Role of Cold Climate and Freeze-Thaw on the Survival, Transport, and Virulence of *Yersinia enterocolitica*

Revised and resubmitted to:

Environmental Science and Technology

November 19, 2013

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Abstract

Surface and near-surface soils in cold climate regions experience low temperature and freeze-thaw (FT) conditions in the winter. Microorganisms that are of concern to groundwater quality may have the potential to survive low temperature and FT in the soil and aqueous environments. Although there is a body of literature on the survival of pathogenic bacteria at different environmental conditions, little is known about their transport behavior in aquatic environments at low temperatures and after FT. Herein, we studied the survival, transport, and virulence of a Gram-negative bacterial pathogen, *Yersinia enterocolitica*, when subjected to low temperature and several FT cycles at two solution ionic strengths (10 and 100 mM) in the absence of nutrients. Our findings demonstrate that this bacterium exhibited higher retention on sand after exposure to FT. Increasing the number of FT cycles resulted in higher bacterial cell surface hydrophobicity and impaired the swimming motility and viability of the bacterium. Moreover, the transcription of *flhD* and *fliA*, the flagellin-encoding genes, and *lpxR*, the lipid A 3'-*O*-deacylase gene, was reduced in low temperature and after FT treatment while the transcription of virulence factors such as *ystA*, responsible for enterotoxin production, *ail*, attachment invasion locus gene, and *rfbC*, O-antigen gene, was increased. *Y. enterocolitica* tends to persist in soil for long periods and may become more virulent at low temperature in higher ionic strength waters in cold regions.

Introduction

One of the main concerns in the land application of animal manure is the survival of microbial contaminants and their transport to groundwater.[1-3] Any environmental factors that extend the growth and survival of pathogens in soil are considered as a threat to public health.[1, 4] Thus, a better understanding of factors that influence the survival of microorganisms in soil is essential to improve land management practices to control the spread of bacterial pathogens.[5]

Temperature is an important environmental factor, having a large effect on survival rates of microorganisms in soil. Previous studies on the survival of pathogens in soil and manure were mainly carried out under constant temperature conditions,[2, 6] but our understanding of microbe survival under varying temperatures, which are more relevant to natural environmental conditions, is limited. Results showed that the survival of pathogens in manure under fluctuating temperature is different compared to survival under constant temperature.[7] For instance, the survival of *Escherichia coli* O157:H7 and *Salmonella* serovar Typhimurium in manure at constant temperature was different from their survival after exposure to fluctuating temperatures with ±4 and ±7°C amplitudes.[7] Therefore, conclusions about pathogen behavior in the environment considering only constant temperatures can lead to errors in the predicted survival time and biased assessment of the risk posed by manure-borne pathogens.

Although considerable research has addressed the survival characteristics of pathogens under different environmental conditions[6, 8-10], there have been few studies on pathogen

survival under cold and FT conditions.[11, 12] Frequent fluctuation of ambient temperature around freezing may cause more rapid rates of microbial pathogen death compared to ambient temperatures.[2, 7] For instance, FT events have been suggested to be the cause of significant loss in viability in *Cryptosporidium parvum* oocysts.[13] During spring melt, microbes attached to soil or entrapped in snow can become resuspended and travel significant distances under saturated conditions and potentially reach groundwater aquifers.[2, 11, 14] Moreover, bacteria that have experienced environmental stresses such as low temperature and FT may exhibit different transport behavior compared to their non-stressed counterparts. The transport behavior of bacteria in groundwater is strongly influenced by cell characteristics[15, 16] such as cell size[17], surface chemistry[18, 19], motility[20-22], and shape[23], which may be affected by environmental stress. Low temperature and temperatures below freezing can affect the membrane and cell surface chemistry of bacteria.[24] For instance, shorter membrane fatty acids and less phosphorylation in the membrane lipids were observed in *Arthrobacter* sp. grown at 4°C compared to 24°C.[24] There are a number of reported laboratory studies on the effects of motility on bacterial transport.[15, 25] There are conflicting results on the role of motility in bacterial transport demonstrating that motility of bacteria can lead to both enhanced[25] or decreased[15, 21] transport rates in soil. For example, higher bacterial deposition onto quartz was observed for motile *Pseudomonas aeruginosa* (*P. aeruginosa*) than a non-motile mutant strain.[26] In another study, Camper et al. found that motile *P. fluorescens* transported farther in a saturated column packed with glass spheres than a non-motile mutant strain of the same bacterium.[21]

Y. enterocolitica is a Gram-negative pathogen with the ability to reproduce over a wide range of temperatures (-5 to 42°C).[27] This bacterium is widely found in nature both in aquatic and animal reservoirs[27] and can be transmitted via contaminated food or water.[27, 28] The primary site of *Y. enterocolitica* pathogenesis is colonization of the intestinal tract as a result of drinking contaminated water.[29] It has been shown that *Y. enterocolitica* can survive 12 weeks in frozen ground pork.[30] However, limited information is available on the survival of *Y. enterocolitica* subjected to low temperatures or FT events in the natural environment.[9]

Virulence factors in *Y. enterocolitica* include lipid A and O-antigen moieties in the lipopolysaccharide (LPS) and adhesion/invasion proteins and enterotoxin encoded by chromosomal and plasmid-encoded virulence genes.[31, 32] Temperature regulates most virulence factors of *Y. enterocolitica* including the structure of LPS lipid A.[33] The number and type of the lipid A fatty acids can vary.[33] For instance, LpxR, a lipid A deacylase, is temperature-regulated and involved in alteration of fatty acids in lipid A.[33, 34] It has been shown that there is an inverse correlation between virulence and flagellum biosynthesis for *Y. enterocolitica*.[35] *Y. enterocolitica* motility is dependent upon *fliA*; a *fliA* mutant is non-motile and does not produce the flagellin protein.[35] FliA expression is temperature-sensitive and Kapatral et al. [35] demonstrated that greater expression of FliA occurred at 25°C than at 37°C, thereby activating a repressor of virulence.[36] In other words, at lower temperature (25°C), *Y. enterocolitica* exhibited greater motility and less virulence.[35] Horne et al.[32] demonstrated that the expression of plasmid-encoded virulence genes is higher at 37°C than at 25°C. They observed higher temperature dependency of expression levels of all plasmid-encoded

virulence genes in a *fliA* mutant compared to the wild type indicating that FliA contributes to temperature regulation of these genes.

The objective of this study was to characterize the changes in survival, virulence and transport in granular media for *Y. enterocolitica* exposed to cold temperatures and FT cycles representative of environmental conditions in cold regions. We studied the survival and transport of *Y. enterocolitica* when subjected to a constant low temperature (10°C) and up to 10 days of FT cycling at two solution ionic strengths (10 and 100 mM). These IS levels were selected to model relevant soil and groundwater conditions.[37] The influence of temperature treatment on virulence factors of *Y. enterocolitica* was also investigated at a specific water chemistry (100 mM KCl, pH 5.7). A representative temperature regime was chosen to simulate temperatures that are experienced over a 10-day period near the end of winter in southern Canada. The extreme temperature range was 10°C and -10°C, a representative temperature profile in surface or near surface soils.[38, 39] This temperature profile is also relevant for other cold climate regions such as polar and high-altitude (e.g. alpine) regions. Viability and culturability of the cells along with other bacterial characteristics such as cell size, surface potential, cell hydrophobicity, and motility were investigated before and after FT treatment. The transport of bacteria exposed to FT was studied using water saturated sand-packed columns. The expression levels of different genes encoding synthesis of flagellin, lipid A deacylase, O-antigen, and selected virulence factors were measured using semi-quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Materials and Methods

Strains and Culture Conditions. A Gram-negative organism, pathogenic *Y. enterocolitica* ATCC 9610 (biosafety level 2) was used as the test bacterium. Details on cell incubation and harvesting are provided in the Supporting Information. The cells were suspended in 10 or 100 mM KCl solution without nutrients (pH 5.7±0.1) at 10^7 cells/mL. The cell suspension was then maintained at 10°C for 16 hours as an acclimatization step to emulate exposure to a cold temperature environment.

Freeze-Thaw Treatment. A representative temperature regime was chosen to simulate temperatures that might be experienced in surface or near-surface soils over a 5 or 10 day period at the end of winter in Quebec, Canada. The temperature range chosen was 10°C and -10°C. The FT cycles consisted of four 8-hour stages of: a constant temperature at 10°C, a gradual decrease from 10°C to -10°C at a computer-controlled constant rate of 2.5°C/h, a constant temperature at -10°C followed by a gradual increase from -10°C to 10°C (at 2.5°C/h).

After acclimatization, the cell suspension was divided into seven 30 mL aliquots for different treatments: (1) Ctrl, no further temperature treatment, cells were used at 10°C immediately following acclimatization; (2) 5 d FT, cells exposed to 5 days of FT cycling in 10 or 100 mM KCl; (3) 10 d FT, cells exposed to 10 days of FT cycling in 10 or 100 mM KCl; (4 and 5) 5 d KCl and 10 d KCl, cells stored at constant temperature of 10°C in 100 mM KCl for 5 and 10 days, respectively (reference samples with no FT); and (6 and 7) 5 d LB and 10 d LB, cells stored at constant 10°C in LB for 5 and 10 days, respectively

(reference samples with no starvation). Samples stored at constant 10°C in KCl or LB for the same amount of time (5 and 10 days) allowed for the evaluation of the incremental contribution of FT and starvation to bacterial survival and virulence, respectively. The cell suspensions in 10 or 100 mM KCl were incubated in a temperature bath (Neslab Thermo Scientific) of a 50% (v/v) ethylene glycol aqueous solution for 5 or 10 days. All samples were centrifuged and resuspended in fresh electrolyte before any characterization experiments. Experiments were performed in duplicate on two different days.

Bacterial Characterization. The culturability of the cells before and after temperature treatment was evaluated by plating on LB-agar medium and incubating for 24 h at 37°C. This temperature was selected to mimic host exposure. Cell viability in terms of membrane integrity was measured using the Live/Dead BacLight Cell Viability Kit (Invitrogen). The measurements were made by adding 50 μL each of SYTO9 and propidium iodide (PI) to an 100 μL aliquot of cell suspension and incubating for 15 min in the dark. Stained bacteria were imaged by fluorescence microscopy (Olympus BX10) with excitation at 480 and 490 nm. Emitted light was examined at 500 nm for identifying the green, viable bacteria, and at 635 nm for the red, cell wall-compromised bacteria.

The electrophoretic mobility (EPM) of the bacteria was measured at 10°C using cells suspended in KCl before and after FT treatment (ZetaSizer Nano ZS, Malvern). These measurements were repeated using two different samples of each bacterial suspension prepared from separate cultures. Measured EPMs were converted to cell zeta potential using the Smoluchowski equation.[40] The hydrodynamic diameter of the same cell suspensions was

measured using dynamic light scattering (DLS) (ZetaSizer Nano ZS, Malvern) over the same range of experimental conditions.

The relative hydrophobicity of the cells was measured before and after FT treatment using the modified microbial adhesion to hydrocarbons (MATH) assay.[41] Samples were prepared by transferring 1 mL of n-dodecane to a glass tube containing 4 mL of bacterial suspension (sampled before or after FT treatment). The tubes were vortexed (vortex mixer, setting 8, Fisher) for 2 min, followed by a 15 min rest period. After this time, allowing for phase separation, a sample of the aqueous bacterial suspension was carefully retrieved using a Pasteur pipette, and 10 μL of this sample was transferred to the Helber cell counting chamber. The bacterial hydrophobicity for each treatment is reported as the percent of total cells partitioned into the hydrocarbon phase. These experiments were performed in triplicate using the whole cell suspension exposed to a given temperature treatment.

Swimming motility was evaluated for each treatment as described previously[42]. Briefly, following each temperature treatment, an aliquot of cells (100 μL) was resuspended in 1 mL fresh LB medium and vortexed. The concentration of the cell suspension was adjusted to 10^8 cells/mL before seeding cells (5 μL) onto the centers of 5-cm-diameter motility plates (tryptone-NaCl with 0.2% w/v agar). *Y. enterocolitica* is not motile at 37°C.[35] Therefore, plates were incubated for up to 24 h at 10°C (the same temperature used in the transport experiments), and the diameters of halos formed due to bacterial migration were measured 24 h after inoculation. These experiments were performed in triplicate using the whole cell suspension exposed to a given temperature treatment.

Preparation of Granular Material and Bacterial Transport Experiments. Transport studies were conducted using an adjustable length glass column of 1 cm inner diameter (GE Life Sciences). The granular material was a quartz sand having mean size of 256 μm (US standard mesh size -50/+70; Sigma-Aldrich). Sand cleaning and drying procedures were similar to previously reported methods.[43] The sand was wet packed into the column with vibration to prevent trapping of air bubbles in the column. The porosity of the water-saturated packed bed was 0.36. All column experiments were conducted at 10°C which is representative of groundwater temperature in Canada.[44] The packed column was equilibrated by injecting 6 pore volumes (PVs) of a background electrolyte solution (10 or 100 mM KCl) at 0.4 mL/min. To avoid any potential changes in the background solution chemistry of the cell suspensions as a result of exposure to low temperature or FT, column experiments were conducted using cells that had been resuspended in fresh electrolyte and equilibrated for 1 h after different temperature treatments. Twelve PVs of the bacterial cell suspension (at concentration C_0) were injected into the column at the same flow rate, followed by a cell-free electrolyte solution for 3 PVs. The effluent cell concentration C was monitored in real-time using UV-visible spectroscopy (Agilent HP8453) at a wavelength of 600 nm using a 1 cm flow-through cell.

Semi-Quantitative Reverse Transcription PCR (qRT-PCR). Cell samples for RNA isolation were obtained from samples before exposure to FT (Ctrl), after FT, and from control samples at 10°C (LB and KCl). To measure the expression of virulence genes, the cells were allowed to equilibrate at 37°C (host environment) for 16 h before RNA extraction.

Total RNA was extracted and purified using the Direct-zol RNA MiniPrep kit (ZYMO Research Corporation) following the manufacturer's instructions and quantified by spectrophotometry using an Eppendorf BioPhotometer Plus (Eppendorf). The RNA concentration was calculated by measuring the absorbance at 260 nm and 300 ng of RNA was used for cDNA synthesis using the M-MLV Reverse Transcriptase Kit (Invitrogen). qRT-PCR was carried out in the ABI Prism 7900 HT thermal cycler (Applied Biosystems) using the Power SYBR Green PCR Master Mix (Applied Biosystems). The list of primers is provided in Table S1. Conditions for qRT-PCR were as follows: 50°C for 2 min, initial denaturation at 95°C for 10 min, and 45 cycles of 15 s at 95°C and 1 min at 60°C. Results were analyzed with SDS software, version 2.2 (Applied Biosystems). Data were normalized to the reference gene *rpoB*. The threshold cycle method ($2^{-\Delta\Delta CT}$)[45] was used to analyze changes in gene expression in a given sample relative to a reference sample (which is a control sample of acclimatized cells, prior to cold temperature or FT treatment). For each sample, qRT-PCR was done in triplicate on the whole cell suspension exposed to a given temperature treatment, and the entire experiment repeated twice with RNA samples extracted from independent cultures.

Results and Discussion

Effect of FT on Bacterial Survival. Studies have shown that freezing temperatures may affect the bacterial cell wall, making it more permeable and thus allowing the leakage of cellular materials and influencing protein synthesis[12, 46, 47] and cell viability.[12, 13] In this study,

viability and culturability of bacteria were measured for Ctrl (cells acclimatized at 10°C) and for cells exposed to 5 and 10 d FT using BacLight Live/Dead and CFU assays, respectively. As presented in Table 1, the culturability of the cells decreased from the initial concentration of 7.8×10^{12} CFU/mL for Ctrl to 1.8×10^{9} and 1.7×10^{9} CFU/mL after 5 and 10 d FT in 10 mM KCl, respectively. In 100 mM KCl, the plate counts yielded 2.9×10^{9} and 1.2×10^{9} CFU/mL after 5 and 10 d FT, respectively. The Live/Dead assay results showed that the reduction in viability was more significant in lower IS ($p<0.05$) (Table 1). The depression of the freezing point is less than 0.1°C for 10 and 100 mM KCl solutions.[48] Thus, the duration of freezing was about the same for both IS. Low cell viability at low IS can be related to high osmotic pressure and electrolyte imbalance across the cell membrane.[49] Overall, the viability of bacteria decreased with increasing number of FT cycles and this reduction was greater in lower IS ($p<0.05$). Viability was assayed using a membrane permeability assay which has been reported as a good indicator of the effects of temperature.[13] Hence, higher viability compared to the culturability may be attributed to the fraction of the bacteria that adapted to the temperature stress and were viable but non culturable (VBNC).

In this study, for cells stored in the same electrolyte at 10°C for the same amount of time without FT treatment, we observed no reduction in viability or culturability (data not shown). Thus, the loss of viability and culturability of the cells can be directly attributed to the temperature variations and FT events. Starvation has been suggested as a principle stress that coliforms encounter in a non-host environment.[11] However, nutrient availability could be considered as a factor in survivability only when the cells are metabolically active, at

temperatures greater than 0°C.[11] Another study showed that cell membrane integrity and cell physiology remains unchanged over long periods of starvation for *E. coli* O157:H7 cells while their resistance against disinfectants increases.[50]

Membrane damage is proposed to be the main cause of cell death after exposure to FT.[51-54] Most studies on FT stresses are done by rapid FT of the bacteria which results in rapid cell death.[55] It has been reported that the first-order die-off rate constants for total coliform bacteria vary between 0.041/d at room temperature and 0.002/d from -15 to -28°C with cooling rates of 2.4°C/h and 1.8°C/h.[11] In another study, for *Y. enterocolitica* and *E. coli* O157:H7 thawed at 7°C for 5 h after storage at -18°C for 28 days, 1.69 and 1.37 log reductions were observed, respectively.[56] In this study, the temperature cycle consisted of constant temperature periods of 8 h at the extreme temperatures and a ramp rate of 2.5°C/h between the temperature extremes. We observed die-off rates (in terms of membrane integrity) of 0.05/d for *Y. enterocolitica* cells exposed to 5 or 10 d FT in 10 mM KCl.

Effect of FT on Bacterial Transport. The transport behavior of bacteria was studied for Ctrl (cells acclimatized at 10°C) and for cells exposed to 5 and 10 d FT. As shown in Figure 1(a, b), increasing the number of FT cycles resulted in higher bacterial retention onto sand grains compared to cells not exposed to FT. The extent of cell retention was greater at higher IS. For example, the percentage of bacterial retention ($1-C/C_0$) was 4% and 23% for the Ctrl cells in 10 and 100 mM KCl, respectively (Figure 1). This was calculated by numerically integrating the area under the breakthrough curves. The

percentage of bacterial retention increased to 40% and 72% after 10 d FT in 10 and 100 mM KCl, respectively, indicating higher bacterial retention after FT treatment.

Figure 1 also shows cell viability in samples of column effluent suspensions taken at different time points during the transport experiments. Overall, cell viability remained in the same range (±10%) during transport of the cell suspension in the sand indicating that there was no significant difference in the deposition behavior of live versus damaged cells ($p<0.05$). The greater extent of bacterial retention after FT treatment can be related to changes in physicochemical properties of the cells such as surface charge, size, motility, and hydrophobicity.[25, 57-61] To explain the role of these properties in transport behavior of bacteria and how they are influenced by low temperature and FT treatment, the results were also compared with two other pre-defined reference conditions (5 and 10 d LB or KCl) for statistical and molecular analyses, as described below.

[FIGURE_1_HERE]

Bacterial Surface Charge and Size. Cell surface charge has been shown to play an important role in bacterial attachment to surfaces[62] and, consequently, bacterial transport behavior; hence, the cell surface (zeta) potential was evaluated from EPM measurements conducted over the range of experimental conditions investigated. The zeta potentials of *Y. enterocolitica* cells are presented in Table 1. The results indicate that bacteria were negatively charged in both IS at pH 5.7. The absolute magnitude of the cell zeta potential decreased with an increase in IS of KCl, as expected based on compression of the electrical double layer resulting in higher bacterial retention onto sand grains in higher IS. Zeta potentials of clean

sand are also negative at these IS (~-30 mV and ~-5 mV for 10 and 100 mM KCl, respectively) at pH 5.6, as reported elsewhere.[63] In both IS, there is no significant change in the cell surface charge as the bacteria are exposed to 10 d FT treatments. Thus, bacterial zeta potential data suggest that factors other than electrostatic forces were involved in controlling the transport behavior of FT-treated *Y. enterocolitica*. A change in cell size may affect bacterial retention by influencing the extent of removal by physical straining and by affecting the likelihood of contact with the grain surface as a result of Brownian diffusion and interception.[64] The hydrodynamic diameters of the cells were measured by DLS. The mean cell hydrodynamic diameter was 1.5±0.1 μm before exposure to FT (Ctrl) and 1.1±0.4 μm after 10 d FT in 100 mM KCl under no nutrient condition (Table 1). Starvation is reported to be responsible for a significant decrease in cell volume.[58] However, 10 d LB samples kept at constant 10°C (no starvation) exhibited the same cell size (1.3±0.3 μm). These results suggest that cell size was not a factor in the change in bacterial retention observed following FT in higher IS.

[TABLE_1_HERE]

Bacterial Motility and Flagellin Expression. Motility has been found to affect the adhesion of bacteria to various surfaces in flowing systems.[22] Some studies report greater attachment of motile bacteria compared to their non-motile counterparts.[15, 21, 26] However, the opposite has also been shown.[20] To examine the influence of FT treatment on bacterial motility in this study, the swimming migration of *Y. enterocolitica* was measured for cells suspended in 100 mM KCl. The higher IS condition was used for these measurements

because bacteria were found to be more viable following FT treatment at the higher salt concentration (Table 1). Evaluation of swimming motility revealed that FT treatment reduced bacterial motility by approximately 84% and 90% after 5 and 10 d, respectively (Figure 2a). Swimming motility of 10 d KCl or LB was less repressed (45%) indicating that FT had a more pronounced effect on motility than the cold temperature treatment (10°C) or starvation ($p<0.05$). It has been reported that for flagellated, rod-shaped bacterium A0500 isolated from the Deep Subsurface Savannah River Collection, when the cells are non-motile at 4°C, their retention onto sand is greater than at 18°C where they are motile.[25] In another study, higher retention of non-motile *Corynebacterium glutamicum* cells was observed on silica sand compared to motile *P. putida* cells.[65] Herein, we also observed a relationship between decreased bacterial motility after FT treatment and higher bacterial retention onto sand.

qRT-PCR was used to examine whether the observed impairment of bacterial motility as a result of FT exposure may be linked to changes in transcriptional regulation of flagellar genes. Specifically, transcription of the genes encoding for the protein flagellin which is critical in the formation of the bacterial flagellum was investigated. There exists a hierarchy in flagellar gene transcription in *Y. enterocolitica*.[32] Regulation of the flagellar regulon takes place at the level of FlhD/FlhC. But environmental factors such as temperature do not always control the flagellar gene regulation at the level of *flhD* expression.[32] For example, temperature affects the synthesis of flagella by affecting transcription of *fliA* but not *flhD*.[32] The expression of two flagellar genes, *flhD* and *fliA*, was measured before (or without)

exposure to FT and after FT treatment (using the whole cell suspension that had been exposed to the temperature treatment, including both viable and non-culturable cells). In Figure 2b, the data are presented as the fold change in mRNA expression relative to the expression under reference condition (Ctrl). In qualitative agreement with the swimming motility measurements, the normalized mRNA relative values for both *flhD* and *fliA* genes decreased after FT treatment. For example, the relative expression of *fliA* gene was reduced to 50% and 30% after 5 and 10 d FT treatments, respectively (Figure 2b). For 5 d LB or KCl, although there was an increase in *flhD* gene expression and no decrease in *fliA* expression, a significant decrease in the corresponding swimming motility was observed. This reduction in motility could be due to the fact that motility is an energy-requiring process;[66] bacteria may be less motile at low temperatures likely in an effort to save energy for their essential metabolic activities. Comparison of 10 d LB or KCl with the 10-day FT samples revealed that the flagellin expression was more repressed after FT treatment ($p<0.05$).

[FIGURE_2_HERE]

Bacterial Hydrophobicity and LPS Alteration. Hydrophobic interactions may also play a role in bacterial retention on surfaces.[18] Analysis of cell surface hydrophobicity employing the modified MATH assay revealed that the hydrophobicity of bacteria increased after 10 d FT. As shown in Figure 3a, bacteria exhibited the lowest hydrophobicity (~10%) in Ctrl (cells acclimatized at 10°C). Thirty percent of bacteria in 10 d KCl or LB partitioned into the hydrocarbon phase or at the hydrocarbon-water interface, whereas this value increased to 50% after 10 d FT suggesting that FT may contribute to alteration of the cell surface

chemistry more significantly than starvation alone. It has been shown that hydrophobic cells such as *Streptococcus salivarius*, *S. thermophilus*, and *Pseudomonas corrugata* displayed higher percentage of adhesion to sand.[67] In another study, hydrophobic strains of *Lactobacillus* and *Streptococcus faecalis* were retained 2 to 3 times more than hydrophilic strains in soil columns.[68] This study also showed more bacterial retention onto sand after FT when the cells are more hydrophobic.

LPS is the major component of the cell wall in Gram-negative bacteria to be involved in structural change of the cellular membrane due to temperature variations.[69] It comprises three parts: the O-antigen, core oligosaccharide, and lipid A.[69] Lipid A forms a hydrophobic moiety of LPS and is responsible for the endotoxic activity of LPS.[34] It is known that *Y. enterocolitica* modulates the fatty acids in lipid A to protect itself under freezing conditions.[12] This alteration in fatty acids can be done with lipid A-modifying enzyme, LpxR.[34] Herein, the expression of *lpxR* gene was measured before and after FT treatment using qRT-PCR. Expression of *lpxR* gene decreased by 60% and 80% after 5 and 10 d FT relative to Ctrl (Figure 3b). Comparison of 10 d LB or KCl with the 10-day FT samples revealed that the *lpxR* expression was more repressed after FT ($p<0.05$). Membrane hydrophobicity can be altered by changes in the fatty acid composition.[70] Repression of *lpxR* results in less lipid A deacylation and longer fatty acids, which should correspond with an increase in cell surface hydrophobicity.[71] Thus, the results of this qRT-PCR experiment are in agreement with the observed increase in cell surface hydrophobicity after FT treatment.

[FIGURE_3_HERE]

Effect of FT on Expression of Selected Virulence Genes and Virulence Factors. It is of interest to evaluate other virulence factors of *Y. enterocolitica* and to examine whether the bacterium could become more virulent after exposure to low temperature or FT. The virulence factors characterized in *Y. enterocolitica* are located within the chromosome and also on a 70 kb virulence plasmid designated pYV which is only detected in virulent strains.[29] Expression of the virulence genes has been shown to be affected by temperature.[72] The expression of different virulence factors (Table S1) was investigated using qRT-PCR in this study.

Several studies have reported that the O-antigen in LPS is involved in the colonization and invasion processes and is required for the proper expression of other outer membrane virulence factors.[73, 74] The *rfbC* gene located within the *rfb* cluster is responsible for the biosynthesis of the O side chain of pathogenic strains of *Y. enterocolitica*.[75] As depicted in Figure 3b, *rfbC* expression was elevated by 5-fold after 10 d FT while its expression was decreased by 20% in 10 d KCl ($p<0.05$). This increased transcription of *rfbC* after 10 d FT could contribute to increased virulence of this bacterium after FT exposure. Commonly used targets located on the virulence plasmid are the *Yersinia* adhesin gene, *yadA*, and a transcriptional activator for many *Yersinia* outer membrane proteins, *virF*.[76] However, PCR targets located on the virulence plasmid are not suitable targets for detection, because the plasmid is unstable and easily lost during laboratory treatment.[77, 78] Therefore, in this study, the expression of two of the chromosomal virulence genes (*ail* and *ystA*) was examined by qRT-PCR. *ail* is the *Y. enterocolitica* attachment invasion locus gene.[76] As shown in Figure

4, expression of *ail* increased by 20- and 30-fold after 5 and 10 d FT. Comparison of these results with those of 5 and 10 d KCl revealed that the increase in *ail* transcript abundance was more significant after FT ($p<0.05$). *ystA* is responsible for the production of a heat-stable enterotoxin in *Y. enterocolitica*.[76] The expression of this gene increased by 4- and 7-fold after 5 and 10 d FT, respectively (Figure 4b). However, this change in transcription was also observed for 10 d LB or KCl suggesting that exposure time at low temperature could also play a role in enhancing bacterial virulence.

Although the virulence of *Y. enterocolitica* was not directly assayed in this study, the changes in transcript abundance of some virulence factors show that this bacterium may become more virulent in low temperature and after FT treatment. In general, the virulence factors that might be significantly affected are specifically the ones that contribute to surface hydrophobicity and attachment such as the number and type of the fatty acids in the lipid A moiety of LPS, O-antigen synthesis and production of attachment invasion locus protein (Ail).

[FIGURE_4_HERE]

We studied how low temperature and repeated FT in upper layers of soil might influence different bacterial properties, as well as bacterial survival and transport behavior, and thus impact the extent of pathogenic contamination of groundwater. *Y. enterocolitica* has been associated with a number of human diseases and can be transmitted via contaminated water or food. This bacterium can survive freezing exceptionally well. Thus, the study of *Y. enterocolitica* fate and transport can help to improve risk assessment and public health

protection. The results of this study show that viability of *Y. enterocolitica* decreased after exposure to FT, but bacteria exhibited greater survival when suspended in a higher IS solution such as that found in certain aquatic environments. Bacterial transport studies revealed greater bacterial retention onto sand grains after exposure to FT, coincident with impaired bacterial motility and increased hydrophobicity. The results showed that these changes in bacterial properties might also be linked to molecular level changes in the transcription of genes associated with selected virulence factors, cell surface biomolecules and structures. Of particular interest, an increase in transcript abundance of virulence genes suggests that *Y. enterocolitica* may become more virulent during the course of FT and after extended exposure to low temperature. Interestingly, after only 5 days exposure to a constant temperature of 10 °C, qRT-PCR experiments revealed greater transcript abundance of *ail* and *rfbC* genes in *Y. enterocolitica*, suggesting that this organism may exhibit greater virulence after incubation at low temperature. Thus, consumers of drinking water produced by domestic wells or drinking water treatment plants in cold climate regions might be at higher risk of infection by *Y. enterocolitica*.

Acknowledgements

This research was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC), the McGill Engineering Doctoral Award Program (MEDA), the Canada Research Chairs (CRC) Program and the Fonds québécois de la recherche sur la

nature et les technologies (FQRNT). The authors also thank Che O'May (McGill) for assistance with manuscript editing.

Supporting Information

Primers used in this study. This information is available free of charge via the Internet at http://pubs.acs.org/.

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Table 1. Characterization of *Y. enterocolitica* before and after freeze-thaw (FT) treatment in 10 and 100 mM KCl (pH 5.7).

	Culturability ($\times 10^9$ CFU/mL)		Viability (%)		Cell diameter (μm)		Zeta potential (mV)	
IS of KCl (mM)	10	100	10	100	10	100	10	100
before FT (Ctrl)	7.8×10^3 ± 0.0	7.8×10^3 ± 0.0	100 ± 0	100 ± 0	1 ± 0.1	1.5 ± 0.1	-8.3 ± 0.5	-2.0 ± 0.6
after 5 d FT	1.8 ± 0.4	2.9 ± 0.5	75 ± 2	89 ± 3	1 ± 0.0	1.4 ± 0.1	-16.1 ± 2.9	-2.9 ± 0.5
after 10 d FT	1.7 ± 0.2	1.2 ± 0.8	47 ± 4	85 ± 2	1 ± 0.1	1.1 ± 0.4	-7.1 ± 0.4	-1.9 ± 0.5

Figure Captions

Figure 1. Breakthrough curves for transport of *Y. enterocolitica* through clean quartz sand at 10°C in (a) 10 mM KCl and (b) 100 mM KCl before FT treatment (Ctrl) (---), after 5 d FT (–Δ–) and after 10 d FT (–○–). BTCs were identical for two replicate experiments. The viability of cells in the column effluent at different time points was measured using the Live/Dead assay and the results are also included on the graphs: Ctrl (■), after 5 d FT (Δ) and after 10 d FT (○). The error bars indicate the standard deviation.

Figure 2. (a) Characterization of the effects of constant cold temperature (10°C) and FT on the swimming motility of *Y. enterocolitica* in 100 mM KCl. (b) Expression of the flagellin encoding genes, *flhD* and *fliA*, for *Y. enterocolitica* before and after FT in 100 mM KCl on the mRNA level by qRT-PCR. Relative mRNA expression of *flhD* and *fliA* was first normalized to that of a housekeeping gene, *rpoB*, and then related to the normalized expression level of the same gene in Ctrl (with no exposure to FT). Results represent mean values ± SD for three independent experiments. Symbols above a bar indicate a statistically significant difference in measured values when compared to Ctrl (determined using Student's t-test, *: $p<0.05$). Symbols are also used to verify significance of differences between FT and reference conditions (5 d and 10 d KCl or LB).

Figure 3. (a) Hydrophobicity of *Y. enterocolitica* in 100 mM KCl calculated as the percent of total cells partitioned into the hydrocarbon phase. (b) Expression (mRNA levels determined by qRT-PCR) of *lpxR,* encoding lipid A 3'-*O*-deacylase, and *rfbC*, O-antigen gene, for *Y. enterocolitica* before and after FT in 100 mM KCl. Relative mRNA expression of *lpxR* and

rfbC was first normalized to that of a housekeeping gene, *rpoB*, and then related to the normalized expression level of the same gene in Ctrl (with no exposure to FT). Results represent mean values ± SD for three independent experiments. Symbols above a bar indicate a statistically significant difference in measured values when compared to Ctrl (determined using Student's t-test, *: $p<0.05$). Symbols are also used to verify significance of differences between FT and reference conditions (5 d and 10 d KCl or LB).

Figure 4. Expression (mRNA levels determined by qRT-PCR) of chromosomal virulence genes, *ystA* and *ail*, for *Y. enterocolitica* before and after FT in 100 mM KCl. Relative mRNA expression of *ystA* and *ail* was first normalized to that of a housekeeping gene, *rpoB*, and then related to the normalized expression level of the same gene in Ctrl (with no exposure to FT). Results represent mean values ± SD for three independent experiments. Symbols above a bar indicate a statistically significant difference in measured values when compared to Ctrl (determined using Student's t-test, *: $p<0.05$). Symbols are also used to verify significance of differences between FT and reference conditions (5 d and 10 d KCl or LB).

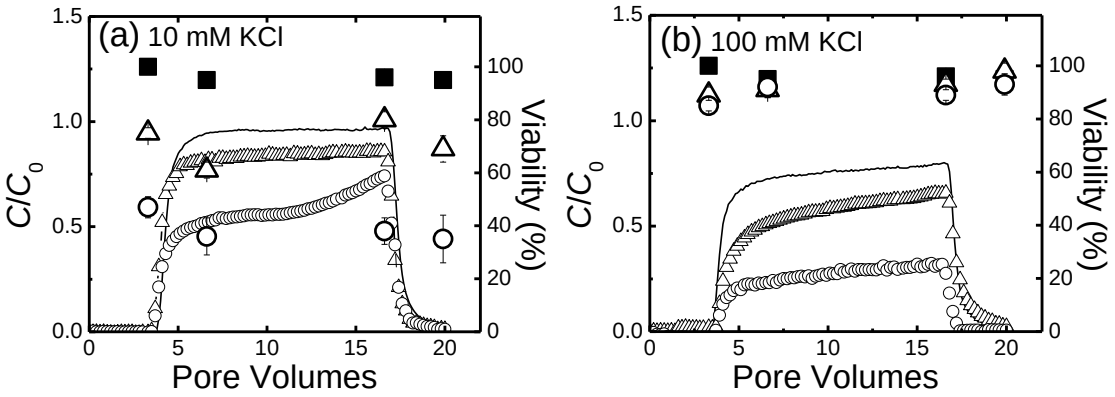


Figure 1.

Asadishad et al.

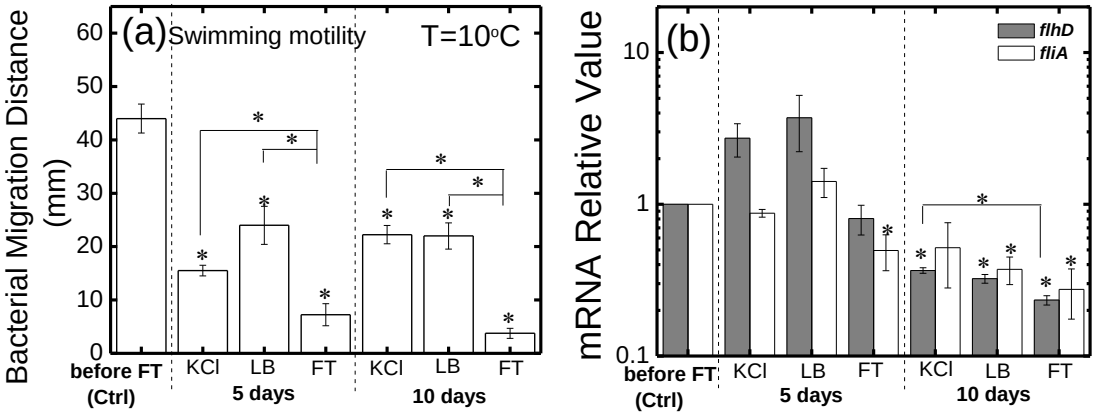


Figure 2.

Asadishad et al.

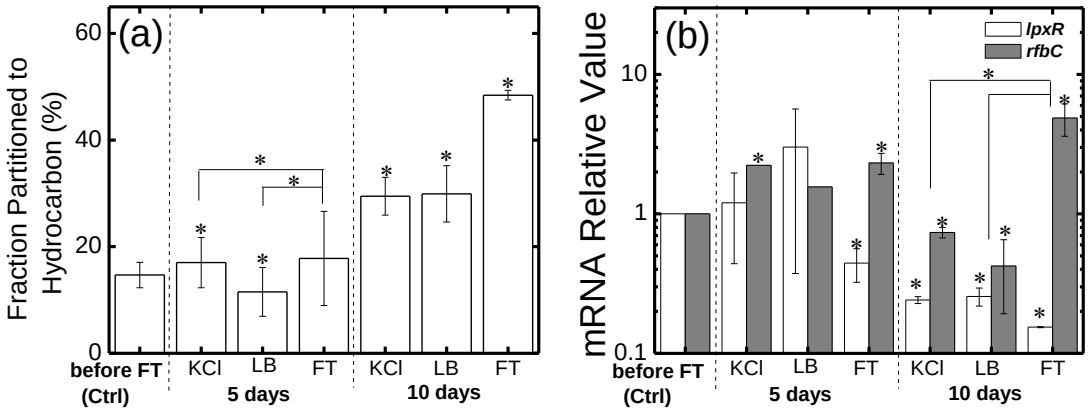


Figure 3.

Asadishad et al.

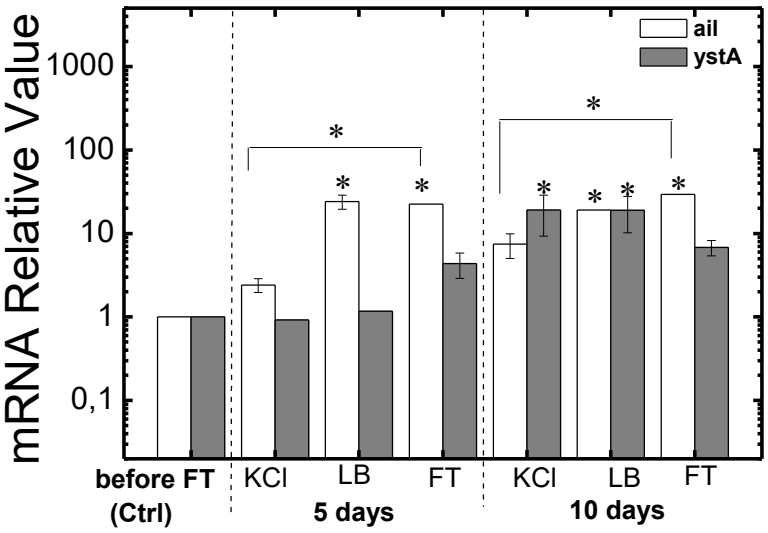
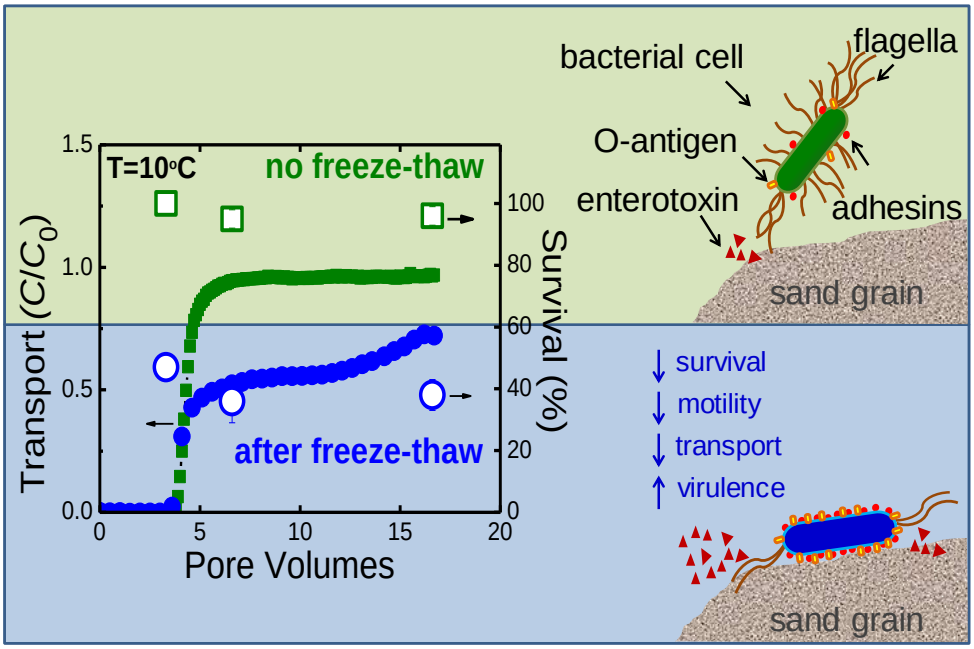


Figure 4.

Asadishad et al.

TOC Graphic


T=10°C
no freeze-thaw
after freeze-thaw
Transport (C/C0)
Survival (%)
Pore Volumes
0.0
0.5
1.0
1.5
0
5
10
15
20
0
20
40
60
80
100
bacterial cell
flagella
O-antigen
enterotoxin
adhesins
sand grain
survival
motility
transport
virulence
sand grain