III secretion apparatus
STM2873	<i>prgI</i>	needle complex major subunit; PrgI protein (SW:PRGI_SALTY); invasion protein; type III secretion apparatus
STM2924	<i>rpoS</i>	RNA polymerase sigma factor RpoS; sigma factors are initiation factors that promote the attachment of RNA polymerase to specific initiation sites and are then released; this sigma factor controls a regulon of genes required for protection against external stresses
STM3084.S		putative regulatory protein; similar to <i>Escherichia coli</i> regulator for <i>uxu</i> operon (AAC77280.1); GntR family
STM3119		putative monoamine oxidase; similar to <i>Escherichia coli</i> putative aldehyde dehydrogenase (AAC74469.1)
STM3161	<i>metC</i>	cystathionine beta-lyase; catalyzes the formation of L-homocysteine from cystathionine
STM3197	<i>glgS</i>	glycogen synthesis protein GlgS; Involved in glycogen synthesis. May be involved in glycogen priming

Table A.5 (Cont.). List of the genes with decreased signal in *S. typhimurium* exposed to ASW for 4 weeks.

Gene symbol	Gene	Gene function
STM3299	<i>greA</i>	transcription elongation factor GreA; necessary for efficient RNA polymerase transcription elongation past template-encoded arresting sites; arresting sites in DNA have the property of trapping a certain fraction of elongating RNA polymerases that pass through, resulting in locked ternary complexes. Cleavage of the nascent transcript by cleavage factors such as GreA or GreB allows the resumption of elongation from the new 3'terminus
STM3353	<i>oadG</i>	oxaloacetate decarboxylase subunit gamma; catalyzes the decarboxylation of oxaloacetate to form pyruvate and carbon dioxide
STM3533		putative transcriptional regulator; similar to <i>Escherichia coli</i> putative regulator (AAC75308.1)
STM3624A		cystathionine gamma-synthase
STM3654		pseudogene; in-frame stop following codon 23
STM3681		putative transcriptional regulator; similar to <i>Escherichia coli</i> possible NagC-like transcriptional regulator (AAC73497.1)
STMsR141	<i>STnc38</i>	
STM3754	0	putative cytoplasmic protein; similar to putative ATP binding protein SugR (gi 4324607)
STM3758	<i>fidL</i>	putative inner membrane protein; FidL (gi 4324611)
STM3873.S	<i>gidB</i>	glucose-inhibited division protein B; probable SAM-dependent methyltransferase
STM3897	<i>yifA</i>	transcriptional regulator HdfR; Negatively regulates the transcription of the flagellar master operon flhDC by binding to the upstream region of the operon
STM3914	<i>rhlB</i>	ATP-dependent RNA helicase RhlB; enables ATP-dependent unwinding of double stranded RNA as a component of the RNA degradosome, a multi-enzyme complex important in RNA processing and messenger RNA degradation
STM4155		putative inner membrane protein
STM4170	<i>hupA</i>	transcriptional regulator HU subunit alpha; histone-like DNA-binding protein
STM4185	<i>aceK</i>	bifunctional isocitrate dehydrogenase kinase/phosphatase protein; catalyzes the phosphorylation/dephosphorylation of the enzyme isocitrate dehydrogenase on a specific serine which regulates activity; unphosphorylated IDH is fully active when cells are grown on glucose while the enzyme becomes phosphorylated and inactive in the presence of acetate or ethanol
STM4503		putative inner membrane protein
STM4558	<i>rimI</i>	ribosomal-protein-alanine N-acetyltransferase; alanine acetyltransferase that specifically acetylates ribosomal protein S18
PSLT013	<i>pefI</i>	regulator; plasmid-encoded fimbriae; orf6
PSLT091	<i>traW</i>	conjugal transfer pilus assembly protein TraW; conjugative transfer
PSLT096	<i>trbE</i>	conjugal transfer protein TrbE

^aThe annotated functions of genes were cited from (13).

Appendix B

Representations of Experimental Set-ups

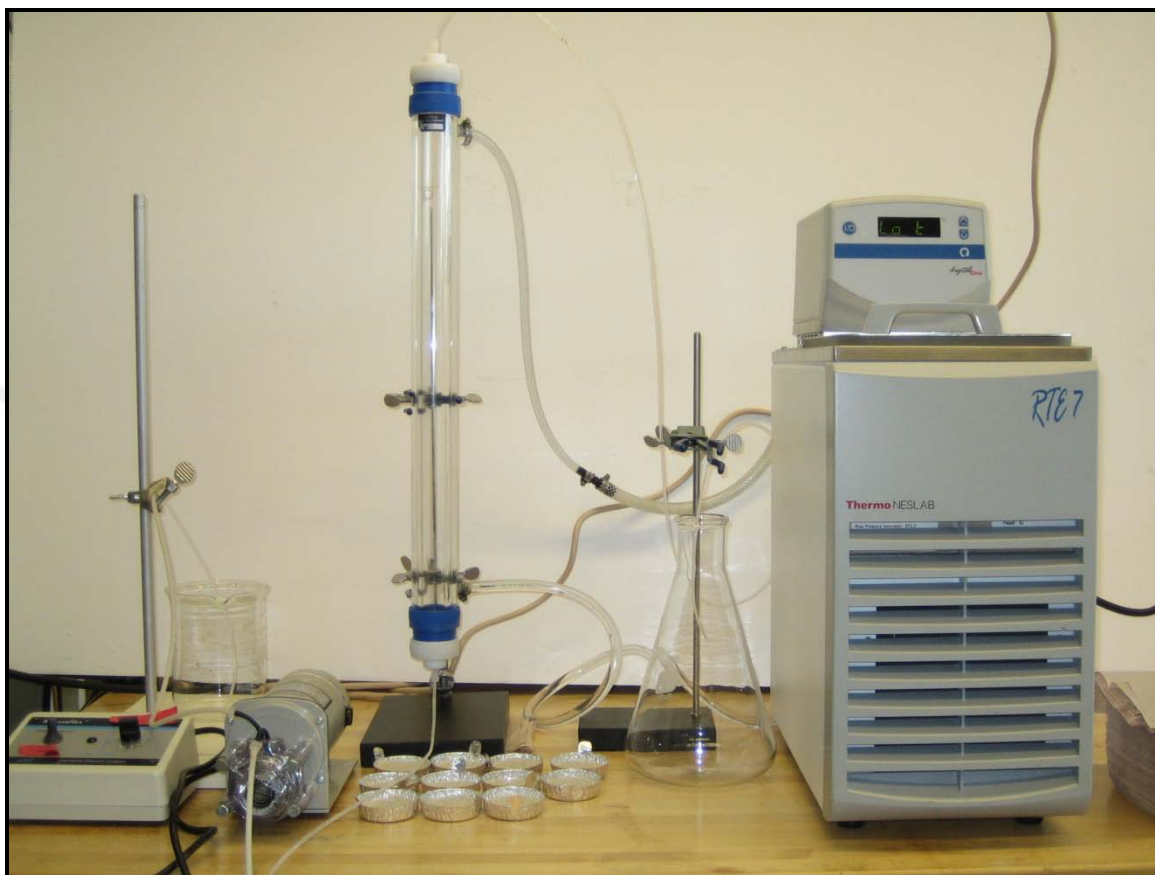


Figure B.1. Picture of the experimental column system used in Chapter 2.

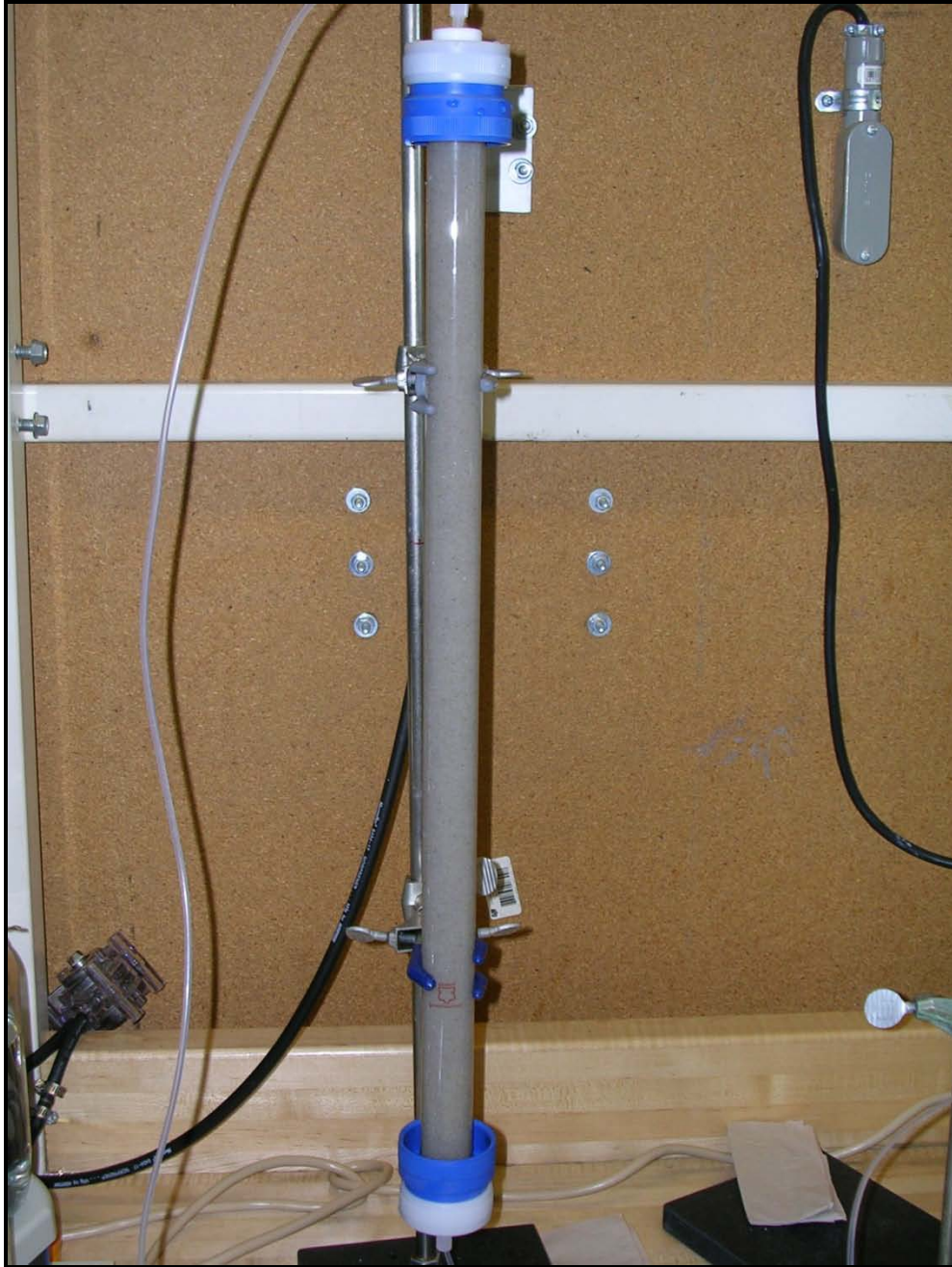


Figure B.2. Picture of the column packed with aquifer sand used in Chapter 2.

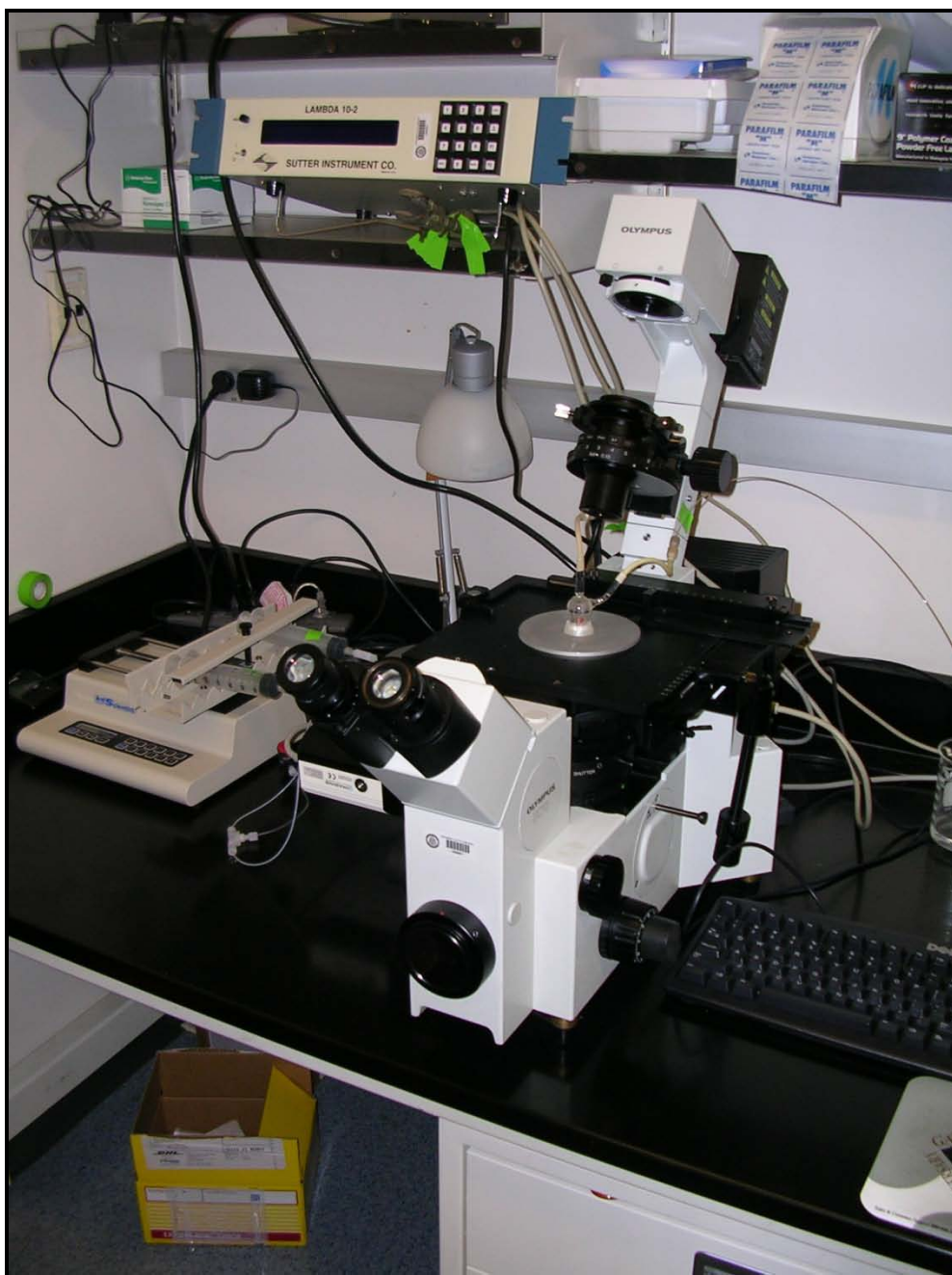


Figure B.3. Picture of the radial stagnation point flow system used in Chapter 3 (Flow cell is seen on the microscope stage).