Published in final edited form as:

Extremophiles. 2015 March ; 19(2): 297–305. doi:10.1007/s00792-014-0714-1.

Bacterial Lipoteichoic Acid Enhances Cryosurvival

Charles V. Rice*, Amy Middaugh, Jason R. Wickham, Anthony Friedline, Kieth J. Thomas III, Erin Scull, Karen Johnson, Malcolm Zachariah, and Ravindranth Garimella

Department of Chemistry and Biochemistry, University of Oklahoma, 101 Stephenson Parkway, Norman, OK 73019

Abstract

Antifreeze proteins in fish, plants, and insects provide protection to a few degrees below freezing. Microbes have been found to survive at even lower temperatures, and with a few exceptions, antifreeze proteins are missing. We show that lipoteichoic acid (LTA), a biopolymer in the cell wall of Gram-positive bacteria, can be added to *B. subtilis* cultures and increase freeze tolerance. At 1% w/v, LTA enables a 50% survival rate, similar to the results obtained with 1% w/v glycerol as measured with the resazurin cell viability assay. In the absence of added LTA or glycerol, a very small number of *B. subtilis* cells survive freezing. This suggests that an innate freeze tolerance mechanism exists. While cryoprotection can be provided by extracellular polymeric substances (EPS), our data demonstrate a role for LTA in cryoprotection. Currently, the exact mode of action for LTA cryoprotection is unknown. With a molecular weight of 3–5 kDa, it is unlikely to enter the cell cytoplasm. However, low temperature microscopy data show small ice crystals aligned along channels of liquid water. Our observations suggest that teichoic acids could protect liquid water within biofilms and planktonic bacteria, augmenting the role of brine while also raising the possibility for survival without brine present.

Keywords

teichoic acid; lipoteichoic acid; wall teichoic acid; peptidoglycan; cryoprotectant; psychrophile; bacteria; extremophile; ice

Introduction

Nature relies on chemistry to maintain liquid water for life at subfreezing temperatures. The current paradigms use thermodynamic and/or kinetic arguments for cryosurvival in mammals, plants and insects.(Hubalek 2003; Price 2007; Yeh and Feeney 1996) Little is known of the cryoprotection chemistry for microbes, the predominate organisms in cold environments.(Feller and Gerday 2003; Wilson et al. 2006) Microbial communities have been discovered in ice,(Junge et al. 2004) snow,(Carpenter et al. 2000) permafrost,(Steven et al. 2007) and at the polar ice caps.(Karl et al. 1999; Priscu et al. 1999) Analyses of core samples show the presence of viable life hundreds of feet below the ice surface. Samples of frozen tundra have yielded various Gram positive, Gram negative, and Archaea species.

*rice@ou.edu.

(Steven et al. 2007; Walker et al. 2006; Wallenstein et al. 2007) Gram-negative *Cryobacterium* and Gram-positive *Enterococci* were found to have a symbiotic interaction. The basis for cryosurvival is unknown, but macromolecules and/or antifreeze proteins were suspected. Bacteria from Arctic sea ice were found in brine veins or attached to particulate surfaces.(Junge et al. 2004) Surface interactions enable the bacteria to remain active at -20°C .(Junge et al. 2006) For Gram positive bacteria, metabolism was observed at a temperature of -10°C .(Junge et al. 2004) Other bacteria were dormant and became quite active when warmed to 37°C .(Junge et al. 2004)

Bacteria, found in nearly all low temperature environments, must prevent ice formation to ensure survival. Cold-adapted psychrophilic bacteria have liquid water present to permit the uptake of nutrients and the exchange of waste products.(Cavicchioli 2006; Price 2000; Price and Sowers 2004) However, most bacteria found at subfreezing temperatures are quiescent and thus cryoprotection is essential to prevent ice formation that could cause cell death. Examination of the respective genomes show that, with only a few exceptions,(Wilson et al. 2006) microbes lack the antifreeze proteins found in fish, plants, and insects. Thus, additional factors must permit liquid water at the microbe/ice interface. These factors may include brine veins, dust/soil particulates, or by endogenous biopolymers such as extracellular polymeric substances (EPS, primarily polysaccharides).(Junge et al. 2006) By forming a capsule around the cell, EPS prevents cell death from osmotic stress, hypersalinity, and mechanical ice damage.(Baker et al. 2010; Collins et al. 2010; Marx et al. 2009) Here, we report the discovery that lipoteichoic acid, a biopolymer in the cell wall of Gram positive bacteria, provides substantial cryoprotection to frozen *Bacillus subtilis* bacteria. Cultures frozen at -20°C in the presence of 1% lipoteichoic acid show a similar freeze tolerance to those frozen with a 1% glycerol solution. The mechanism of lipoteichoic acid cryoprotection may include the ability of lipoteichoic acid to protect liquid water at temperatures significantly below freezing. Fluorescence microscopy images show that lipoteichoic acid alters the shape and size of ice crystals, provides liquid water in pockets and channels, and aids in sequestering bacteria into these domains. In this way, lipoteichoic acid could provide Gram positive bacteria with the capability to maintain liquid water within the peptidoglycan cell wall and the immediate extracellular space. The numerous phosphate, hydroxyl, amine, and carboxyl groups of teichoic acid (Figure 1) provide the opportunity for hydrogen bonds, ionic forces, and van der Waals interactions with water and/or ice crystals. Similar rationales are used to explain the properties of other known cryoprotectants.(Chao et al. 1997; Mao and Ba 2006; Scotter et al. 2006) Phillips *et al.* showed that 15% glycerol, a common cryoprotectant, ensured $> 80\%$ survivability for microorganisms frozen at -70°C for one year.(Ludlam et al. 1989) Other non-permeable cryoprotective agents, such as polymers and polysaccharides, create a thin layer of liquid water to prevent extracellular ice from contacting the cell surface.(Hubalek 2003)

Materials and Methods

Bacterial Cell Growth and Cryosurvival Assay

Cultures of *B. subtilis* 1A578 were prepared via inoculation from a frozen culture and allowed to incubate in 20 mL LB broth overnight with 20 μL 1% chloramphenicol. A

portion of these cells were transferred to a solution of 20 mL LB broth and 20 μ L chloramphenicol and cells were harvested during mid-log phase growth (OD_{600} of 0.8 to 1.0). The culture was diluted with LB to an OD_{600} of 0.2 and aliquots transferred to Eppendorf tubes. Thus, excess nutrients were available for cell growth after thawing. Prior to freezing, samples with 1% and 10% glycerol cryoprotectant were prepared by adding 5 μ L or 50 μ L of glycerol (Aldrich, Inc.) to 495 or 450 μ L of cells. Another sample was prepared without additives and the tubes immediately placed in a -20 °C laboratory freezer. The 1% LTA sample was prepared by transferring 500 μ L of cells to a glass vial containing 5 mg of lipoteichoic acid (psLTA ultrapure, Invivogen, Inc.) followed by vigorous vortex mixing. This solution was transferred to an Eppendorf tube and immediately placed in the freezer. The LTA for this commercial sample was delivered as a pure lyophilized sample after isolation and purification from bacterial growth. According to the supplier, the LTA sample was obtained from *Staphylococcus aureus* bacteria. *S. aureus* produces an identical LTA molecule to that of *B. subtilis*. The purification method for psLTA is superior to that used in the production of *B. subtilis* LTA commercial samples. (Morath et al. 2002) The chemical structure of the LTA used in this work was verified with nuclear magnetic resonance spectroscopy. As shown in the supplemental data (Figure S1), the proton spectrum was analyzed using a 500 MHz Varian VNMRS spectrometer.

The Cell-Titer Blue® Cell Viability Assay was used to measure cell growth and determine the viability of cells before and after freezing. The fluorescence-based system is a quantitative measure of cell metabolism and hence viability. Resazurin, which does not have fluorescence properties, is metabolically reduced to resorufin, which emits light at 590 nm. Emission intensity correlates to the number of viable cells in a sample. The resazurin dye, also known as Alamar Blue, has proven to be an accurate test of a cell's viability. (Promega 2009) Growth of the 0.2 OD_{600} culture was evaluated by adding 100 μ L of cells to 3 wells of a black 96-well flat-bottom microtiter plate along with 20 μ L of Cell-Titer Blue® Cell Viability Assay. On the same plate, a control set of wells was prepared by adding 100 μ L of LB broth and 20 μ L of the assay solution. This plate was covered and placed in an incubator at 37 °C for two hours with readings taken every 30 minutes to ensure usual bacterial growth. Fluorescence intensity data were collected with a Cary Eclipse fluorescence spectrophotometer at an excitation wavelength of 560 nm and emission filter of 590 nm. After cold storage (1 or 4 weeks), the Eppendorf tubes were taken from the freezer, thawed, and plated in the manner described above. The cell growth was monitored for 8 hours with readings at regular time intervals.

Fluorescence Spectroscopy

Variable temperature fluorescence spectroscopy was used to confirm the quenching of rhodamine-B in ice. Fluorescence spectra were taken using modular spectrophotometer (USB 2000 FLG, Ocean Optics Inc.) consisting of a gated spectrofluorometer (350-1000 nm), a pulsed xenon light source excitation light source (45 mJ/pulse output, 220-750 nm), 2 fiber optic cables, and a temperature controlled cuvette holders with 4 optical windows. The optical cables were positioned at a 90° angle on the cuvette holder. Each spectrum is collected in a few seconds. The peak maximum and linewidth were determined using the Ocean Optics Software (OOIbase32). The dye was mixed with either water or aqueous

solutions of DNA (5% w/v), LTA (1% w/v) or NaCl (1 M). The solutions were placed in the cuvette and the temperature lowered by pumping chilled water/glycerol through the cuvette holder. The temperature was maintained using a temperature controller (model TC-125, Quantum Northwest, Inc.).

Fluorescence Microscopy

B. subtilis cells were isolated in mid-log growth phase, pelleted, and suspended in 10 mM NaCl solution to prevent osmotic shock, and mixed with rhodamine-B dye. A 5 μ L drop was placed onto a glass microscope slide and covered with a glass coverslip. Microscopy experiments were performed with a Nikon TE2000-E inverted epi-fluorescence microscope. In this configuration, the lens and light beam are underneath the sample, which allows us to cool the microscope slide from the top. The slides were cooled using a home-built brass manifold with ports to circulate a chilled glycerol/water solution. A thin wire thermocouple was placed between the brass manifold and the surface of the microscope slide and the temperature recorded. Using a 60x oil-immersion lens, a thin layer of oil was maintained between the lens surface and the slide coverslip. This prevented frost buildup at the lens/sample interface, which otherwise would severely hinder the fluorescence experiments. In our experience, after 30–45 minutes, water would penetrate the oil layer, create a frost layer, and make it impossible to bring the image into focus. Thus, we would change samples after 20 minutes of work.

Results

Bacterial cryoprotection was evaluated with a cell viability assay based on the metabolic reduction of resazurin to its fluorescent analog resorufin. Assays based on binding to nucleic acids or bioluminescence from ATP produced large background signals from interactions with the phosphodiester LTA biopolymer. After thawing, the samples were mixed with the resazurin reagent, distributed in a 96-well plate, and placed into an incubator. Over an 8 hour period, fluorescence intensity increased from cell growth and resazurin metabolism (Figure 2). Rapid growth is seen for cells frozen with 10% and 1% glycerol, reaching maximum intensity after 3 hours. These results are not surprising as glycerol, a known cryoprotectant, has been used extensively to protect frozen stock since the 1950's. For bacteria frozen in the presence of 1% LTA, cell growth begins immediately after thawing and reaches maximum intensity after 3 hours. The log-phase growth mimics that of the 1% glycerol sample; however, the transition to stationary phase and death phase occurs faster than the 1% glycerol data for unknown reasons. Regardless, 1% LTA is providing cryoprotection although the survival mechanism is not fully understood. Cryoprotection from both glycerol and LTA is less effective for bacteria frozen for a longer time period. After 4 weeks at -20 °C, the data in Figure 2 show that the 10% glycerol stock sample generates about 1/2 of fluorescence intensity as the same solution frozen for 1 week. As the resazurin content is the same in all samples, this indicates that the number of viable bacteria decreases with freezing time. This is not unexpected given the rich history of adding glycerol to frozen bacterial cultures. The 1% glycerol and 1% LTA samples also show decreased survival after 4 weeks, but nonetheless also demonstrate the ability of LTA to act as a natural cryoprotection agent.

If LTA is a natural cryoprotectant, then bacteria should survive freezing without any additives. This is indeed the case. Data presented in Figure 2 shows cell growth of a thawed *B. subtilis* culture frozen in saline solution without additional glycerol or LTA. The fluorescence intensity is very low and does not increase until 5 hours of incubation. The apparent lag phase growth could indicate freeze-stress recovery that does not involve resazurin metabolism or, alternatively, a very low number of survivors whose metabolic fluorescent signal cannot be detected until the cell density increases. Although we cannot distinguish between the two possible causes of low signal, these data are consistent with growth curves recorded using a 0.01% inoculation from frozen stock. While salt solutions can lower the freezing point of water, a 10 mM solution should have a negligible effect, lowering the freezing point by 0.04 °C.(Atkins and De Paula)

Bacterial survival requires limiting contact with damaging ice crystals. This can occur by either changing the size/shape of ice crystals or creating a protective layer of liquid water. LTA may enable one or both of these mechanisms and fluorescence microscopy was used to visualize the ice/water mixture. While selecting a fluorescent dye, we discovered a brief report in a book from 1957 describing the quenching of rhodamine-B in ice.(Szent-Györgyi 1957) This phenomenon was verified with fluorescence emission spectra collected in our lab (Figure 3). When rhodamine-B is immobilized in ice (Figure 3B), the electrons in rhodamine-B are able to occupy a triplet state, frustrating fluorescence processes. The emission spectra of Figure 3 clearly show the loss of signal, which is restored upon the addition of 1% w/v LTA (Figure 3C). However, DNA or NaCl solutions do not restore signal intensity, suggesting that the LTA functions as a cryoprotectant in a non-colligative manner (see supplemental data Figure S3). Emission quenching of rhodamine-B in ice is also seen in the two-dimensional microscope images (Figure 4A). After LTA addition, the rhodamine-B fluorescence intensity is large (Figure 4B). Compared to Figure 4A, we estimate a nearly thousand fold increase in light intensity, calculated from a 10x difference in image brightness and the 100 fold intensity enhancement of Figure 4A. The unexpected nature of Figure 4B was the size and shape of the liquid water fraction. One could imagine that large ice crystals would form and segregate the LTA/dye/water into large pockets. Instead, we observe microscopic ice crystals on the scale of a few microns in diameter and length. These data demonstrate that LTA creates a highly connected network of small water-filled cavities between ice crystals. Such an arrangement could contribute to microbial survival by permitting nutrient/waste exchange in the liquid water and enable the formation of microbial communities along the water channels.(Rohde and Price 2007)

Cultures of *B. subtilis* were transferred to a saline solution to prevent death from osmotic shock. When mixed the bacteria, the rhodamine-B dye is able to enter the cells and stain the interior. From a room temperature image (see supplemental data, Figure S2), the bacteria absorb nearly all of the dye from solution. At -20 °C, the 10 mM NaCl solution is frozen(Atkins and De Paula) yet dye emission is observed within the bacterial cells (Figure 4C) because of liquid water in the cytoplasm, cell wall (peptidoglycan and TA), or both. We suspect liquid water in both regions because both the cytoplasm and teichoic acid in the cell wall protect liquid water. However, when additional LTA is added, the image taken at -20 °C shows the bacteria restricted to areas between ice crystals (Figure 4D). These areas

resemble the channels of LTA in water (Figures 4B). These images are reproduced in the supplemental data for further inspection (Figures S4A-D).

The fluorescence microscopy images show that the water is present in long connected channels between ice crystals. These features would enable life to exist at subfreezing temperatures (Rohde and Price 2007) by 1) alleviating osmotic pressure, 2) allowing the exchange of nutrients/waste products, 3) permitting bacterial motility along the water channels, 4) permitting the exchange of biochemical signals to allow cell-to-cell communication and quorum sensing. The presence of liquid water would also allow for DNA repair processes and perhaps other enzymatic events. (Cavicchioli 2006; Rothschild and Mancinelli 2001) Low temperatures would slow normal processes; though some cold-induced enzymes would be able to function. (Cavicchioli 2006; Feller and Gerday 2003; Rothschild and Mancinelli 2001) Even for quiescent microbes, antifreeze LTA properties may have a survival role for extremophiles found in ice-core samples. (Cavicchioli 2002; Clarke et al. 2007; Hoover et al. 2003; Horneck 2000; Mueller et al. 2005; Nghiem et al. 1993; Pikuta et al. 2006a; Pikuta et al. 2003a; Pikuta et al. 2003b; Pikuta et al. 2007a; Pikuta et al. 2006b; Pikuta et al. 2005; Pikuta et al. 2007b)

Discussion

The presence of microbes in frozen ecosystems (Carpenter et al. 2000; Feller and Gerday 2003; Hoover et al. 2003; Junge et al. 2004; Karl et al. 1999; Pikuta et al. 2006a; Pikuta et al. 2003a; Pikuta et al. 2003b; Pikuta et al. 2007a; Pikuta et al. 2006b; Pikuta et al. 2005; Pikuta et al. 2007b; Priscu et al. 1999; Steven et al. 2007; Wilson et al. 2006) and the possibility for extraterrestrial life in the polar ice caps of Mars (Cavicchioli 2002; Horneck 2000) will benefit by defining the conditions under which life can exist. (Price 2007; Rothschild and Mancinelli 2001) Liquid water must be protected within/around these life forms and genetic mapping is often used to determine if an organism has antifreeze proteins. (D'Amico et al. 2006) Although some bacteria have antifreeze proteins (Duman and Olsen 1993; Gilbert et al. 2005; Gilbert et al. 2004; Kawahara 2002; Kawahara et al. 2007; Kawahara et al. 2001), the use of AFPs is not widespread. Psychrophilic bacteria lack AFPs, and freeze tolerance is attributed to unfreezable water when confined to small spaces. (Mindock et al. 2001) Even if AFPs are present, the freeze protection is only effective to a few degrees below zero. (Yeh and Feeney 1996) Our data show that LTA provides an natural innate mechanism to maintain liquid water in the cell wall without the need for brine veins, although brine/LTA may have mutual benefits to the microbe.

Teichoic acids, WTA and LTA, compose 50% of the dry weight of the cell wall. (Neuhaus and Baddiley 2003) The discovery that teichoic acid permits liquid water at sub-zero temperatures adds to the list of functions for teichoic acid. (Neuhaus and Baddiley 2003) Without additional LTA, the cells survive but require many hours before cell growth is measureable. This result suggests a low, but non-zero, number of survivors. Survival occurs because of many factors, including the presence of teichoic acids (LTA and WTA) in their cell walls. It is also possible that survival may be greater in nature where lipoteichoic acid is excreted from cells, and the extracellular concentration can be significant, especially within biofilm environments. (Brock and Reiter 1976; Ekstedt and Bernhard 1973; Hussain et al.

1991; Hussain et al. 1993; Jones et al. 2005; Tojo et al. 1988) Within biofilms, LTA binds water and forms cell-to-cell contacts.(Hasty et al. 1992; Mattsson et al. 2004; Weidenmaier et al. 2003) The latter event involves a “grappling hook” action that precedes the transfer of cell signaling molecules.(Dunny and Leonard 1997; DUNNY et al. 1995; Leonard et al. 1996) Additionally, it is widely believed that LTA plays a significant role in bacterial adhesion, an event that precedes biofilm formation.(Gross et al. 2001; Rose 2000) Extracellular polymeric substances (EPS) are a major component of biofilms, and EPS has been shown to be an effective cryoprotectant.(Baker et al. 2010; Collins et al. 2010; Junge et al. 2006; Krembs et al. 2002; Krembs and Engel 2001; Marx et al. 2009; Raymond and Fritsen 2001) Although EPS alone could provide a biofilm with cryosurvival properties, the presence of LTA in biofilms would be an additional benefit to the microbial communities. The samples in this study reached -20 °C in a few minutes; a biofilm would not have time to mature nor would it be possible to have sporulation. However, fluorescence microscopy images show the bacteria organized into groups, mimicking a small biofilm cluster. Such organization would enable natural cooperative cryosurvival strategies between adjacent bacteria and could involve localized secretion of EPS, LTA, or both.

Discovering antifreeze properties for bacterial teichoic acid and demonstrating enhanced survival when extra LTA is present fills a void in cryobiology. Currently, we do not know the mechanism by which liquid water is protected by teichoic acid. One possibility is the use of NMR spectroscopy; a methodology was used to understand the properties of antifreeze proteins.(Chao et al. 1997; Krishnan et al. 2005; Mao and Ba 2006; Scotter et al. 2006; Tsvetkova et al. 2002; Yeh and Feeney 1996) Additional insight regarding the role of teichoic acids in low temperature biochemistry could be obtained using mutants with teichoic acid deletions. These strains should exhibit increased freeze mortality. Gründling and Schneewind demonstrated the complete deletion of LTA, which caused *S. aureus* to stop growing.(Grundling and Schneewind 2007) The remaining cells showed enlarged cell size, thicker walls, and misplaced septa for cell division. These traits would complicate examination of freeze-induced mortality in the LTA-absent mutants and therefore LTA-absent mutants are unlikely towards understanding cryoprotection from LTA.

Conclusions

Life processes at sub-zero temperatures require liquid water. The discovery of viable organisms in extreme environments has required new chemical and biochemical explanations of the manner by which liquid water is protected. These explanations also impact the search for extraterrestrial life, for instance on the polar ice caps of Mars, by helping to define the conditions under which life can exist.(Price 2007; Rothschild and Mancinelli 2001) Genetic mapping has been used to determine if an organism has antifreeze proteins.(D'Amico et al. 2006) These studies show that only a few Gram-positive bacteria have the genetic makeup to express antifreeze proteins. *B. subtilis* is not among this group. Gram-positive bacteria can use salts, nucleic acids, and lipids as antifreeze agents in the cytoplasm and membrane bilayer. Our data show teichoic acid provides a mechanism to maintain liquid water and that bacterial survival is greatly enhanced when teichoic acids are present in the extracellular space. As teichoic acid is ubiquitous in Gram-positive bacteria, it can be used to ensure survival of individual bacteria and biofilm communities. In the latter,

teichoic acids are excreted into the extracellular space where it could play a symbiotic role in cryoprotection.

Teichoic acid has diverse chemical functional groups that may contribute to its ability to protect liquid water; however, an exact chemical explanation for this new function. It is possible that the mechanism of bacterial lipoteichoic acid cryoprotection shares enthalpic and entropic properties of cryoprotectants found in higher forms of life. At the onset of freezing, frogs excrete large amounts of glucose to lower the freezing point of water while preventing the formation of large ice crystals. Fish, plants, and insects inhibit ice formation with antifreeze proteins (AFP) that bind to the growth face of ice crystals. Enthalpic contributions are accompanied by entropic factors from a disorganized chemical system. (Chao et al. 1997) These factors slow the kinetics of crystallization such that AFPs perform their task at very low concentration, characterized as a non-colligative depression of the freezing point. It is possible that the teichoic acid biomolecules share these properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work is supported by the University of Oklahoma and the National Institutes of Health (R01-GM090064). We are grateful to Dr. Jim Henthorn, OU Health Sciences Center, for assisting with the fluorescence microscopy experiments. Jason R. Wickham is currently an Assistant Professor at Northwestern Oklahoma State University. He was supported during this project as a Graduate Assistance in Areas of National Need (GAANN) Fellow via a Department of Education Grant No. P200A030196 awarded to the Department of Chemistry and Biochemistry, University of Oklahoma.

References

- Atkins, PW.; De Paula, J. Physical chemistry. 9th edn.. W.H. Freeman; New York: 2010.
- Baker MG, Lalonde SV, Konhauser KO, Foght JM. Role of Extracellular Polymeric Substances in the Surface Chemical Reactivity of *Hymenobacter aerophilus*, a Psychrotolerant Bacterium. *Appl Environ Microb*. 2010; 76:102–109. doi:10.1128/aem.02006-09.
- Brock JH, Reiter B. Chemical and Biological Properties of Extracellular Slime Produced by *Staphylococcus-Aureus* Grown in High-Carbohydrate. High-Salt Medium *Infect Immun*. 1976; 13:653–660.
- Carpenter EJ, Lin SJ, Capone DG. Bacterial activity in South Pole snow. *Appl Environ Microb*. 2000; 66:4514–4517.
- Cavicchioli R. Extremophiles and the search for extraterrestrial life. *Astrobiology*. 2002; 2:281–292. [PubMed: 12530238]
- Cavicchioli R. Cold-adapted archaea. *Nat Rev Microbiol*. 2006; 4:331–343. [PubMed: 16715049]
- Chao HM, et al. A diminished role for hydrogen bonds in antifreeze protein binding to ice. *Biochemistry-U.S.* 1997; 36:14652–14660.
- Clarke A, Johnston NM, Murphy EJ, Rogers AD. Introduction. Antarctic ecology from genes to ecosystems: the impact of climate change and the importance of scale *Philosophical Transactions of the Royal Society. B-Biological Sciences*. 2007; 362:5–9.
- Collins RE, Roca G, Deming JW. Persistence of bacterial and archaeal communities in sea ice through an Arctic winter. *Environ Microbiol*. 2010; 12:1828–1841. doi:10.1111/j.1462-2920.2010.02179.x. [PubMed: 20192970]
- D'Amico S, Collins T, Marx JC, Feller G, Gerday C. Psychrophilic microorganisms: challenges for life. *Embo Rep*. 2006; 7:385–389. [PubMed: 16585939]

- Duman JG, Olsen TM. Thermal Hysteresis Protein-Activity in Bacteria. Fungi, and Phylogenetically Diverse Plants Cryobiology. 1993; 30:322–328.
- Dunny G, Leonard B. Cell-cell communication in gram-positive bacteria. ANNU REV MICROBIOL. 1997; 51:527–564. [PubMed: 9343359]
- DUNNY G, LEONARD B, HEDBERG P. PHEROMONE-INDUCIBLE CONJUGATION IN ENTEROCOCCUS-FAECALIS -INTERBACTERIAL AND HOST-PARASITE CHEMICAL COMMUNICATION. J Bacteriol. 1995; 177:871–876. [PubMed: 7860595]
- Ekstedt RD, Bernhard JM. Preparation and Characterization of a Slime Layer Material Produced by Staphylococcus-Aureus P Soc Exp. Biol Med. 1973; 142:86–91.
- Feller G, Gerday C. Psychrophilic enzymes: Hot topics in cold adaptation. Nat Rev Microbiol. 2003; 1:200–208. [PubMed: 15035024]
- Gilbert JA, Davies PL, Laybourn-Parry J. A hyperactive, Ca²⁺-dependent antifreeze protein in an Antarctic bacterium. Fems Microbiology Letters. 2005; 245:67–72. [PubMed: 15796981]
- Gilbert JA, Hill PJ, Dodd CER, Laybourn-Parry J. Demonstration of antifreeze protein activity in Antarctic lake bacteria. Microbiology-Sgm. 2004; 150:171–180.
- Gross M, Cramton SE, Gotz F, Peschel A. Key role of teichoic acid net charge in Staphylococcus aureus colonization of artificial surfaces. Infect Immun. 2001; 69:3423–3426. [PubMed: 11292767]
- Grundling A, Schneewind O. Synthesis of glycerol phosphate lipoteichoic acid in Staphylococcus aureus. P Natl Acad Sci USA. 2007; 104:8478–8483.
- Hasty DL, Ofek I, Courtney HS, Doyle RJ. Multiple adhesins of streptococci. Infect Immun. 1992; 60:2147–2152. [PubMed: 1587582]
- Hoover RB, Pikuta EV, Bej AK, Marsic D, Whitman WB, Tang J, Krader P. Spirochaeta americana sp nov., a new haloalkaliphilic, obligately anaerobic spirochaete isolated from soda Mono Lake in California. International Journal of Systematic and Evolutionary Microbiology. 2003; 53:815–821. [PubMed: 12807206]
- Horneck G. The microbial world and the case for Mars Planetary and Space. Science. 2000; 48:1053–1063.
- Hubalek Z. Protectants used in the cryopreservation of microorganisms. Cryobiology. 2003; 46:205–229. [PubMed: 12818211]
- Hussain M, Hastings JGM, White PJ. Isolation and Composition of the Extracellular Slime Made by Coagulase-Negative Staphylococci in a Chemically Defined Medium. J Infect Dis. 1991; 163:534–541. [PubMed: 1995727]
- Hussain M, Wilcox MH, White PJ. The Slime of Coagulase-Negative Staphylococci -Biochemistry and Relation to Adherence Fems. Microbiol Rev. 1993; 104:191–208.
- Jones KJ, Perris AD, Vernallis AB, Worthington T, Lambert PA, Elliott TSJ. Induction of inflammatory cytokines and nitric oxide in J774.2 cells and murine macrophages by lipoteichoic acid and related cell wall antigens from Staphylococcus epidermidis. J Med Microbiol. 2005; 54:315–321. [PubMed: 15770014]
- Junge K, Eicken H, Deming JW. Bacterial activity at -2 to -20 degrees C in Arctic wintertime sea ice. Appl Environ Microb. 2004; 70:550–557.
- Junge K, Eicken H, Swanson BD, Deming JW. Bacterial incorporation of leucine into protein down to -20 degrees C with evidence for potential activity in sub-eutectic saline ice formations. Cryobiology. 2006; 52:417–429. [PubMed: 16647051]
- Karl DM, Bird DF, Bjorkman K, Houlihan T, Shackelford R, Tupas L. Microorganisms in the accreted ice of Lake Vostok. Antarctica Science. 1999; 286:2144–2147.
- Kawahara H. The structures and functions of ice crystal-controlling proteins from bacteria. Journal of Bioscience and Bioengineering. 2002; 94:492–496. [PubMed: 16233340]
- Kawahara H, et al. A novel, intracellular antifreeze protein in an Antarctic bacterium, Flavobacterium xanthum. Cryoletters. 2007; 28:39–49. [PubMed: 17369961]
- Kawahara H, Li JP, Griffith M, Glick BR. Relationship between antifreeze protein and freezing resistance in Pseudomonas putida GR12-2. Current Microbiology. 2001; 43:365–370. [PubMed: 11688802]

- Krembs C, Eicken H, Junge K, Deming JW. High concentrations of exopolymeric substances in Arctic winter sea ice: implications for the polar ocean carbon cycle and cryoprotection of diatoms. *Deep-Sea Res Pt I*. 2002; 49:2163–2181.
- Krembs C, Engel A. Abundance and variability of microorganisms and transparent exopolymer particles across the ice-water interface of melting first-year sea ice in the Laptev Sea (Arctic). *Mar Biol*. 2001; 138:173–185.
- Krishnan VV, Lau EY, Tsvetkova NM, Feeney RE, Fink WH, Yeh Y. Characterization of the restricted rotation of the dimethyl groups in chemically N-terminal C-13-labeled antifreeze glycoproteins: A temperature-dependent study in water to ice through the supercooled state. *J Chem Phys*. 2005; 123
- Leonard B, Podbielski A, Hedberg P, Dunne G. Enterococcus faecalis pheromone binding protein, PrgZ, recruits a chromosomal oligopeptide permease system to import sex pheromone cCF10 for induction of conjugation. *P Natl Acad Sci USA*. 1996; 93:260–264.
- Ludlam HA, Nwachukwu B, Noble WC, Swan AV, Phillips I. The Preservation of Microorganisms in Biological Specimens Stored at -70-Degrees-C. *J Appl Bacteriol*. 1989; 67:417–423. [PubMed: 2584170]
- Mao YG, Ba Y. Insight into the binding of antifreeze proteins to ice surfaces via C-13 spin lattice relaxation solid-state. *NMR Biophys J*. 2006; 91:1059–1068.
- Marx JG, Carpenter SD, Deming JW. Production of cryoprotectant extracellular polysaccharide substances (EPS) by the marine psychrophilic bacterium Colwellia psychrerythraea strain 34H under extreme conditions. *Canadian Journal of Microbiology*. 2009; 55:63–72. doi:10.1139/w08-130. [PubMed: 19190702]
- Mattsson E, Hartung T, Morath S, Egesten A. Highly purified lipoteichoic acid from Staphylococcus aureus induces procoagulant activity and tissue factor expression in human monocytes but is a weak inducer in whole blood: Comparison with peptidoglycan. *Infect Immun*. 2004; 72:4322–4326. [PubMed: 15213184]
- Mindock CA, Petrova MA, Hollingsworth RI. Re-evaluation of osmotic effects as a general adaptive strategy for bacteria in sub-freezing conditions. *Biophysical Chemistry*. 2001; 89:13–24. [PubMed: 11246742]
- Morath S, Stadelmaier A, Geyer A, Schmidt RR, Hartung T. Synthetic lipoteichoic acid from Staphylococcus aureus is a potent stimulus of cytokine release. *J Exp Med*. 2002; 195:1635–1640. doi:10.1084/Jem.20020322. [PubMed: 12070290]
- Mueller DR, Vincent WF, Bonilla S, Laurion I. Extremotrophs, extremophiles and broadband pigmentation strategies in a high arctic ice shelf ecosystem. *Fems Microbiol Ecol*. 2005; 53:73–87. [PubMed: 16329931]
- Neuhaus F, Baddiley J. A continuum of anionic charge: Structures and functions of D-Alanyl-Teichoic acids in gram-positive bacteria. *MICROBIOL MOL BIOL R*. 2003; 67:686.
- Nghiem SV, Yueh SH, Kwok R, Nguyen DT. Polarimetric Remote-Sensing of Geophysical Medium Structures. *Radio Science*. 1993; 28:1111–1130.
- Pikuta EV, Hoover RB, Bei AK, Marsic D, Whitman WB, Krader PE, Tang J. Trichococcus patagoniensis sp nov., a facultative anaerobe that grows at -5 degrees C, isolated from penguin guano in Chilean Patagonia. *International Journal of Systematic and Evolutionary Microbiology*. 2006a; 56:2055–2062. [PubMed: 16957099]
- Pikuta EV, Hoover RB, Bei AK, Marsic D, Detkova EN, Whitman WB, Krader P. Tindallia californiensis sp nov., a new anaerobic, haloalkaliphilic, spore-forming acetogen isolated from Mono Lake in California Extremophiles. 2003a; 7:327–334.
- Pikuta EV, Hoover RB, Bei AK, Marsic D, Whitman WB, Cleland D, Krader P. Desulfonatrum thiodismutans sp nov., a novel alkaliphilic, sulfate-reducing bacterium capable of lithoautotrophic growth. *International Journal of Systematic and Evolutionary Microbiology*. 2003b; 53:1327–1332. [PubMed: 13130014]
- Pikuta EV, Hoover RB, Tang J. Microbial extremophiles at the limits of life Critical. *Reviews in Microbiology*. 2007a; 33:183–209.

- Pikuta EV, Itoh T, Krader P, Tang J, Whitman WB, Hoover RB. *Anaerovirgula multivorans* gen. nov., sp nov., a novel spore-forming, alkaliphilic anaerobe isolated from Owens Lake, California. USA International Journal of Systematic and Evolutionary Microbiology. 2006b; 56:2623–2629.
- Pikuta EV, Marsic D, Bej A, Tang J, Krader P, Hoover RB. *Carnobacterium pleistocenium* sp nov., a novel psychrotolerant, facultative anaerobe isolated from permafrost of the Fox Tunnel in Alaska. International Journal of Systematic and Evolutionary Microbiology. 2005; 55:473–478. [PubMed: 15653921]
- Pikuta EV, et al. *Thermococcus thioeducens* sp nov., a novel hyperthermophilic, obligately sulfur-reducing archaeon from a deep-sea hydrothermal vent. International Journal of Systematic and Evolutionary Microbiology. 2007b; 57:1612–1618. [PubMed: 17625204]
- Price PB. A habitat for psychrophiles in deep Antarctic ice. P Natl Acad Sci USA. 2000; 97:1247–1251.
- Price PB. Microbial life in glacial ice and implications for a cold origin of life. Fems Microbiol Ecol. 2007; 59:217–231. [PubMed: 17328118]
- Price PB, Sowers T. Temperature dependence of metabolic rates for microbial growth, maintenance, and survival. P Natl Acad Sci USA. 2004; 101:4631–4636.
- Priscu JC, et al. Geomicrobiology of subglacial ice above Lake Vostok. Antarctica Science. 1999; 286:2141–2144.
- Promega. CellTiter-Blue Cell Viability Assay. Promega Corporation; Madison, WI: 2009.
- Raymond JA, Fritsen CH. Semipurification and ice recrystallization inhibition activity of ice-active substances associated with Antarctic photosynthetic organisms. Cryobiology. 2001; 43:63–70. [PubMed: 11812052]
- Rohde RA, Price PB. Diffusion-controlled metabolism for long-term survival of single isolated microorganisms trapped within ice crystals. P Natl Acad Sci USA. 2007; 104:21021–21021. vol 104, 16592, 2007 doi:DOI 10.1073/pnas.0710898105.
- Rose R. The role of calcium in oral streptococcal aggregation and the implications for biofilm formation and retention. Biochimica et Biophysica Acta-General Subjects. 2000; 1475:76–82.
- Rothschild LJ, Mancinelli RL. Life in extreme environments. Nature. 2001; 409:1092–1101. [PubMed: 11234023]
- Scotter AJ, Marshall CB, Graham LA, Gilbert JA, Garnham CP, Davies PL. The basis for hyperactivity of antifreeze proteins. Cryobiology. 2006; 53:229–239. [PubMed: 16887111]
- Steven B, Briggs G, McKay CP, Pollard WH, Greer CW, Whyte LG. Characterization of the microbial diversity in a permafrost sample from the Canadian high Arctic using culture-dependent and culture-independent methods. Fems Microbiol Ecol. 2007; 59:513–523. [PubMed: 17313587]
- Szent-Györgyi, A. Bioenergetics. Academic Press; New York: 1957.
- Tojo M, Yamashita N, Goldmann DA, Pier GB. Isolation and Characterization of a Capsular Polysaccharide Adhesin from *Staphylococcus-Epidermidis*. J Infect Dis. 1988; 157:713–722. [PubMed: 3279138]
- Tsvetkova NM, et al. Dynamics of antifreeze glycoproteins in the presence of ice. Biophys J. 2002; 82:464–473. [PubMed: 11751333]
- Walker VK, Palmer GR, Voordouw G. Freeze-thaw tolerance and clues to the winter survival of a soil community. Appl Environ Microb. 2006; 72:1784–1792.
- Wallenstein MD, McMahon S, Schimel J. Bacterial and fungal community structure in Arctic tundra tussock and shrub soils. Fems Microbiol Ecol. 2007; 59:428–435. [PubMed: 17313585]
- Weidenmaier C, Kristian SA, Peschel A. Bacterial resistance to antimicrobial host defenses -An emerging target for novel anti-infective strategies? Curr Drug Targets. 2003; 4:643–649. [PubMed: 14577655]
- Wilson SL, Kelley DL, Walker VK. Ice-active characteristics of soil bacteria selected by ice-affinity. Environ Microbiol. 2006; 8:1816–1824. [PubMed: 16958762]
- Yeh Y, Feeney RE. Antifreeze proteins: Structures and mechanisms of function. Chem Rev. 1996; 96:601–617. [PubMed: 11848766]

Figure 1.

Chemical structure of teichoic acids found in the cell wall of Gram positive bacteria. The polyphosphate backbone is very long, with 40-50 repeat units dependent upon the species of bacteria. The many different constituents of teichoic acid facilitate chemical interactions with various ionic species, water, and the cell wall peptidoglycan. The random distribution of sidechain groups produces a disordered polymer lacking secondary structure. This high degree of conformational freedom provides an entropic mechanism to keep water in a disordered state.

Figure 2.

A cell viability assay shows that lipoteichoic acid provides protection against freeze death. In absence of additives, cell growth does not enter log phase until many hours after thawing. When cells are stored for 1 week or 4 weeks at -20 °C, protection by LTA resembles that of 1 % glycerol. Whereas glycerol enters the cell to reduce osmotic stress, the mode of action of LTA involves the protection of liquid water in the extracellular space.

Figure 3.

Fluorescence emission spectra of Rhodamine-B dissolved in water. At room temperature, the emission peak is apparent (A). When frozen, the emission is quenched (B). The emission spectrum resembles that of pure ice, which occurs because the incident light scatters off the ice crystals (see supplemental data). When mixed with LTA at 1% w/v, the emission peak is restored at -20 °C because LTA allows the presence of liquid water.

Figure 4.

The presence of lipoteichoic acid, LTA, ensures the presence of liquid water as shown by the strong fluorescence emission of the Rhodamine-B dye in these fluorescence microscope images. The dark regions are occupied by pure water ice crystals. Image (A) was taken with Rhodamine-B dye dissolved in water. The light magnification in (A) is 100x the other images (B-D), demonstrating that the fluorescence intensity is negligible. With LTA present, channels of liquid water are present, separating the ice crystals (B). *B. subtilis* bacteria enable Rhodamine-B emission in liquid water. The fluorescence microscope image shows that the bacteria aggregate in clusters and channels between ice crystals (C). When 1% w/v LTA is added to the droplet of *B. subtilis*, the arrangement changes dramatically (D). The bacteria now reside in channels, similar to that seen with LTA itself (B).