Transport, Motility, Biofilm Forming Potential and Survival of *Bacillus subtilis* Exposed to Cold Temperature and Freeze-Thaw

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Abstract

In cold climate regions, microorganisms in upper layers of soil are subject to low temperatures and repeated freeze-thaw (FT) conditions during the winter. We studied the effects of cold temperature and FT cycles on the viability and survival strategies (namely motility and biofilm formation) of the common soil bacterium and model pathogen *Bacillus subtilis*. We also examined the effect of FT on the transport behavior of *B. subtilis* at two solution ionic strengths (IS: 10 and 100 mM) in quartz sand packed columns. Finally, to study the mechanical properties of the bacteria-surface bond, a quartz crystal microbalance with dissipation monitoring (QCM-D) was used to monitor changes in bond stiffness when *B. subtilis* attached to a quartz substrate (model sand surface) under different environmental conditions. We observed that increasing the number of FT cycles decreased bacterial viability and that *B. subtilis* survived for longer time periods in higher IS solution. FT treatment decreased bacterial swimming motility and the transcription of flagellin encoding genes. Although FT exposure had no significant effect on the bacterial growth rate, it substantially decreased *B. subtilis* biofilm formation and correspondingly decreased the transcription of matrix production genes in higher IS solution. As demonstrated with QCM-D, the bond stiffness between *B. subtilis* and the quartz surface decreased after FT. Moreover, column transport studies showed higher bacterial retention onto sand grains after exposure to FT. This investigation demonstrates how temperature variations around the freezing point in upper layers of soil can influence key bacterial properties and behavior, including survival and subsequent transport.

Keywords: *Bacillus subtilis, survival, transport, biofilm formation, motility, bond stiffness*

1. Introduction

Microorganisms in upper layers of soil experience frequent changes in environmental conditions, the most notable being temperature fluctuations (Budde et al., 2006). Bacteria in near-surface soil likely experience extreme temperature conditions during the course of a day and seasonal changes, especially in cold climate regions, will subject them to freeze-thaw (FT) conditions (Walker et al., 2006; Yergeau and Kowalchuk, 2008). Bacteria present in soils or biosolids applied to soils can become re-suspended under saturated conditions such as spring melt, potentially reaching groundwater aquifers (Adhikari et al., 2007; Stoddard et al., 1998). The contamination of groundwater supplies by bacterial pathogens can pose a high risk to public health (Curriero et al., 2001; Rose, 1997). Thus, it is important to investigate how cold temperature and FT might affect bacterial survival and transport in natural subsurface environments.

Bacillus subtilis is a Gram positive soil bacterium that is commonly found in the upper layers of soil (Vlamakis et al., 2013). *B. subtilis* is a good model organism for the study of the transport behavior of Gram positive pathogenic bacteria due to its ease of cultivation, non-pathogenic nature, accessible genetic profile and agricultural relevance (Jiang et al., 2005; Vlamakis et al., 2013). *B. subtilis* has been used as a surrogate for bacterial pathogens in drinking water supplies in several studies (Greenberg et al., 2010; Pang et al., 2005; Sinclair et al., 2012). In particular, *B. subtilis* spores have been used as an indicator of *Cryptosporidium parvum* transport in engineered and riverbank filtration systems (Stimson et al., 2009). *B. subtilis* has also been widely accepted as a non-lethal surrogate for *B. anthracis*, the causative agent of anthrax (Nicholson and Galeano, 2003). Cold temperature can substantially influence the transcriptional profiles of *B. subtilis* as well as its survival and transport behavior (Budde et al., 2006; Jiang et

al., 2005; Neale and Chapman, 1970). For instance, growth at 15°C profoundly alters the physiology of *B. subtilis* in comparison to cells grown at 37°C (Budde et al., 2006; Neale and Chapman, 1970).

B. subtilis employs different strategies and mechanisms to withstand adverse environmental conditions which are crucial for bacterial survival; most notably, motility, matrix production, and the general stress response (Hoffmann and Bremer, 2011; Klein et al., 1999; Lopez et al., 2009). Changes in fatty acid branching pattern in membrane lipids have been reported as an adaptation response for *B. subtilis* to survive cold shock from 37 to 15°C (Klein et al., 1999). In another study, this cold shock response in *B. subtilis* has been characterized by rapid induction and accumulation of stress proteins (Beckering et al., 2002). The flagellum is essential for active movement of bacteria in an aqueous environment and for chemotaxis and plays an important role in interaction with surfaces (Senesi et al., 2004). Biofilms are surface-associated microbial communities that provide constituent cells with some shelter, protecting them from adverse conditions (Davey and O'Toole, 2000). However, studies on the influences of cold temperature and FT on these response strategies are scarce in the literature (Beal et al., 2001; Kato et al., 2002; Yamamoto and Harris, 2001).

In this study, we investigate the role of cold temperature and FT on the survival and transport of *B. subtilis*. The cells were subjected to constant cold temperature (10°C) or several FT cycles at two solution ionic strengths (10 and 100 mM) and their survival strategies (*e.g.*, motility and biofilm formation) were monitored. The temperature range chosen was 10°C to -10°C, a representative temperature profile in upper layers of soil (Burn and Smith, 1988; Zhang et al., 2005) that is experienced over a 10-day period near the end of winter in southern Canada Viability and culturability of the cells along with other physical properties such as cell size and

cell surface (zeta) potential were investigated before and after FT treatment. The adhesion properties and transport of bacteria exposed to FT were studied using quartz crystal microbalance with dissipation monitoring (QCM-D) and water saturated sand-packed columns, respectively. The expression levels of selected genes encoding proteins related to motility and adhesins were measured using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

2. Materials and Methods

2.1. Strains and Culture Conditions

A Gram positive, non-pathogenic strain of *B. subtilis*, ATCC 6633A, 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 10^9 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.

2.2. Freeze-Thaw (FT) Treatment

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, (3) 10 d FT, cells exposed to 10 days of FT cycling, (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 temperature in LB for 5 and 10 days, respectively (reference samples with no starvation). Samples stored at constant 10°C in KCl or in LB for 5 and 10 days allow for the evaluation of the contribution of FT and starvation

to bacterial properties, respectively. The FT cycles consisted of consecutive, four 8-hour stages of: a constant temperature at 10°C, a gradual decrease from 10°C to -10°C (at a 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). More details on FT treatment and on cell characterization are included in the Supporting Information.

2.3. Motility and Biofilm Assays and Quantitative Reverse Transcription PCR (qRT-PCR)

Bacterial swimming and swarming motilities were evaluated for each experimental treatment. Biofilm formation was examined with the standard microtitre plate model. Quantitative Reverse Transcription PCR (qRT-PCR) was used to measure the expression of genes implicated in motility and biofilm formation. Details regarding the experimental methods are provided in the Supporting Information.

2.4. Quartz Crystal Microbalance with Dissipation Monitoring (QCM-D)

QCM-D (E4, Q-Sense AB) was employed to investigate the mechanical properties of the bonds between bacteria and a model silica surface. The central component of the QCM-D is the quartz crystal sensor that senses any mass depositing on its surface through shifts in its resonance frequencies (Δf) (fundamental and, up to the 13^{th}, odd overtones) and dissipation factors (ΔD) (i.e. dissipative energy losses measured through oscillation decay time). Details on the QCM-D experiments and interpretation of QCM-D data are included in the Supporting Information.

2.5. Bacterial Transport Experiments

Transport studies were conducted using an adjustable length glass column of 1 cm inner diameter (GE Life Sciences) packed with quartz sand. Details regarding the experimental methods are provided in the Supporting Information.

3. Results and Discussion

3.1. Effect of FT on Bacterial Survival

Viability and culturability of bacteria were measured for Ctrl (cells acclimatized at 10°C for 16 h with no exposure to FT) 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 significantly decreased from 2×10^9 CFU/mL for Ctrl to 200 and 1 CFU/mL after 5 and 10 d FT in 10 mM KCl, respectively. In 100 mM KCl, the plate counts yielded 8.7×10^4 and 6.7×10^2 CFU/mL after 5 and 10 d FT, respectively. The Live/Dead assay results showed 100% and 30% reduction in viability after 10 d FT in 10 and 100 mM KCl, respectively (Table 1). Freezing temperatures have been previously reported to markedly affect bacterial cell membrane integrity, allowing leakage of cellular material and influencing cell viability (Calcott and MacLeod, 1975; Kato et al., 2002; Walker et al., 2006; Yamamoto and Harris, 2001). It is suggested that membranes are primarily damaged when cells are frozen and thawed (Mazur, 1966) and viability and membrane damage can be correlated (Calcott and MacLeod, 1975). Herein, the BacLight Live/Dead results confirmed the loss in membrane integrity after FT treatment. The reduction in viability and culturability was more significant in lower IS ($p<0.05$). Low cell viability at low IS can be related to high osmotic pressure and electrolyte imbalance across the cell membrane (Record Jr et al., 1998). This result was in agreement with our previous study on *Yersinia enterocolitica*,

where bacterial survival decreased when increasing the number of FT cycles (Asadishad et al., 2013).

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3.2.Effect of FT on Bacterial Survival Strategies

We studied the effect of cold temperature and FT treatment on two *B. subtilis* survival strategies, motility and biofilm formation, which contribute to the transport of bacteria to a surface, their initial adhesion onto a surface, and matrix production.

3.2.1. Swimming Motility and Flagellin Expression

To examine the influence of FT treatment on bacterial motility, the swimming migration of *B. subtilis* 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 KCl concentration (Table 1). Evaluation of swimming motility revealed that FT treatment reduced bacterial motility by approximately 61% and 89% after 5 and 10 d, respectively (Figure 1a). Swimming motility of reference samples 5 d KCl and 5 d LB was less repressed (~35%) indicating that FT had a more significant effect on motility than the cold temperature treatment (10°C) and starvation ($p<0.05$), respectively. However, for 10-day temperature treatments, the motility impairment was in the same range for reference samples maintained in LB or KCl and FT-treated samples.

The flagellum is essential for bacterial movement and plays an important role in bacterial interaction with surfaces (Branda et al., 2004). Several genes are responsible for activity and growth of the flagellum in *B. subtilis* (Bardy et al., 2003). The *hag* gene, required for swimming motility, encodes the flagellar filament structural protein flagellin (Mirel and Chamberlin, 1989) and *fliG* gene encodes the flagellar motor switch (Blair et al., 2008).

The effect of FT on the transcription of flagellum and motor switch encoding genes was investigated using qRT-PCR. The transcription of two flagellar genes, *hag* and *fliG*, was measured after acclimation at 10°C, after additional exposure to 10°C in KCl and LB media, and after FT treatment. The data are presented as the fold change in mRNA expression relative to the expression under control condition (Ctrl) in Figure 1b. In qualitative agreement with the swimming motility measurements, the transcription values for the *hag* gene decreased after FT treatment. The relative transcription of *hag* gene was reduced by 35% and 80% after 5 and 10 d FT treatments, respectively (Figure 1b). For 5 d LB or KCl, although there was an increase in *hag* gene transcription, significant decrease in the corresponding swimming motility was observed ($p<0.05$). This reduction could be due to the fact that motility is energy intensive (Bardy et al., 2003); and bacteria appeared to 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 freeze-thawed samples revealed that the flagellin transcription was more decreased after FT treatment ($p<0.05$ for *hag*). For the *fliG* gene, there was no reduction observed under all conditions except for 5 d LB or KCl and 10 d FT where the transcription values decreased to less than 50% compared to Ctrl. This could be another contributing factor to the observed decreased motility for 5 d LB or KCl. Overall, the results showed that the FT treatment had a greater effect on the transcription of *hag* gene compared to the *fliG* gene.

3.2.2. Swarming Motility and Surfactin and SwrA Expression

It has been reported that unlike the wild type, the laboratory strains of *B. subtilis* do not swarm (Patrick and Kearns, 2009). Herein, evaluation of swarming motility revealed that *B. subtilis* minimally swarmed under all conditions and FT treatment had no effect on bacterial swarming motility. Swarming motility requires other genetic features such as surfactant

production to reduce surface tension and an increase in flagellar number per cell (Patrick and Kearns, 2009). Surfactin, a lipopeptide surfactant produced by *B. subtilis*, increases surface wettability and facilitates sliding (Abushady et al., 2005). The *sfp* gene is involved in surfactin production (Ghelardi et al., 2012). SwrA is also essential for swarming motility in *B. subtilis* (Calvio et al., 2005). It activates flagellar biosynthesis gene expression and increases the number of flagella on the cell surface (Ghelardi et al., 2012).

The effect of FT on the transcription of surfactin and SwrA encoding genes was investigated. The transcription levels of *sfp* and *swrA* were measured before (or without) exposure to FT and after FT treatment. In Figure 1c, the data are presented as the fold change in mRNA expression relative to the expression under control condition (Ctrl). Although no change in swarming motility was observed, the normalized mRNA relative values for *sfp* gene increased for all conditions except for 10 d KCl and 10 d FT. For example, the relative transcription of *sfp* gene increased by 2-fold after 5 d FT treatment which was significantly higher compared to 5 d KCl ($p<0.05$) (Figure 1c). In contrast, the transcription of *swrA* was decreased by 80% and 60% after 5 and 10 d FT treatments, respectively (Figure 1c). Expression of both *sfp* and *swrA* genes are necessary for swarming motility (Ghelardi et al., 2012). In Ctrl (cells acclimatized at 10°C), the swarming migration of the bacteria was at the same level as the temperature-treated cells (reference conditions in LB or KCl and FT samples) suggesting that even before exposure to FT, the transcription level of *swrA* and *sfp* might not be high enough for bacteria to produce sufficient amount of surfactin or increase the number of flagella for swarming.

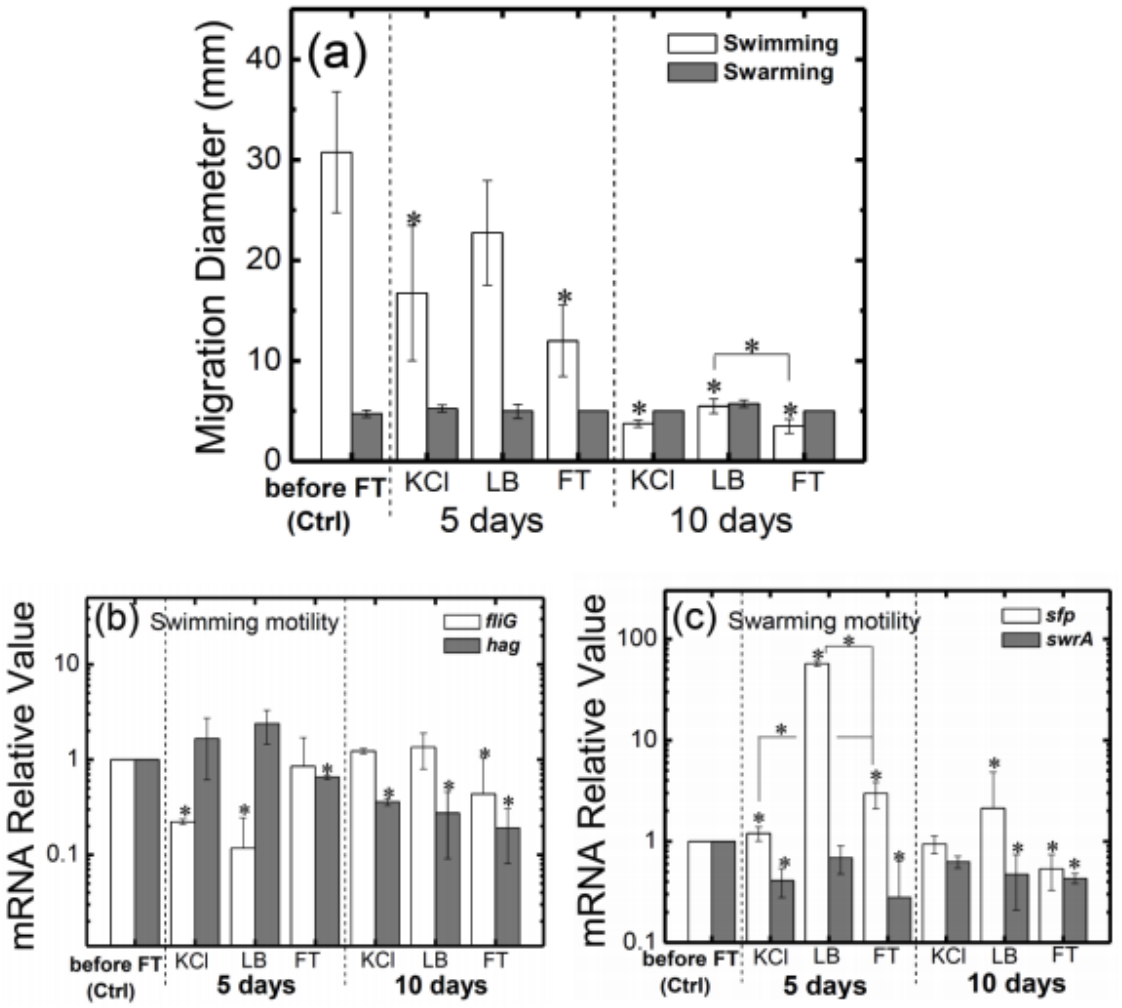

Fig. 1. (a) Characterization of the effects of constant cold temperature (10 °C) and FT on the swimming and swarming motility of *B. subtilis* in 100 mM KCl. (b-c) Relative mRNA expression of (b) the flagellin encoding genes, *fliG* and *hag*, and (c) the surfactin and SwrA encoding genes, *sfp* and *swrA*, for *B. subtilis* before and after FT in 100 mM KCl. Relative mRNA expression of genes 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).

3.2.3. Biofilm Formation and Extracellular Matrix Production

We studied the effect of cold temperature and FT on *B. subtilis* biofilm formation. Although the bacterial growth remained above 80% of that of Ctrl, the biofilm formation was substantially decreased after 10 d FT ($p<0.05$) (Figure 2a). As shown in Figure 2a, the biofilm formation decreased to 66% and 3% of the Ctrl after 5 and 10 d FT, respectively.

B. subtilis modulates biofilm formation using a number of secreted compounds, one of which is the lipopeptide, surfactin (Cosmina et al., 1993). A mutant with deletion in a surfactin synthase gene deficient in surfactin production failed to form robust biofilm (Shank and Kolter, 2011). As discussed above, surfactin production did not stop after temperature treatment and except for 10 d FT, there was a general increase in *sfp* gene expression. Thus, other factors must be involved in the observed decreased biofilm formation after FT treatment.

Biofilms of *B. subtilis* consist of chains of cells that are held together predominately by an extracellular matrix of exopolysaccharide (EPS) and the protein TasA (Branda et al., 2006). EPS is produced by enzymes encoded by the *eps*A-O operon and the gene encoding TasA is located in the *yqxM* (tapA)-*sipW*-*tasA* operon (Serrano et al., 1999). Another extracellular polymer, γ-polyglutamic acid, has been shown to play a role in cellular adherence to solid surfaces (Budde et al., 2006). The genes responsible for the synthesis of γ-polyglutamic acid are *pgsBCA* (Branda et al., 2006).

In this study, the effect of FT on the transcription of EPS and TasA protein (*epsE*, *tasA*) as well as a γ-polyglutamic acid encoding gene, *pgsB*, was measured before (or without) exposure to FT and after FT treatment. The results show that there was an 11-fold increase in *epsE* transcription level after 5 d FT whereas after 10 d FT, the relative transcription of *epsE* gene was substantially decreased to <1% (Figure 2b). This is in agreement with the biofilm results which

showed very low biofilm formation for cells after 10 d FT (Figure 2a). For 5 d and 10 d reference samples in LB or KCl, the *epsE* expression was also in qualitative agreement with the observed biofilm formation. In these experiments, *tasA* transcription levels exhibited 1.1-fold and 2.3-fold increase after 5 and 10 d FT, respectively (Figure 2c). These results show that a longer period under freezing and thawing conditions had significant effect in reduction of EPS whereas the production of TasA protein increased. It has been reported that the absence of TasA protein or EPS can result in lack of robust biofilm while the absence of both components can cause failure in biofilm formation (Branda et al., 2006). Here, we observed significant reduction in *epsE* transcription after 10 d FT treatment which likely contributed to the lack of robust biofilm.

In Figure 2d, the relative transcription of *pgsB* gene showed no reduction after FT treatment. *pgsB* expression increased by 12-fold and 2-fold after 5 and 10 d FT, respectively. *pgsB* is involved in synthesis of γ-polyglutamic acid which has been reported to function as a cryoprotectant (Bhat et al., 2013). It should be noted that γ-polyglutamic acid does not contribute significantly to the extracellular matrix (Lopez et al., 2009).

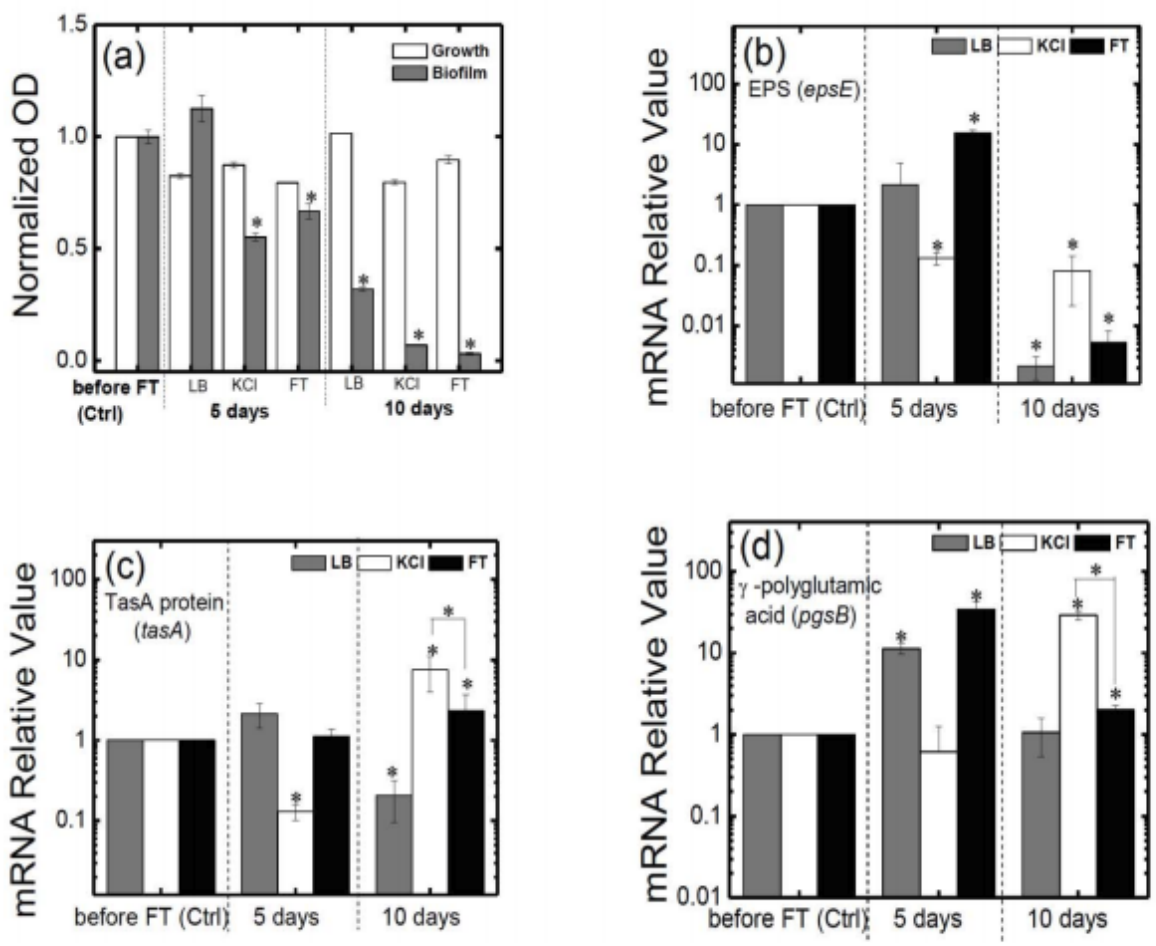

Fig. 2. (a) Characterization of the effects of freeze-thaw on *B. subtilis* biofilms and planktonic growth in microtitre plate wells at 24h. Normalized OD = $[OD-OD_{blank}]/[OD_{Ctrl}-OD_{blank}]$. (b-d) Expression levels of genes responsible for the production of extracellular matrix. Relative mRNA expression of (b) exopolysaccharide gene, *epsE*, (c) TasA protein, *tasA*, and (d) γ-polyglutamic acid via *pgsB* for *B. subtilis* before and after FT in 100 mM KCl. Relative mRNA expression of genes 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).

3.3. Effect of FT on Mechanical Properties of the Bacterial Bond with the Surface using QCM-D

To better understand the effect of FT on the adhesive bond strength between attached *B. subtilis* cells and the silica surface, QCM-D experiments were conducted with Ctrl cells (acclimatized at 10°C) and cells exposed to 5 d and 10 d FT in two solution ISs. Figure 3a-b presents the frequency and dissipation shifts of the third overtone as a function of time for *B. subtilis* adhering to silica coated QCM-D surfaces, before and after FT, while suspended in 10 and 100 mM KCl. The increase in IS resulted in larger frequency and dissipation shifts indicating more bacterial deposition, which is expected based on compression of the electrical double layer (Tufenkji, 2006). For both IS, FT treatment resulted in larger frequency and dissipation shifts, which is in agreement with higher bacterial retention observed for *B. subtilis* in the column experiments, as described in section 3.4 below. The median bacterial number densities at the surface were 5.4×10^5, 4.2×10^5 and 6×10^5 cells cm^{-2} at 10 mM KCl for before FT, 5d FT, and 10d FT, respectively, and increased to 9.6×10^5 and 8.4×10^5 cells cm^{-2} at 100 mM KCl for before FT and 5d FT, respectively. For the treatment of 10d FT in 100 mM KCl, cell aggregates were observed on the crystals and therefore, reliable estimates of bacteria counts could not be obtained.

The measured negative frequency shifts (Figure 3a-b) indicate that for *B. subtilis* deposition onto silica, the QCM-D response was mainly dominated by the bacteria's inertia, and not by deformation of their bond to the surface (Olsson et al., 2012b). In this case, a decrease in bond stiffness would increase the magnitude of the negative frequency shift per individual bacterium, (Olsson et al., 2012a). In Figure 3c, the frequency shifts were divided by the median bacterial number density at the surface for each temperature treatment. In both ISs, the negative frequency

shift per attached cell increased after FT. If the mass of individual bacteria is assumed to be the same before and after FT, then these results suggest that the stiffness of the bacterial bond to the surface decreased after exposure to FT.

Bond stiffness has been linked to the strength of the bond (Olsson et al., 2012b), and thus, possibly to the bacteria's ability to withstand sheer induced detachment forces. Hence, from these QCM-D results, we hypothesize that even if *B. subtilis* became "stickier" with FT (as demonstrated in the column experiments, see section 3.4 below), its bond with the surface was weakened, which might enhance the risk of bacterial detachment from the surface when subjected to a liquid flow. This observation, along with the significant reduction in EPS production, might also explain the lack of robust biofilm formation under FT conditions.

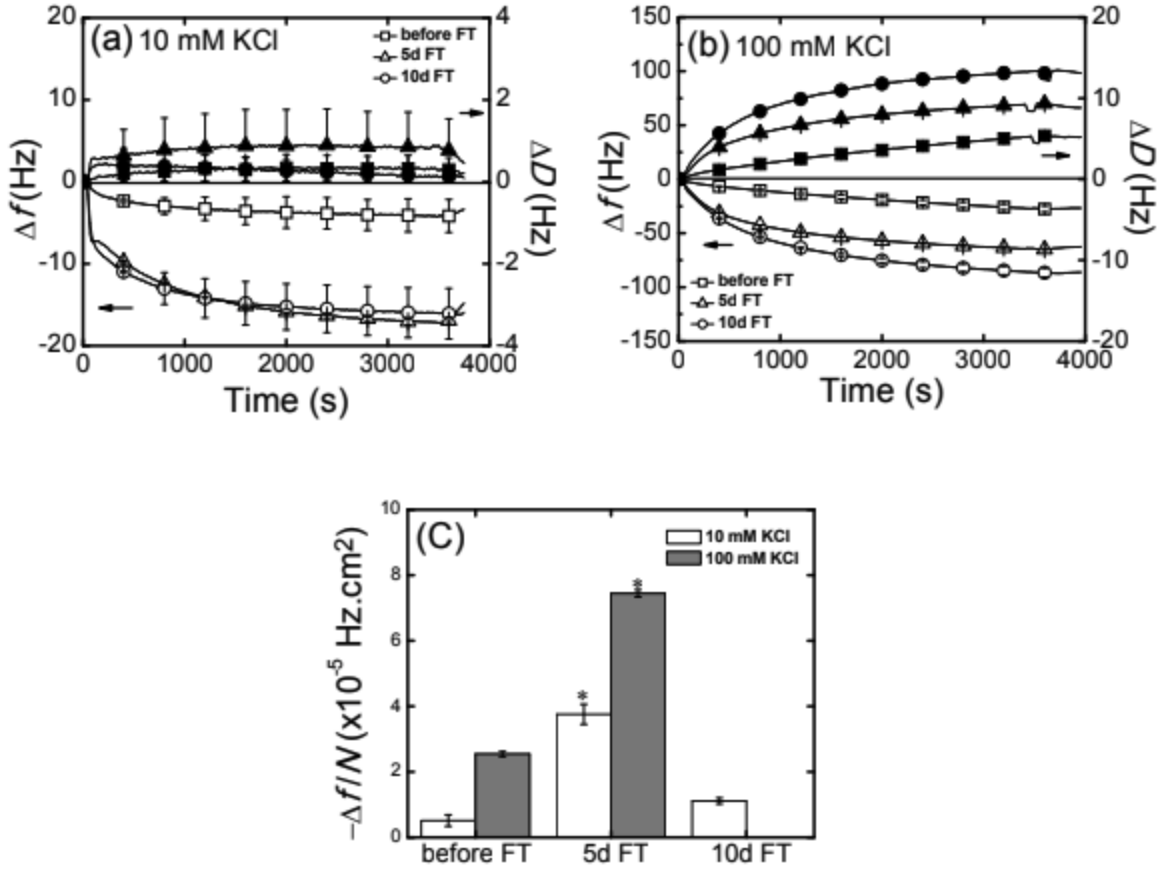

Fig. 3. Effect of FT on bacterial adhesion properties using QCM-D. (a-b) Frequency (open symbols) and dissipation (solid symbols) shifts at 3rd overtone for *B. subtilis* in (a) 10 mM KCl and (b) 100 mM KCl before FT and after 5 d and 10d FT. The results are the average of two experiments. (c) Normalized frequency shift ($\Delta f/N$) at 3rd overtone for *B. subtilis* in 10 and 100 mM KCl before FT treatment and after 5 d FT. *N*: represents the number density of the deposited bacteria at the surface determined by fluorescence microscopy and ImageJ software. Because of the presence of cell aggregates on the crystal for 10d FT in 100 mM KCl, the *N* could not be obtained for that condition. Results represent mean values ± SD for two independent experiments. Symbol above a bar indicate a statistically significant difference in measured values when compared to Ctrl (before FT) (determined using Student's t-test, *: $p<0.05$).

3.4. 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 4a, 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 5% and 16% for the control cells (Ctrl) in 10 and 100 mM KCl, respectively (Figure 4). Here, C/C_0 is evaluated by numerically integrating the area under the breakthrough curves (BTCs). The percentage of bacterial retention increased to 42% and 37% after 10 d FT in 10 and 100 mM KCl, respectively. Figure 4 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 difference in the deposition behavior of live versus damaged cells. The greater extent of bacterial retention after FT treatment may be related to changes in the physicochemical properties of the cells such as surface charge, size, motility, and molecular composition of the cell wall (Abu-Lail and Camesano, 2003; Haznedaroglu et al., 2008; Kjelleberg and Hermansson, 1984; McCaulou et al., 1995; Tufenkji, 2007).

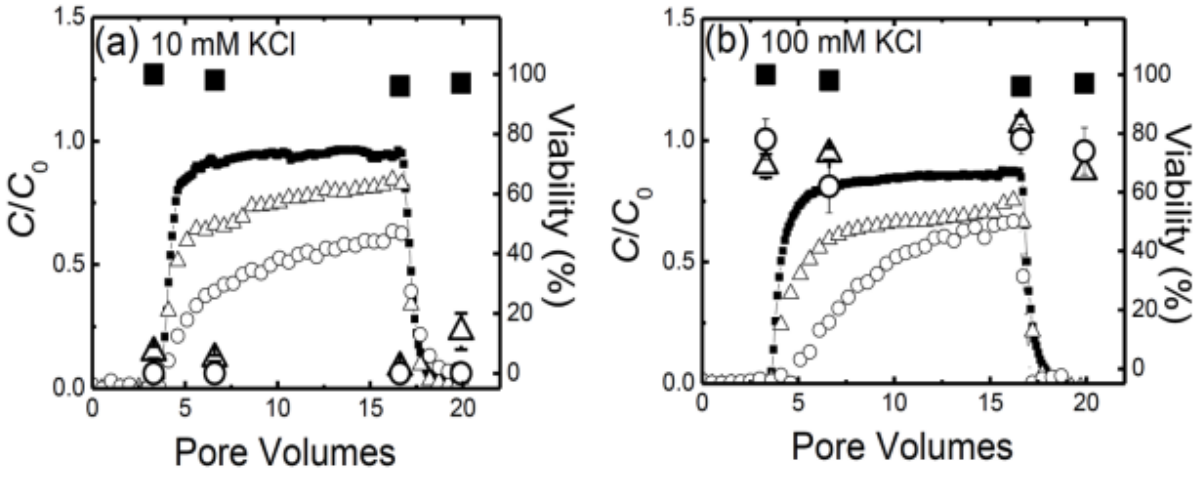

Fig. 4. Breakthrough curves (BTC) for transport of *B. subtilis* through clean quartz sand at 10°C in (a) 10 mM KCl and (b) 100 mM KCl before FT treatment (Ctrl) (–■–), after 5 d FT (–Δ–) and after 10 d FT (–○–). BTCs were identical for two replicate experiments. The viability of cells in the column 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.

The bacterial surface (zeta) potentials were evaluated from EPM measurements conducted over the range of experimental conditions and 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 (Tufenkji, 2006) resulting in greater bacterial retention onto sand grains in higher IS. Zeta potentials of clean sand are also negative at these conditions (~ -30 mV and ~ -5 mV for 10 and 100 mM KCl, respectively) at pH 5.7, as reported elsewhere (Tufenkji et al., 2004). In both IS, the absolute zeta potential decreased after 5 d FT but increased to values higher than its initial value (~ - 20 mV) after 10 d FT. This variation in cell surface charge could be due to secretion of EPS and cryoproteins. If classical DLVO (Derjaguin and Landau, Verwey and Overbeek) interactions were controlling the bacterial transport behavior, we would expect the cells exposed to 5 d FT to exhibit higher retention than the 10 d FT cells; however, inspection

of Figure 4 reveals that this is not the case. Thus, bacterial zeta potential data suggest that factors other than electrostatic forces were involved in controlling the transport behavior of FT-treated *B. subtilis*.

The hydrodynamic diameters of the cells were measured by DLS. The cell size did not change much through FT treatments, with hydrodynamic diameters of 1±0.1 μm before exposure to FT (Ctrl) and 0.9±0.02 μm after 10 d FT in 100 mM KCl under the no nutrient condition (Table 1). These results suggest that cell size did not contribute to higher bacterial retention observed following FT. In higher IS, the cell size reduced to 0.5 μm after 5 d FT but the cells regained their original size (~1μm) after 10 d FT. This size reduction after 5 d FT could contribute to the observed increased bacterial deposition (as much as 16%) due to increased Brownian diffusion of the smaller cells compared to the cells that were not exposed to FT (Ctrl).

The motility of the bacteria was measured as described above. A reduction in bacterial swimming motility was observed after exposure to FT (Figure 1a). Some studies report greater attachment of motile bacteria compared to their non-motile counterparts (Becker et al., 2004; Camper et al., 1993; Kerchove and Elimelech, 2008). However, the opposite has also been shown (Camesano and Logan, 1998). Herein, we observed a coincidence between decreased bacterial motility after FT treatment and higher bacterial retention onto sand (Figure 4).

Table 1. Characterization of *B. subtilis* cells before and after freeze-thaw (FT) treatment in 10 and 100 mM KCl (pH 5.7). Results represent mean values ± SD for three independent experiments.

	Culturability (CFU/mL)		Viability (%)		Cell diameter (μm)		Zeta potential (mV)	
KCl (mM)	10	100	10	100	10	100	10	100
before FT (Ctrl)	2.0×10^{9} ± 0.0	8.3×10^{8} ± 4.2×10^{8}	100 ± 0	100 ± 0	1.7 ± 0.2	1.0 ± 0.1	-27.9 ± 0.5	-21.6 ± 0.4
after 5d FT	200.0 ± 18.9	8.7×10^{4} ± 2.3×10^{4}	7 ± 2	78 ± 7	0.9 ± 0.0	0.5 ± 0.0	-15.8 ± 0.6	-14.7 ± 1.8
after 10d FT	1.0 ± 0.0	6.7×10^{2} ± 1.4×10^{2}	0 ± 0	69 ± 4	0.8 ± 0.1	0.9 ± 0.0	-36.9 ± 0.4	-29.9 ± 0.8

4. Conclusions

This study shows how temperature variations around freezing point 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. Soil is the main habitat of *B. subtilis* and therefore, *B. subtilis* can be subject to cold temperature and seasonal FT processes. *B. subtilis* has been widely accepted as a representative surrogate for bacterial pathogens, particularly, *B. anthracis* (Nicholson and Galeano, 2003). Thus, the study of *B. subtilis* fate and transport as a surrogate can help to improve risk assessment and public health protection. The results of this study show that viability of *B. subtilis* decreased after exposure to FT, but bacteria exhibited greater survival when suspended in a higher IS solution. Common survival strategies of *B. subtilis* such as motility and biofilm formation were significantly influenced. Impaired motility and lack of robust biofilm formation were observed after 10 d of FT treatment. Bacterial transport studies revealed greater bacterial retention onto sand grains after exposure to FT, however, the strength/stiffness of the bacteria-silica bond was found to weaken after FT. Weak bonds between bacteria and the substratum may lead to detachment of cells from surface or near surface soil during spring melt resulting in subsurface transport and risk of bacterial contamination.

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Appendix A. Supporting Information

Strain and culture conditions and primers designed for this study and some of the experimental methods. This information can be found at http://dx.doi.org/.

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