5-2009

Natural Ice-Nucleating Bacteria Increase the Freezing Tolerance of the Intertidal Bivalve Geukensia demissa

Alexander M. McCorkle
Connecticut College, amccorkl@conncoll.edu

Natural ice-nucleating bacteria increase the freezing tolerance of the intertidal bivalve *Geukensia demissa*

Alexander McCorkle

Advisor: Dr. Stephen Loomis

April 2009

Abstract

Instead of avoiding freezing, freeze tolerant invertebrates actively initiate controlled ice nucleation at relatively high sub-zero temperatures in extracellular compartments. Most produce proteinaceous ice-nucleators in their hemolymph, however the intertidal bivalve mollusc *Geukensia demissa* lacks this ability. Instead it utilizes at least one strain of ice-nucleation active (INA) bacteria, *Pseudomonas fulva,* present in seawater, to induce crystallization in the pallial fluid that fills its mantle cavity. In this study, two additional INA bacteria strains were isolated from the palial fluid of *Geukensia demissa*: *Psychrobacter* sp. and *Shewanella* sp. The ice-nucleation activity of both strains was characterized and *Psychrobacter* was found to consistently induce nucleation at temperatures 1-3°C higher than *Shewanella*. Based on 16S rRNA sequencing, neither of these bacteria have yet been identified. The effects of *Psychrobacter* on the freeze tolerance of summer-acclimatized *Geukensia* were assessed and compared to the freeze tolerance of winter-acclimatized specimens. This assessment was accomplished through whole-organism death experiments involving 12-hour periods of exposure to sub-zero temperatures and cell viability tests using a LIVE/DEAD sperm viability kit (Molecular Probes, Inc, Eugene, OR). Adding INA bacteria to summer-acclimatized *Geukensia* reduced their LT50 from -12.5°C to -15.0°C. The LT50 of winter-acclimatized specimens was determined to be -16.5°C. This result may be explained by the presence of cryoprotectants and multiple strains of bacteria in the winter-acclimatized specimens. Gill cell viability tests resulted in an average of 12% greater damage in summer-acclimatized *Geukensia* without added bacteria at -13.5°C, but no significant differences at -10°C and -15°C. This study is, to our knowledge, the first time that a bacterium has been shown to increase the survival of a freeze tolerant animal.

1. Introduction

1.1 Bacterial Ice Nucleation: A Review

History of Ice Nucleation

Although ice formation was first studied in the 1800's, the concept of ice nucleating particles was not elucidated until the mid-20th century (Vali 1995). Research into ice nucleation was initially conducted primarily within the context of atmospheric phenomena through cloud chamber studies in which water vapor was frozen homogeneously. Curiously, scientists in this field ended up playing a substantial role in the discovery of biological ice nucleation. As the idea became better established it began to cross over into biological studies as early as 1954 (Lucas 1954). It is now a fundamental concept in cryobiology -- the study of the effects of cold temperatures on living organisms.

The process of ice nucleation may have been a well established concept, but it was not until the 20th century that scientists realized that certain particles could actually mimic ice crystals and initiate the nucleation of ice at high subzero temperatures. Efficient ice-nucleating particles capable of limiting the supercooling of water began to be identified in the 1950's. Inorganic iodine compounds such as AgI, minerals like CuS and later multiple organic compounds were found to cause ice to form at relatively high temperatures despite low concentration of nuclei (Vali 1995). Still, it was not until the 1970's that biological ice nucleation, or ice nucleation as a result of compounds produced by a living organism, was found to exist (Upper and Vali 1995). Though many organic ice nucleators had previously been identified, biological ice nucleation remained elusive and largely unsuspected (Upper and Vali 1995). Two teams of researchers simultaneously discovered them when they found that water turbid from decay nucleated at significantly higher temperatures than it did when fresh. One

team was looking for organic ice nucleators responsible for initiating atmospheric ice while the second was attempting to understand the biological source of frost damage to corn. Both found their answer in the then unlikely source of a microorganism. *Pseudomonas syringae* was identified as the responsible bacteria, becoming the first known ice-nucleation active (INA) bacteria (Lindow et al. 1975; Maki et al. 1974). Since then, more species of diverse genera have been shown to be ice nucleation active.

P. syringae has become something of a model organism in the study of INA bacteria. It has high INA, causing ice nucleation as high as -2°C. The genes responsible for its activity have been isolated and extensively analyzed, providing much of the current knowledge of the structure and function of ice nucleating proteins. These genes were the first to be transferred to *E. coli,* causing them to express ice nucleation activity (Ice^+) and helping to elucidate various facts about the mechanism of ice nucleation. In addition, *P. syringae* has been genetically modified to be used, along with *P. fluorescens,* as a method of controlling frost damage to plants by Ice^+ strains of the bacteria.

The very discovery of INA bacteria spanned an unusual set of scientific fields -- a characteristic of research into the subject that continues to be true to this day. Due to the ubiquity of both water and microbes on the earth, biological ice nucleation has been shown to be important to many different systems and there are surely others that have yet to be discovered. Ice nucleation is still not completely understood and the extent to which ice nucleating bacteria affect the biological and physical world is only just beginning to be uncovered. INA bacteria have been found in plants, animals and distributed in the environment. Additionally, humans have found various novel uses for them including preventing frost damage to plants, making artificial snow and frozen food preservation (Gurian-Sheman and Lindow 1993).

This review will discuss the mechanism by which bacterial ice nucleation occurs, the role of INA bacteria in biological systems at an ecological and physiological scale, ice-nucleating protein and gene properties and current applications of INA bacteria.

Mechanism of ice nucleation, ice-nucleating proteins and physiology of INA bacteria

The overall mechanism by which INA bacteria act upon water to nucleate ice is well understood, though many of the details remain speculation. Much research was directed towards understanding bacterial ice nucleation in the 80's and mid 90's but since then fewer advances have been made, as evidenced by the fact that recent articles about the subject primarily cite research published before 1995.

To understand bacterial ice nucleation, a basic understanding of supercooling and nucleation in water is required-- although the freezing point of water is 0°C, pure water can supercool to -40°C before homogeneous nucleation randomly occurs and ice forms. Below 0°C, water molecules have very little movement but do not immediately crystallize due to the energy required for ice to form. Homogeneous nucleation in pure water occurs when it becomes energetically favorable for the liquid to be in a crystallized form. However, unless water is meticulously purified, it invariably contains at least some tiny particles that act as sites of nucleation, orienting water molecules so that they form ice crystals in the process of heterogeneous ice nucleation. Though particles can cause ice nucleation, it is worth noting that it still does not occur until a critical embryo size has been reached. Ice molecules may become oriented in crystalline form but spontaneous ice nucleation does not result until they have reached a certain size. For homogeneous nucleation at -5°C, 45,000 water molecules are required in an embryo though this number can drop to 600 or lower when ice nucleators are

present. Generally, minimally impure water will freeze spontaneously before -15°C. This freezing point can be elevated by molecules that bear similarities in structure to crystalline water, such as the aforementioned inorganic and organic ice nucleators.

The mechanism by which INA bacteria cause ice nucleation in water is the production of ice-nucleating proteins (INPs). These proteins are thought to mimic the crystalline structure of water in a similar fashion to non-biological ice nucleators. The many hydrogen bonds in these proteins provide an effective template for ice in the context of the tertiary structure of the protein. So far, six different genes that transcribe INPs have been sequenced from six different bacterial strains. The genes that have been isolated thus far are known as *inaW* from *Pseudomonas fluorescens* (Warren et al. 1986), *inaZ* from *Pseudomonas syringae* (Green and Warren 1985), *inaA* and *inaU* from *Erwinia* (*Pantoea*) *ananatis* (Abe et al. 1989, Michigami et al. 1994), *iceE* from *Erwinia herbicola (Pantoea agglomerans)* (Warren and Corotto 1989), and *inaX* from *Xanthomonas xampestris* (Zhao and Orser 1990). These genes have been extensively studied and various models have been proposed for the tertiary structure of their proteins. They are all highly conserved between species and genera, leading to the hypothesis that the Ina^+ gene only evolved once and has since been horizontally transmitted among diverse species and genera (Edwards et al. 1994).

Each isolated INP has been shown to be composed of three domains. A hydrophobic N-domain containing phosphatidylinositol serves to anchor the INP to cell membranes and does not appear to be important to ice nucleation (Warren 1995, Kozloff 1991). It is followed by a much longer R-domain (on the order of 1000 base pairs) that makes up more than two thirds of the protein and is composed almost entirely of a repeated sequence of eight amino acids. Based on the fact that length of the R-domain seems to be correlated with higher ice-nucleation activity

and that it is largely hydrophilic, it is assumed that ice nucleation occurs on this section of the protein (Kawahara 2008). Last, a short, hydrophilic C-terminal has been shown to be critical to ice nucleation by means of substituting amino acids (Michigami et al. 1995).

Though the tertiary structure of INPs has been difficult to discern due to their very large size, some known characteristics of INPs have allowed us to draw conclusions about their structure and arrangement on bacteria. All previously isolated INPs are undoubtedly located within bacterial outer membranes and reliant upon them for their structure. It is well documented that when their membranes have been compromised, INA bacteria almost completely lose their nucleation ability (Kajava 1995, Maki et al. 1974). Thus, it has been concluded that the tertiary structure of INPs is reliant upon a membrane anchor. Almost all INA bacteria in the literature thus far are gram-negative, as would be expected due to their possession of an outer membrane. One exception is a gram-positive *Exiguobacterium* with moderate INA isolated from Siberian permafrost (Ponder et al. 2005). No mechanism has yet been proposed to explain this ability, though the existence of such a bacteria poses significant questions with regards to the structure and evolution of INPs.

As stated earlier, the middle domain of INP has been proposed as the site of ice nucleation and is also the site of most interest to tertiary structure prediction due to its size. It is accepted that this repetitive region consists largely of β-strands (Kajava 1995). Based on this prediction, various models have been proposed for the overall structure of the protein. Although there is currently no foolproof method for the prediction of protein tertiary structure, most of the models proposed bear similarities and are in agreement as to the basic structure of the molecules. It can be deduced that these proteins possess a regular structure, or one that is composed of repeated structures, based on the repetitive nature of the primary structure. As previously

mentioned, the central domain is composed of 8-residue repetitive sections. These can be extended to both a 16-residue or 48-residue consensus sequence. Current proposed models have suggested helical structures with some variation of this number of residues (Kajava and Lindow 1993, Warren and Corotto 1986, Mizuno 1989, Graether and Jia 2001). Recently, NMR measurements were made which revealed that the 16-residue model is the most accurate, with each repeating sequence forming a circular loop (Kumaki et al. 2008). The implications of these data for INP structure has yet to be revealed.

Perhaps equally important in determining the structure of INPs is the knowledge of their function. Because INPs cause ice nucleation, their structure must be such as to cause water molecules to move into an orientation favorable for the formation of an ice nucleus. This orientation of water is caused by chemical binding, probably though hydrogen bonds in amino acids in the central domain. Other proteins known as anti-freeze proteins (AFPs) also bind to water in a similar fashion, although they inhibit the formation of ice crystals rather than promote them. Recently, it has been proposed that INPs are essentially long, repeated AFPs composed of β-helical folds that interact with water through a TXT amino acid motif (Graether and Jia 2001). Even more recently, a 96-residue polypeptide generated from *Pseudomonas syringae* INP sequence has been shown to possess ice-binding ability, further lending support to a model involving the hexagonal bi-pyramidal form of AFPs (Kobashigawa et al. 2005). As a much larger protein, INPs probably cause ice crystals to reach critical nucleus size quickly by binding many together at once rather than binding to water molecules and preventing them from binding to one another as AFPs do. Earlier research has demonstrated that aggregation highly increases the activity of INPs (Burke and Lindow 1990, Kajava and Lindow 1993), further lending support to the idea that large, repeated groups of AFP-like proteins could cause ice nucleation.

Though the structure of INP is well understood, its absolute structure is still unknown. We know that it is in a shape that allows it to bind water molecules, and this is probably accomplished through hydrogen bonds in the highly conserved center of the molecule. Though only a few proteins have been isolated up to this point, the discovery of new species of bacteria can be expected to yield new proteins that will provide further insights into the structure of this unique protein.

Ecology of INA bacteria

While the ability of INA bacteria to raise the temperature of ice nucleation is an interesting novelty in and of itself, their ecological role in the environment provides just as many questions as their physiological characteristics. While the presence of an ice^{+} phenotype is somewhat puzzling as an evolutionary advantage, there are several habitats to which INA bacteria are well adapted. By modifying their habitat, they are able to accomplish several survival strategies.

Most INA bacteria have been found first on plants, and their interactions with those plants are well studied. To date, at least ten different species isolated from the leaves and other aspects of plant phyllosphere are known to contain strains of INA bacteria. These species include *Pseudomonas syringae* (Maki et al. 1974), *P. pluorescens* (Kaneda 1986), *P. viridiflava* (Obata et al. 1989), *P. chlororaphis* (Schnell et al. 1991), *Erwinia herbicola* (Synonym: *Pantoea agglomerans*) (Lindow et al. 1978), *E. ananas* (Goto et al. 1989), *E. uredevora* (Synonym: *Pa. ananatis*) (Obata et al. 1990), *P. aeruginosa* (Hazra et al. 2004) and *Xanthomonas campestris* (Kim et al. 1987). Of these, *P. syringae* is probably the most widely distributed on plants and also most active (Lindow 1983).

Most of the aforementioned bacteria are pathogenic to the plants from which they were isolated. Pathogenicity has so far been identified as the most common reason bacteria are able to nucleate ice. Frost injury, for example, is a common method by which INA bacteria modify the plants they inhabit. By elevating the temperature at which frost forms on leaves, bacteria can cause unseasonal damage to plants, causing them to release nutrients that allow the bacteria to multiply (Hirano and Upper 1995). Experiments using ice^+ and ice^- *P. syringae* on oat leaves have shown that frost damage increases and more bacterial cells are present after incubation at -5°C with ice^+ bacteria (Buttner and Amy 1989). INA alone is therefore an advantage for bacteria living on plant leaves. INA bacteria are in fact responsible for the frost sensitivity of most plants (Lindow 1983). The damage that INA bacteria inflict to the leaves they inhabit is probably also a benefit to their pathogenicity. Several studies have showed that pathogenic INA bacteria display increased levels of infection following frost (Panagopoulos and Crosse 1964, Klement et al. 1984, Vigouroux 1989).

Because not all INA bacteria are plant pathogens and some are not associated with plants at all, there must be other advantages to the Ice^+ phenotype aside from plant pathogenicity. It has been suggested that this adaptation could actually be a benefit to the bacteria's host, allowing water to freeze at relatively warm temperatures and providing freezing tolerance (Upper and Vali 1995). This suggestion is possible though somewhat far-fetched, as INA bacteria have yet to be shown as a mechanism for freeze tolerance in any organism in published literature. Though the following study suggests this very correlation, it is more likely that the INA bacteria present in the palial fluid of *Geukensia demissa* occur incidentally due to their presence in seawater.

The presence of INA bacteria in seawater is a relatively unexplored concept. Arctic sea ice has become a point of interest for researchers of INA bacteria, and at least one INA bacteria

has been successfully isolated from Antarctic sea ice (Obata et al. 1999). Contrarily, Junge et al. (2008) recently tested at least 15 strains of bacteria from Arctic sea ice for INA and found no reasonable activity in any of them. This study used an atmospheric chamber to measure nucleation temperatures, as the researchers were primarily concerned with the effect of sea ice bacterium on weather phenomena. Substrate may have an impact on the INA of bacteria. INA bacteria that are active in the atmosphere may be unculturable by conventional means, though it is increasingly estimated that they play an important part in the formation of snow in the atmosphere. The role of INA bacteria in weather phenomena is not well understood, but it has been shown that biological ice nucleators are active in at least 69% of snow and can be carried long distances in the atmosphere without the need for substrates (Christner et al. 2008).

It is possible that INA bacteria use their ice nucleating ability to protect themselves in their cold habitats.

Other unique habitats in which INA bacteria have been found are the gut of the frog *Rana sylvatica* (Lee et al. 1995), the land snail *Helix pomatia* (Nicolai et al. 2005), mackerel (Chen, Chiou and Jiang 2002), the gills of the bivalve *Geukensia demissa* (Loomis and Zinser 2001) and Siberian permafrost (Ponder et al. 2005). As researchers continue to look for INA bacteria in seemingly unlikely places, more unique relationships are uncovered. One interesting example is a possible connection between INA bacteria in the guts of the prey of the house spider *Achaeranea tepidariorum*, which may also increase the spider's supercooling point (Tanaka and Watanabe 2003). INA bacteria have also been successfully isolated from the guts of the beetles *Ceratoma trifurcate* and *Hippodamia convergens* (Lee et al. 1991) and the sub-Antarctic beetles *Hydromedion sparsutum* and *Perimylops antarcticus* (Worland and Block 1999).

Applications of INA bacteria

INA bacteria have a number of current and proposed applications, including natural pesticide, frost prevention, food uses and artificial snow. One application that has been successfully instituted is the creation of an Ice^- mutant of *P. syringae*, known as Frostban, designed to be applied to crops and outcompete the indigenous Ice^+ strains, thereby preventing them from causing frost damage (Skirvin et al. 2000). This application has met resistance since its inception due to the view that adding a genetically modified variant of the bacteria to large amounts of crops could drastically alter the ecosystem. The idea has been successfully marketed and used with no ill effects in the form of Blightban, an Ice^- variant of *P. fluorescens*.

Ski resorts have been using artificial snow seeded with INA bacteria since 1987. The increased nucleation temperature, higher than with other non-biological nucleators, allows the snow to be cooled to much lower temperatures and to be sustained more easily (Skirvin 2000).

Food processing has also benefited from the INA bacteria *Xanthomonas campestris* because it is not potentially pathogenic to humans like some other INA genera. Additionally, because they are not genetically engineered they are not covered by many laws regarding bacteria and can be used without as much federal regulation (Skirvin 2000). One of their primary uses is to improve the texture of frozen foods. Freezing at higher temperatures allows a more gradual crystallization process and therefore less damage is caused. This application and the others previously mentioned are currently the only ones in use, but the future may hold other interesting uses for INA bacteria. One example is weather control. As the influence of INA bacteria on atmospheric conditions is explored and elucidated, new applications will surely be discovered.

1.2 Freezing tolerance in animals

Animals living in environments that reach sub-zero temperatures have evolved many different adaptations to survive freezing. Generally, these adaptations either allow the animal to avoid freezing entirely or to tolerate freezing of its extracellular fluids. Some common avoidance mechanisms include migration, as seen in some bird species, thick coats of fur in many larger mammals, and cryoptrotectant antifreeze proteins in some insects, fish and marine invertebrates. These animals are classified as freeze-intolerant because although the fluids of some can supercool, ice nucleation within the organism is lethal. A less common mechanism of survival in freezing environments is freeze tolerance. Species that have evolved mechanisms of freeze tolerance include the land snail *Helix pomatia*, the wood frog *Rana sylvatica*, larvae of the gall fly *Eurosta solidaginis*, hatchlings of some turtles, and the organism studied in this research, the bivalve mollusc *Geukensia demissa* (Loomis 1995, Nicolai 2005, Storey 1984, Yi 2003).

Many freeze tolerant animals are capable of surviving the crystallization of more than 50% of their extracellular body water. Crystallization within cells is invariably damaging as the ice crystals expand within the cell, rupturing plasma membranes. No animals yet discovered can recover from complete freezing, although larvae of the midge *Polypedilum vanderplanki* have been shown to display the ability to survive complete desiccation (Watanabe 2002). Extracellular crystallization, while also potentially lethal, is manageable through a number of strategies which allow cellular metabolism to continue and prevent damage to cell membranes. In freeze tolerant invertebrates, nucleation usually occurs in the hemolymph. Adaptations in these organisms prevent cell death caused by dehydration, ischemia, mechanical distortion and lethal ion concentrations in the cell (Loomis 1995, Murphy and Pierce 1975).

Freeze tolerant organisms are adapted to control the point at which ice nucleation occurs. They are capable of supercooling intracellularly, but unlike freeze intolerant organisms they contain mechanisms that raise the temperature of extracellular ice-nucleation instead of lowering it. While such a mechanism seems intuitively counterproductive, freezing at a higher subzero temperature allows the organism to ensure that ice crystallization occurs extracellularly first and to acclimate to the gradual formation of crystals as opposed to sudden, complete crystallization that occurs at lower temperatures below -10°C. Most freeze tolerant invertebrates produce ice-nucleating proteins in their hemolymph. These proteins contain molecular structures that bind to water molecules and induce the formation of a crystalline lattice.

Ice-nucleating activity helps to prevent the dehydration of cells. As pure water forms into ice in extracellular compartments, it increases the concentration of solutes in the unfrozen portion of the fluids, forming an osmotic potential across cell membranes. Water diffuses out of the cells, eventually leading to dehydration and cessation of metabolic activity, probably due to a combination of lethal ion concentration, mechanical disruption of the cell membrane, and drastic shrinking of the cell (Loomis 1995, Murphy and Pierce 1975). Nucleation at higher temperatures allows time for osmotic potentials to be regulated and ensures that ice forms outside of cells. As a result of the increased solute concentration in the cytoplasm, the cell's freezing point is depressed and intracellular ice nucleation becomes less likely. Acclimation to higher salinities has been shown to significantly increase the freezing tolerance of *Geukensia*, lowering the temperature at which lethality occurs (Murphy and Pierce 1975).

Additionally, cryoprotectant molecules such as sorbitol, glycerol, trehalose and certain amino acids accumulate in the cells prior to freezing (Loomis 1995). These molecules further depress the freezing point of the cytoplasm, allowing intracellular fluids to supercool while

extracellular fluids are frozen.The increased presence of Ca^{2+} during freezing may also contribute to survival of the cell by binding to the plasma membrane and stabilizing it (Murphy 1977).

In *Geukensia demissa,* ice formation occurs in the pallial fluid that fills the mantle cavity and it is initiated by INA bacteria present in seawater (Loomis and Zinser 2001). *Geukensia* are sessile intertidal bivalve molluscs and are found embedded in the peat of the upper intertidal ranges of salt marshes. They are regularly exposed to sub-zero temperatures for extended periods of time during low tides in winter months. The freezing tolerance of *Geukensia* has been extensively studied but until recently their ice-nucleation mechanism was unknown. They have been shown to contain at least one strain of ice-nucleating bacteria, *Pseudomonas fulva,* upon which their ice-nucleation ability is dependent (Loomis and Zinser 2001). The pallial fluid of *Geukensia* does not supercool to temperatures lower than -1º C before nucleation occurs and the organism can survive temperatures as low as -13.76º C (Murphy and Pierce 1975, Loomis and Zinser 2001).

Geukensia are currently unique in that no similar freeze tolerance mechanism relying on INA bacteria has been firmly described in another organism. INA bacteria are associated with some species of insect, but these species increase cold hardiness by evacuating bacteria from their gut prior to freezing rather than relying on them for nucleation (Lee and Costanzo 1998). Several species of INA bacteria have also been isolated from the gut of the freeze tolerant frog *Rana sylvatica* and a similar mechanism has been proposed, but no further research has been published (Lee 1995).

It is highly likely that more than one species of bacteria is responsible for the freeze tolerance present in *Geukensia*. In this study, several strains of bacteria isolated from pallial

fluid are assessed for their INA. Their impact on the freeze tolerance of *Geukensia* is compared to the natural freeze tolerance of the organism when acclimatized to cold weather and when acclimatized to warm weather and without bacterial ice nucleators.

2.Methods

2.1 Isolation of INA bacteria

Three strains of bacteria with potential ice-nucleation activity were isolated from the pallial fluid of *Geukensia* (by Dalin Chan, unpublished data).

2.2 Determination of bacterial growth equations

Serial dilutions were performed on each unknown bacterial strain in LB broth to a factor of six. Initial absorbace (A620) was recorded. Nutrient agar plates prepared with 28 ppt artificial seawater were inoculated and grown with bacteria at each dilution. Resulting colonies were counted and the relationship between optical density and cell density was determined.

2.3 Antibiotic effectiveness

In anticipation of the possible use of antibiotics to remove microbes from control *Geukensia*, bacterial lawns were prepared using a swab and streaking technique. Paper discs were soaked in Penicillin G (Benzyl Penicillin) and Steptomycin sulfate at concentrations of 25000, 50000 and 100000 units/mL. The discs were placed onto the lawns and size of the resulting halo was recorded.

2.4 Determination of ice-nucleation activity

Ice-nucleation activity of each bacterial strain was determined by placing 20 droplets of broth culture onto the surface of an aluminum plate connected to a Peltier device, which enabled the temperature of the plate to be reduced at approximately 1°C/min. The nucleation point of each droplet was recorded. These droplet assays were performed on each bacteria culture at varying cell densities and on sterile LB broth.

To determine the relationship of temperature to bacterial ice-nucleator production, triplicates of *Psychrobacter* sp. and *Shewanella* sp. bacteria cultures were incubated at 5, 10, 15,

20 and 25°C. All cultures were diluted to a concentration of 10^8 bacterial cells/mL before droplet assays were conducted.

2.5 Characterization of bacteria and optimal growth temperature

Bacteria were gram-stained and their morphology was observed. Cultures were grown aerobically in broth medium over a period of 48 hours, after which their absorbance (620 nm) was measured to determine cell concentration, using previously determined equations. Cultures were incubated at 5, 15, 25 and 40°C.

2.6 Amplification and sequencing of bacterial DNA

Bacterial cells were lysed using freeze-heatshock cycles and 16S rRNA was amplified with the forward primer 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse 1492r (5'-GGTTA-CCTTGTTACGACTT-3') primers using PCR. Amplified DNA was sent to Harvard DF/HCC DNA Resource Center (http://dnaseq.med.harvard.edu/) for sequencing. Phylogeny for *Shewanella* sp. and *Psychrobacter* sp. was reconstructed using PHYLIP and bootstrap values were determined for each branch. Sequences selected in BLAST (ncbi.nlm.nih.com) for their similarities or differences to our sequences were aligned with ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

To reconstruct phylogenies with PHYLIP, distance matrices were first calculated using DNADIST. Next, lineages were constructed with NEIGHBOR using archaea EU284666.1 as an outgroup.

Bootstrap values were calculated using the SEQBOOT program in PHYLIP, assuming 100 data sets. NEIGHBOT was run once again, using archaea EU284666.1 as the outgroup and using 100 data sets. Last, CONSENSE was used to assign bootstrap values to the tree. The consensus type was chosen to be majority rule.

2.7 Attempted isolation of Ice^{+} genes

DNA was extracted from the *Shewanella* and *Psychrobacter* cultures using Powersoil DNA kit (MO BIO Laboratories, Inc. 2746 Loker Ave. West, Carlsbad, CA). PCR was run with primers previously designed from *inaA* and *inaE* genes, isolated from *Erwinia ananas* and *P. fluorescens*, respectively (Watanabe and Sato 1998). These sequences were IceE1-R (5´-GGT-TTAGACATGAAAGAAGACAAGG-3´), IceE2-L (5´-TTATTCTTCGGGTTTATTCACGA-TA-3´), IceE1-1R (5´-GATTTCAATCGTTTGTGTCGGCTGC-3´), IceE1-IL (5´-GCAGGCT-ACGGCAGTTCGCTGACCA-3´) and IceE1-L (5´-CCTCTGTTATGGCGATTATTCTTCG-3´) (Watanabe and Sato 1998). These primers were used according to previously successful PCR conditions (Watanabe and Sato 1998).

In addition, two primers designed based on *inaZ* cloned from *Pseudomonas putida* were used with *Psychrobacter* and *Shewanella* DNA. These primers were 5´-ATCCAGTCATCGTC-CTCGTC-3´ and 5´-CAAGTGTCACGTTACCGGTG-3´ (Keikhasaber et al. 2007). PCR was run with 57°C annealing temperature, according to previous methods.

2.8 *Geukensia* collection

Geukensia specimens were collected from an estuarine salt marsh in Beebe Cove, Noank, CT during summer and winter seasons from 2007-2009. Seawater was collected from a nearby dock and the *Geukensia* were maintained in a laboratory aquarium with water at room temperature in the summer and 5°C for winter acclimatized specimens.

2.9 Determination of ciliary response to freezing

Small portions of the gills of live summer-acclimatized *Geukensia* specimens were dissected. Their removal was accomplished through severance of the anterior and posterior adductor muscles. The base of the gill, from which the filaments “stem,” was severed so that a

small portion could be removed. The portion was then placed under a microscope. A strobe light was positioned next to the stage. The cilia of the gills were observed under the microscope and their beat frequency was determined by adjusting the strobe light until its frequency synchronized with the beat frequency of the cilia and they appeared to stop moving in accordance with the methods of Chilvers (2000).

To determine the effect of freezing on the cilia, whole organisms were kept at -20°C for 0, 24, 48 and 72 hours. Their cilia were dissected and the rate of ciliary beating determined.

2.10 Mean survival of whole organisms

The LT50 of summer-acclimatized and winter-acclimatized *Geukensia* was determined through a series of 12 hour periods during which the specimens were held at sub-zero temperatures to simulate low tide. Summer-acclimatized *Geukensia* received two different treatments before LT50 testing. Pallial fluid was first drained by drilling two holes in the shell of the *Geukensia*: one directly opposite from the anterior adductor mussel and one at the posterior apex of the shell. Holes were drilled directly where the valves met. The pallial fluid was drained from the mantle cavity as completely as possible and artificial seawater (28ppt, Instant Ocean) was pipetted in. This process was repeated twice for control specimens and once for the second group, into which *Psychrobacter* bacterium and seawater solution was subsequently pipetted.

The holes drilled into the shells were stopped with putty after the bacteria solution was added. The seawater was prepared in this fashion by centrifuging bacterial cultures (10^8 bacteria/mL) decanting the broth and dissolving the pellets in artificial seawater (28ppt). The resulting solution possessed high ice-nucleating activity. *Psychrobacter* sp. was chosen over *Shewanella* sp. due to its greater ice nucleation activity. Winter-acclimatized *Geukensia* were left untreated.

Ten *Geukensia* were placed into a jacketed glass beaker, into the sides of which ethylene glycol was pumped by a Neslab RTE-140 cooling device. Insulation materials and a fan ensured that the air temperature within the chamber was equal to the temperature of the ethylene glycol. The temperature was reduced at 1°C/min from 20°C, then held for 12 hours at that temperature. Thermocouple probes monitored the temperature of four specimens in each cycle. At the end of this period, *Geukensia* were transferred into the aquarium for an additional 12 hours. If their adductor muscles were capable of contraction after this full cycle, they were considered to have survived. The cycle was repeated with *Geukensia* of each treatment at varying temperatures.

2.11 Gill cell viability

Gill cell viability was assessed with the LIVE/DEAD sperm viability kit (Molecular Probes, Inc, Eugene, OR) using the methodology of Yi and Lee (2003) as a basis for our own methods. The sperm viability kit contained 1mM SYBR 14 dye in DMSO and 2.4 mM propridium iodide solution in H_2O. To assess cell viability, ten *Geukensia* were first incubated in the cooling chamber previously described for 12 hours at varying temperatures. Five were treated with bacteria and five were controls. After the 12-hour period, the specimens were removed, thawed and piece of their gills were dissected, using previously described methods, so as not to damage the filaments. The gill samples were placed individually onto microscope slides and 0.5μL of SYBR14 dye in 100μL filtered artificial seawater (28ppt) were added. The ten samples were incubated in this manner for 15 minutes, then 4μL of propidium iodide solution in 100μL of filtered artificial seawater (28ppt) were added. The samples were incubated for a further 15 minutes.

The samples were removed, covered with a cover slide marked with a grid of 36 equal quadrats and observed under an epifluorescent microscope (Olympus BH-2) (Yi and Lee 2003).

Live, undamaged cells fluoresced green while dead and damaged cells fluoresced red. The squares on the cover slide were assigned numbers based on their position and numbers were randomly selected. If gill was present in the section, the sample was photographed (Fig. 1). Triplicates of photographs were taken for each sample. Total green and red cells were counted in each photograph. A t-test (R) was used to determine differences between gills with and without ice-nucleating bacteria.

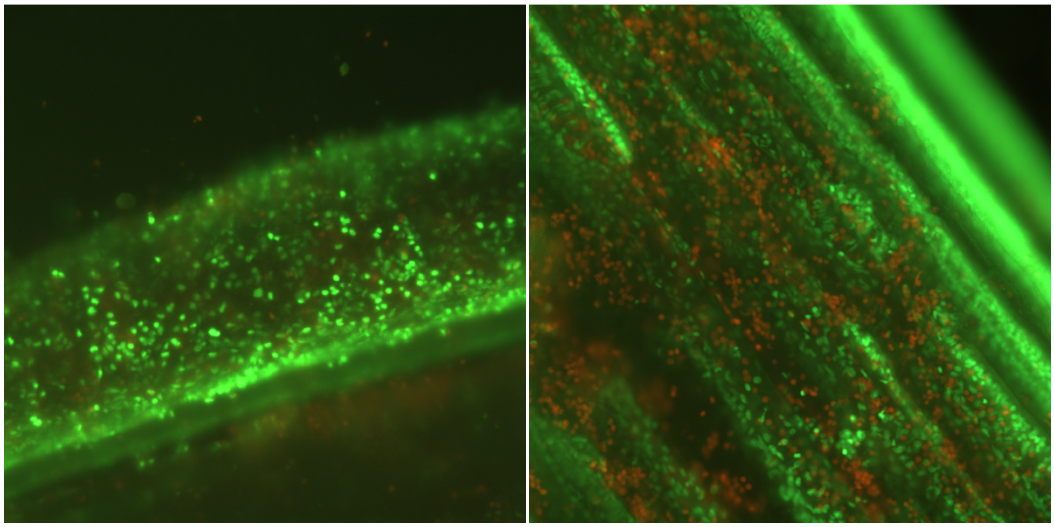

Figure 1. Photographs of gill filament samples after application of LIVE/DEAD sperm viability kit.

This procedure was repeated in February-April 2009 with winter-acclimatized *Geukensia* at -15, -16, -16.5 and -17°C.

3. Results

3.1 Bacterial growth equations

Psychrobacter sp. was present at higher cell densities at lower ABS than *Shewanella* sp., but was slightly lower in cell density than “INA A” (Figs. 2, 3, 4).

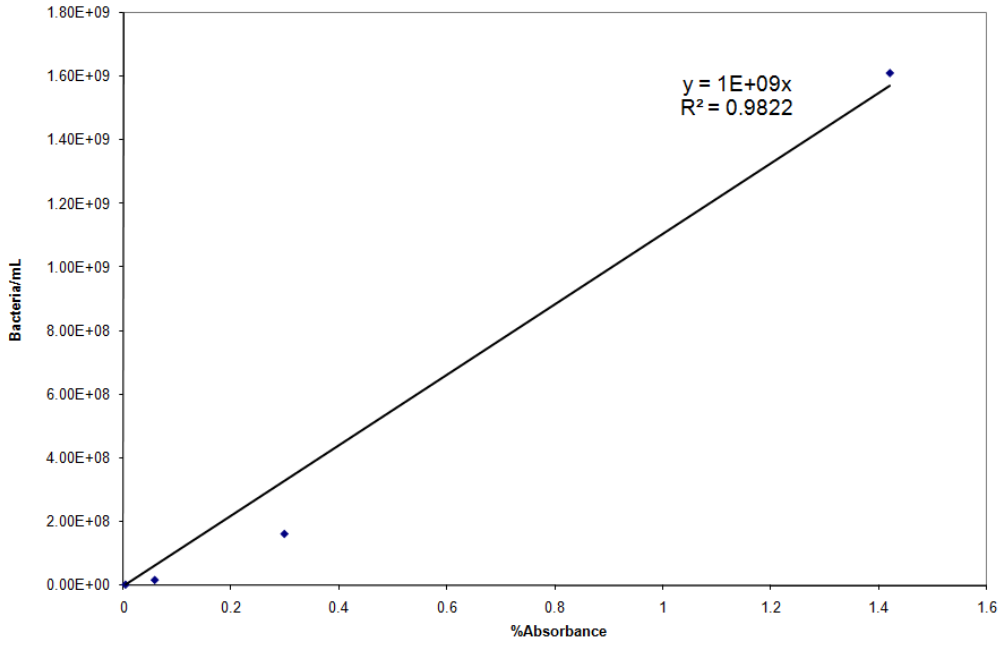

Figure 2. Cell density of *Psychrobacter* sp. bacteria in LB broth medium against ABS.

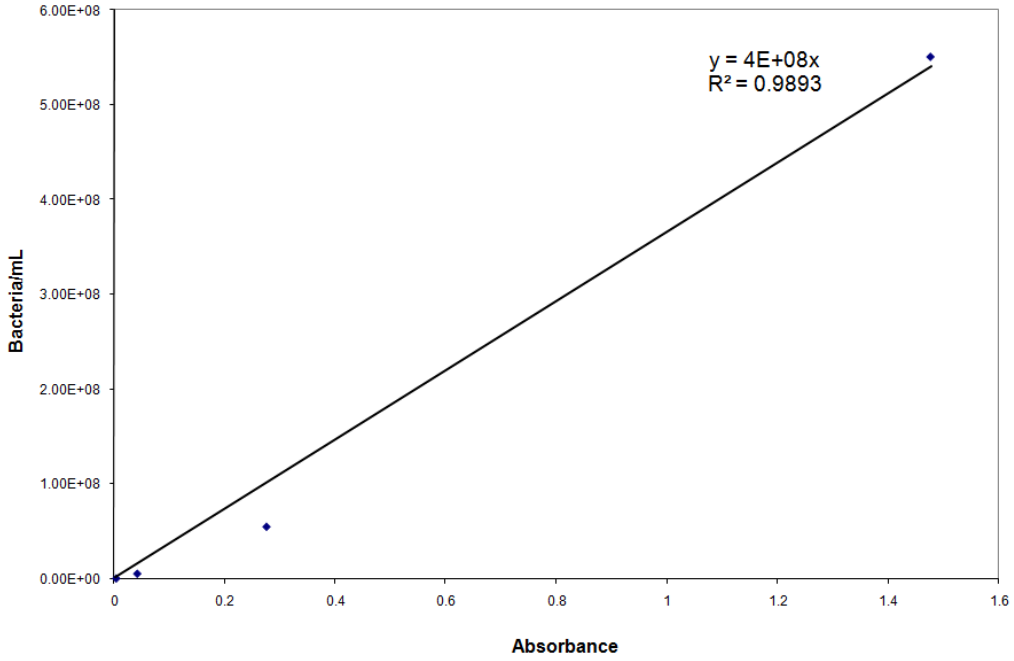

Figure 3. Cell density of *Shewanella frigidimarina* in LB broth medium against ABS.

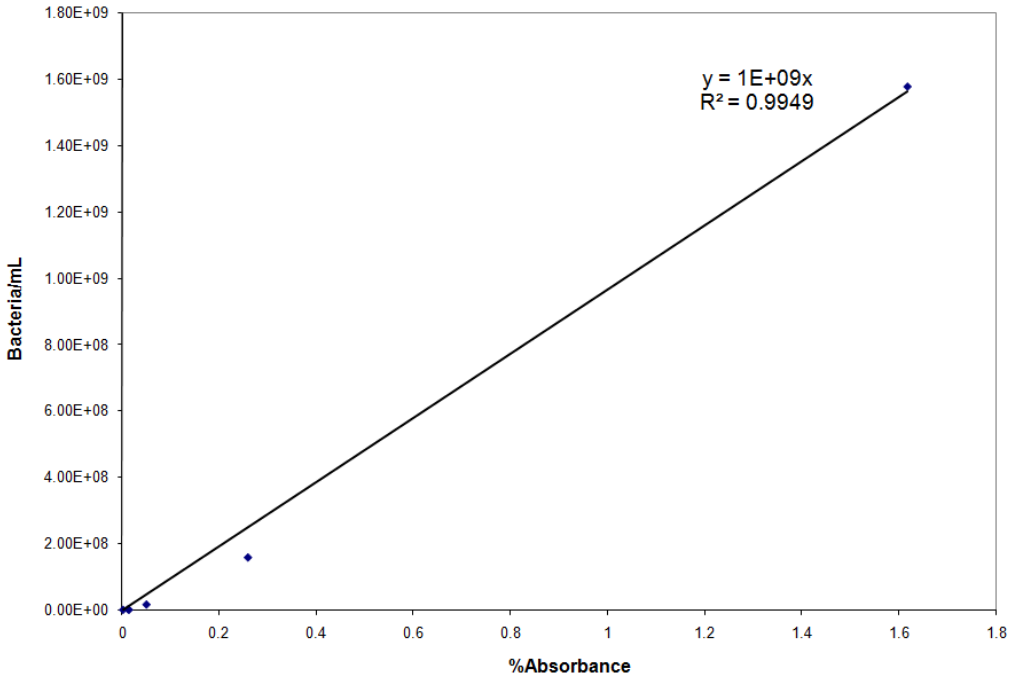

Figure 4. Cell density of unknown bacteria 'INA A' in LB broth medium against ABS.

3.2 Antibiotic effectiveness

The antibiotics were mostly equally effective on each bacterial strain (Fig. 5). Penicillin G was significantly more effective on "INA A" than on *Psychrobacter* sp.

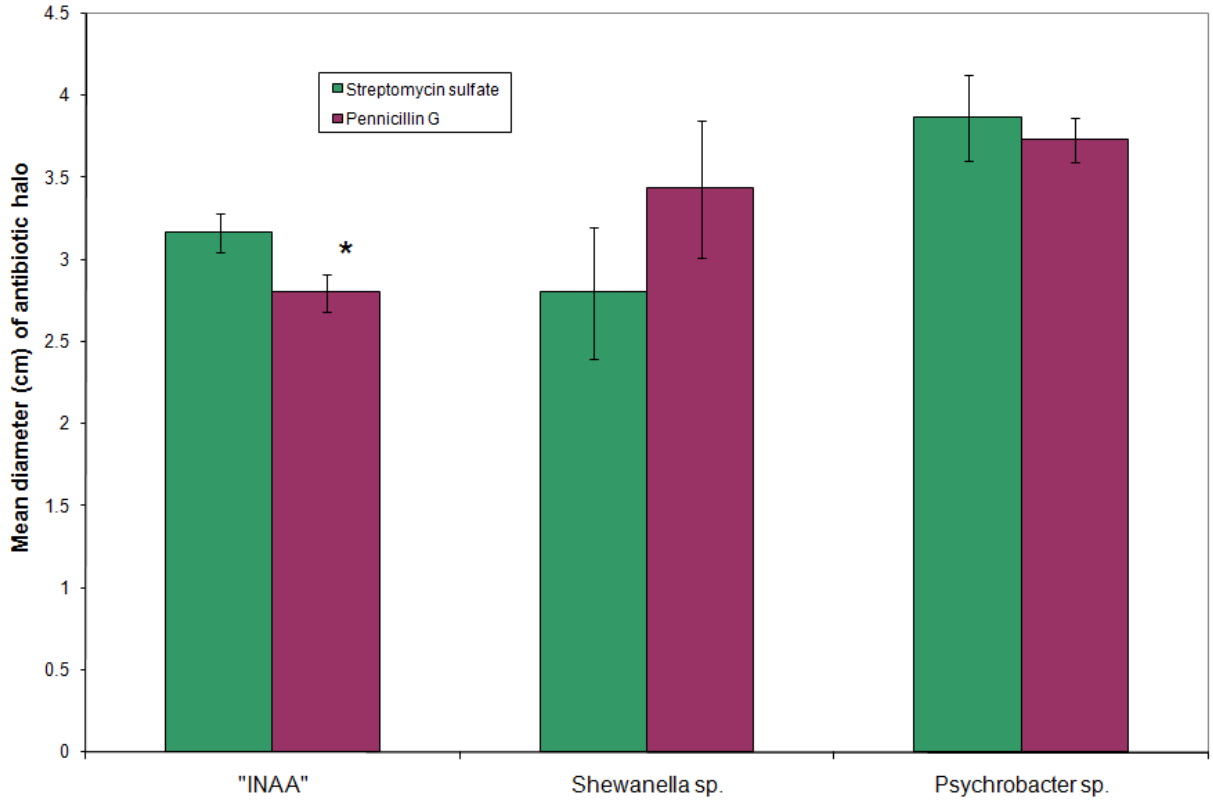

Figure 5. The mean diameter of antibiotic halos. An * indicates significant differences between antibiotics ($p<0.05$).

3.3 Ice-nucleation activity

Two of the isolated bacteria strains, *Psychrobacter* sp. and *Shewanella* sp., displayed INA consistently even at lower cell densities (Figs. 6, 7).

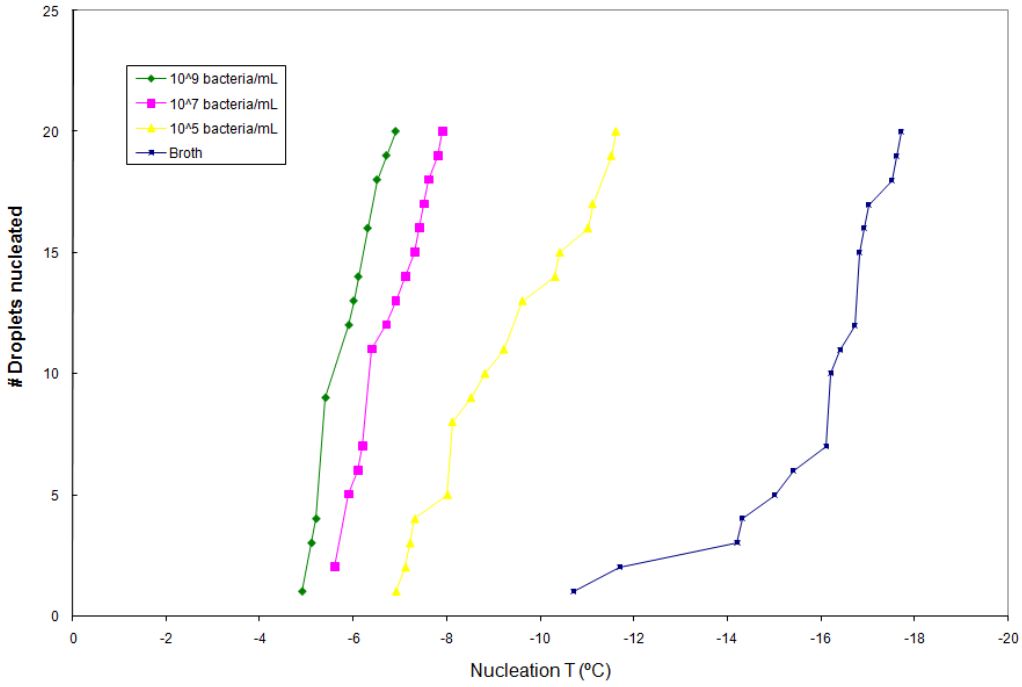

Figure 6. The ice-nucleation profile of *Psychrobacter* broth cultures at varying dilution factors.

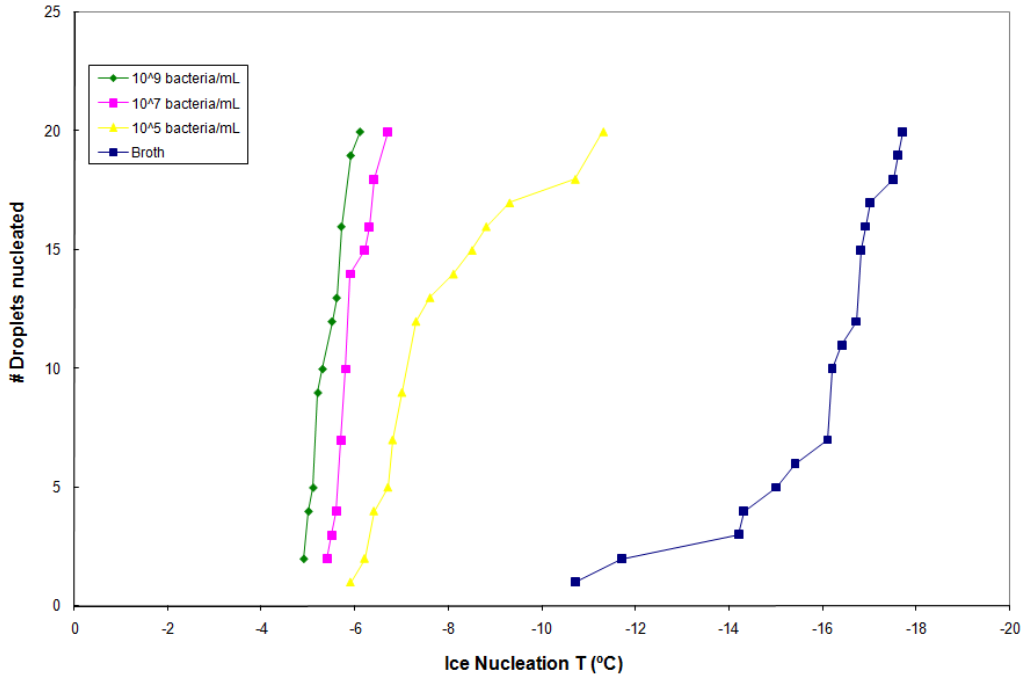

Figure 7. The ice-nucleation temperatures for 20 droplets of *Shewanella frigidimarina* broth cultures at varying dilution factors.

Unidentified bacterial strain “INA A” did not display any significant INA (Fig. 8).

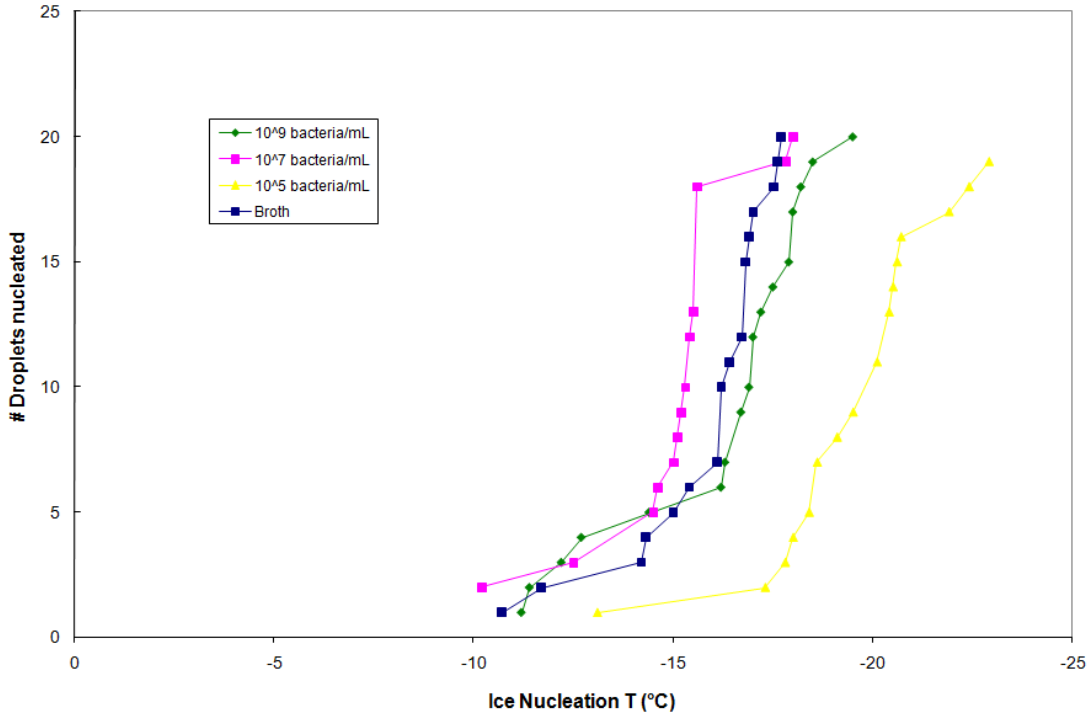

Figure 8. The temperature at which 20 droplets of bacterial broth cultures (INA A) of varying dilution factors nucleated on a cold plate reduced in temperature at 1°C/min.

INA was similar for *Psychrobacter* sp. and *Shewanella* at all dilutions. On average, undiluted (10^9 bacteria/mL) *Psychrobacter* sp. cultures nucleated at -5.91ºC and *Shewanella* nucleated at -5.43ºC. *Psychrobacter* sp. was used in subsequent LT50 and cell viability experiments due to its higher concentration and faster growth rate.

3.4 Characterization of bacteria and optimal growth

Both *Shewanella* sp. and *Psychrobacter* sp. were found to be gram-negative rods. *Psychrobacter* display motility. Both species are aerobic, growing more quickly when shaken.

Both bacteria were moderately psycrophilic, as they displayed maximal growth between 15 and 25°C (Figs. 9 and 10). The bacteria did not grow at 40°C.

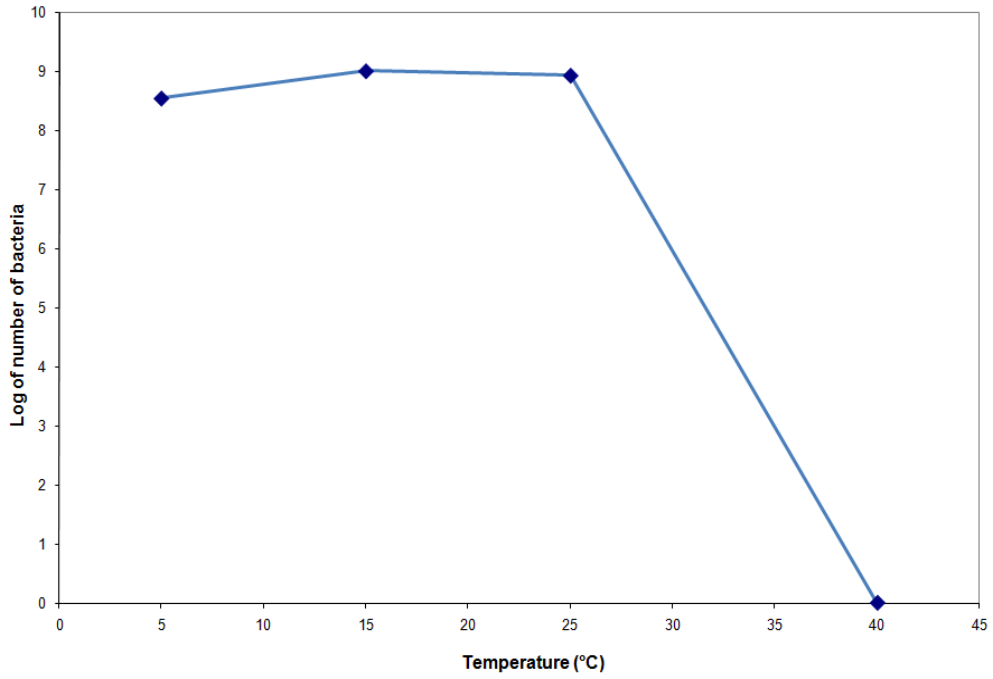

Figure 9. Growth (log of number of bacteria) of *Phychrobacter* sp. at temperatures ranging from 5 to 40°C after 48 hours of incubation.

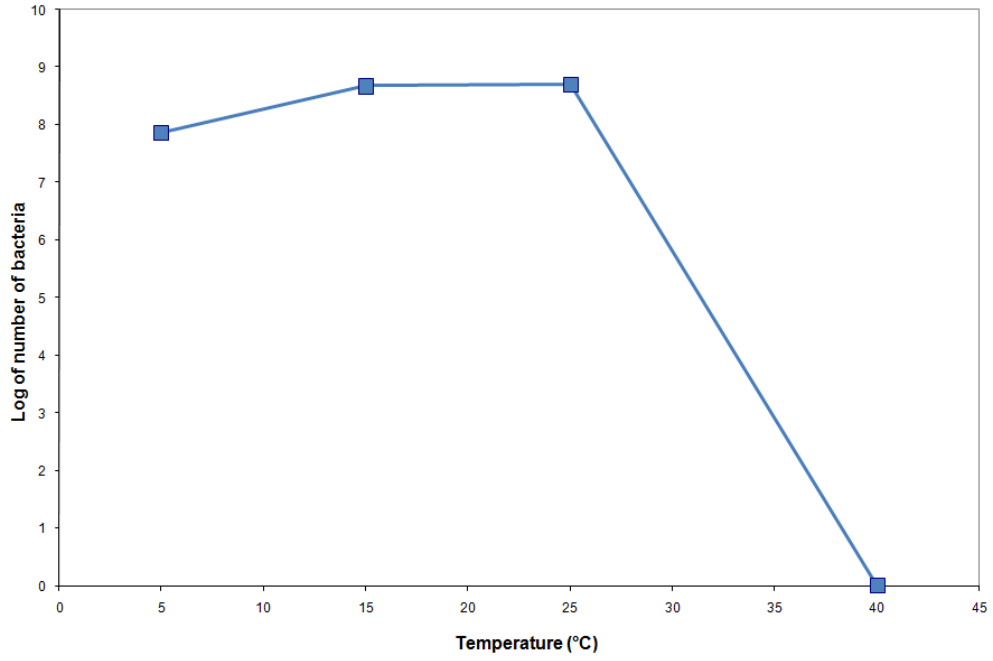

Figure 10. Growth (log of number of cells) of *Shewanella* sp. at temperatures ranging from 5 to 40°C.

3.5 DNA sequences

A phylogenetic tree shows that *Psychrobacter* sp. is most closely related to *Psychrobacter glacincola* and an unidentified species. When sequenced with 1492r primers, the species is much less similar to other strains (Fig. 11).

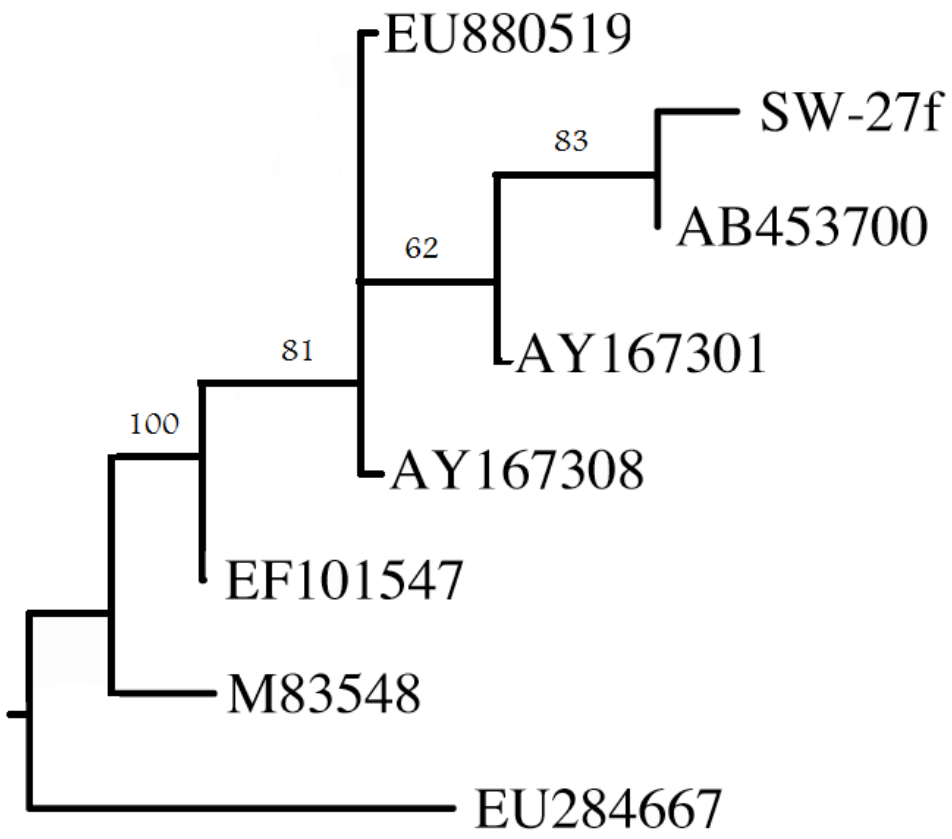

Figure 11. Phylogeny reconstructed from unidentified *Psychrobacter* sp. partial sequence in this study, various other species of *Psychrobacter*, and other distantly related species for outgroups. **Key:** SW -27f = unidentified specimen 27f primer sequence; AY1673011 = *Psychrobacter glacincola* strain ANT9253 16S ribosomal RNA gene, partial sequence; AB4537001 = *Psychrobacter* sp. T-3-2 gene for 16S rRNA, partial sequence; EF1015471 = *Psychrobacter aquimaris* strain KOPRI24929 16S ribosomal RNA gene, partial sequence; EU8805191 = *Psychrobacter nivimaris* strain P24a 16S ribosomal RNA gene, partial sequence; AY1673011 = *Psychrobacter glacincola* strain ANT9221 16S ribosomal RNA gene, partial sequence; M835482 AQ = *Aquifex pyrophilus* 16S ribosomal RNA gene, partial sequence; EU2846671 = Uncultured archaeon clone IP1SAT-H9 16S ribosomal RNA gene, partial sequence. Bootstraps values are provided for each branch.

Shewanella sp. also showed high similarity to other strains in that genus when a phylogeny was reconstructed, but like many other distinct species in its genus does not appear to be differentiable based on 16S rRNA (Fig. 12). As with *Psychrobacter*, the 1492r primers produced radically different results.

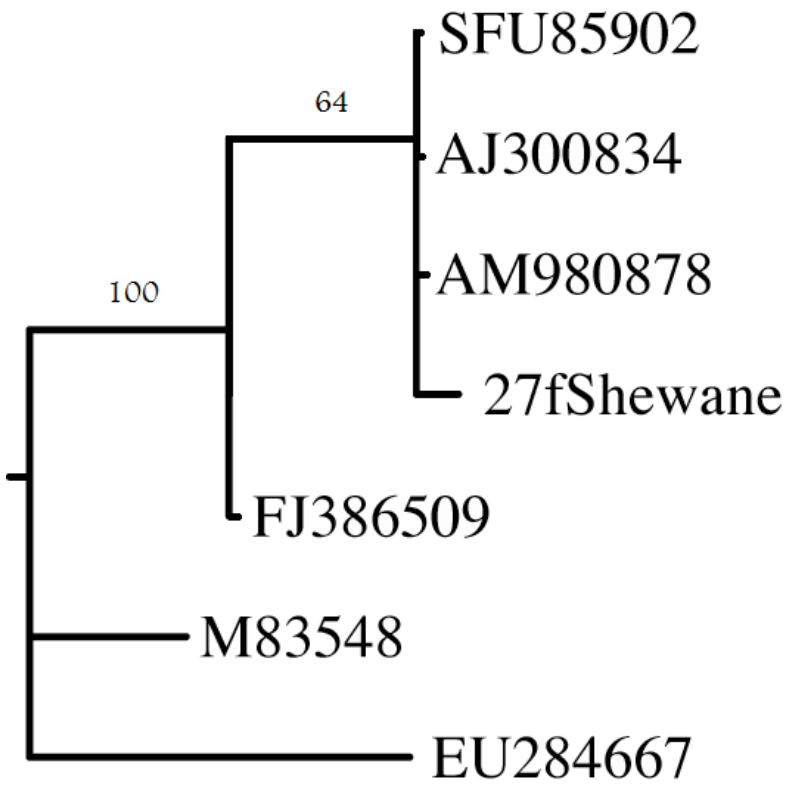

Figure 12. Phylogeny reconstructed from 27f and 1492r primer sequences of unidentified *Shewanella* sp. from this study and other *Shewanella* strains, along with other outgroups. **Key:** 27fShewane = unidentified *Shewanella* sp. partial sequence 27f primer; FJ386509.1 = *Shewanella* sp. Q-2 16S ribosomal RNA gene, partial sequence; AM90878.1 = *Shewanella vesiculosa* partial 16S rRNA gene, strain M; SFU85902 = *Shewanella frigidimarina* ACAM 584 16S ribosomal RNA gene, partial sequence; AJ300834.1 = *Shewanella livingstonis* 16S rRNA gene, strain LMG19866T; M83548.2 = AQF16SRRN *Aquifex pyrophilus* 16S ribosomal RNA gene, partial sequence; EU284667.1 = Uncultured archaeon clone IP1SAT-H9 16S ribosomal RNA gene, partial sequence. Bootstrap values are included.

3.6 Decreasing bacterial ice-nucleator production at higher incubation temperatures

The average nucelation temperature of *Psychrobacter* sp. was higher than *Shewanella* sp. at all temperatures (Fig. 13). With increasing incubation temperature, INA decreased substantially in both bacteria.

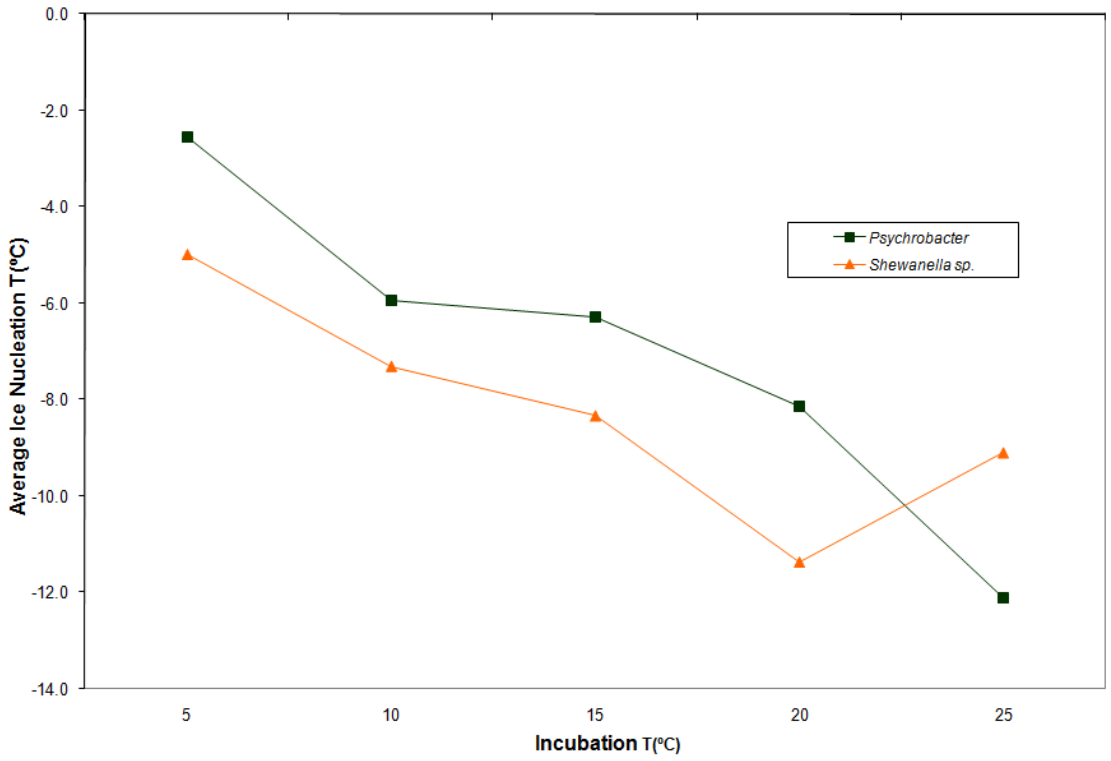

Figure 13. The average ice nucleation temperature (°C) of bacterial broth cultures incubated at temperatures ranging from 5 to 25°C during 20-droplet freezing assays.

3.7 Attempted isolation of ice^{+} genes

No PCR products were detected with any of the primers for either species of bacteria using conditions that were successful for previous studies (see Watanabe and Sato 1998 and Keikhasaber et al. 2007).

3.8 Ciliary response to freezing

The mean ciliary beat frequency for untreated *Geukensia* was determined to be 13.5 beats/sec. The cilia of *Geukensia* exposed to -20ºC conditions for 24 hours or greater periods of times were damaged extensively, and were not measurable using strobe technique (Table 1).

Table 1. The time *Geukensia* were exposed to -20ºC conditions and the condition of their cilia as observed under a microscope.

Time (hrs)	Observations
0	13.5 beats/second
24	Uncoordinated beating. Visible damage.
48	Few areas display movement. Highly damaged.
72	Completely degraded. No individual cilia visible.

3.9 Whole organism survival curves and LT50

The LT50 of each of the three treatments was significantly different ($p<0.01$). *Geukensia* without bacteria were the least freeze tolerant, while winter-acclimatized were the most capable of surviving freezing at the lowest temperatures of the three treatments (Table 2).

Table 2. LT50 of each *Geukensia* of each treatment type.

Treatment	LT50 (℃)
Control	-12.5
Psychrobacter sp. added	-15.0
Winter acclimatized	-16.5

The survival curves of each treatment display results consistent with the LT50's determined by PRISM (Fig. 14, Table 2)

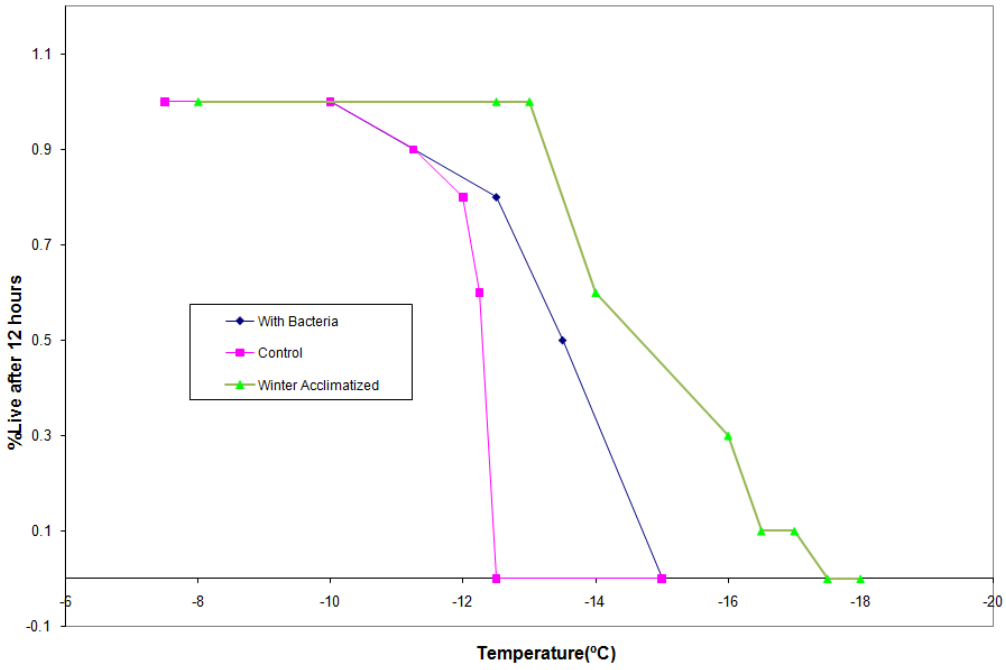

Figure 15. Whole organism survival curve for summer acclimatized *Geukensia* with *Psychrobacter* sp. added to their mantle cavity, summer-acclimatized without bacteria (control) and winter-acclimatized.

3.10 Gill cell viability

Damage was significantly greater ($p<0.05$) in specimens washed of palial fluid than in those with *Psychrobacter* sp. added at -13.5°C (Fig. 15). The percentile difference in damage was not significant between *Geukensia* at -10, 13.5, or -15°C.

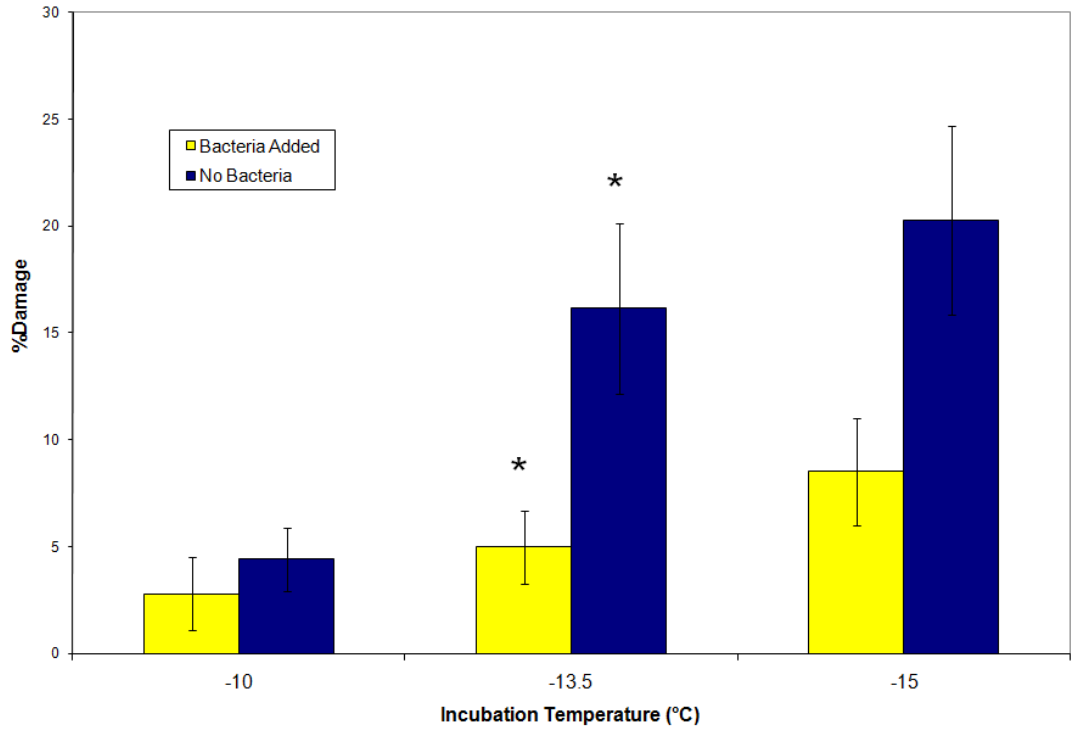

Figure 15. The mean % damage for *Geukensia* gill cells at the indicated temperature and with *Psychrobacter* or no bacteria. An * indicates signficance ($p<0.05$).

For winter-acclimatized *Geukensia*, adding *Psychrobacter* reduced damage significantly ($p<0.05$) at -15°C (Fig. 16). At other temperatures, damage was less though not significantly in specimens with bacteria added.

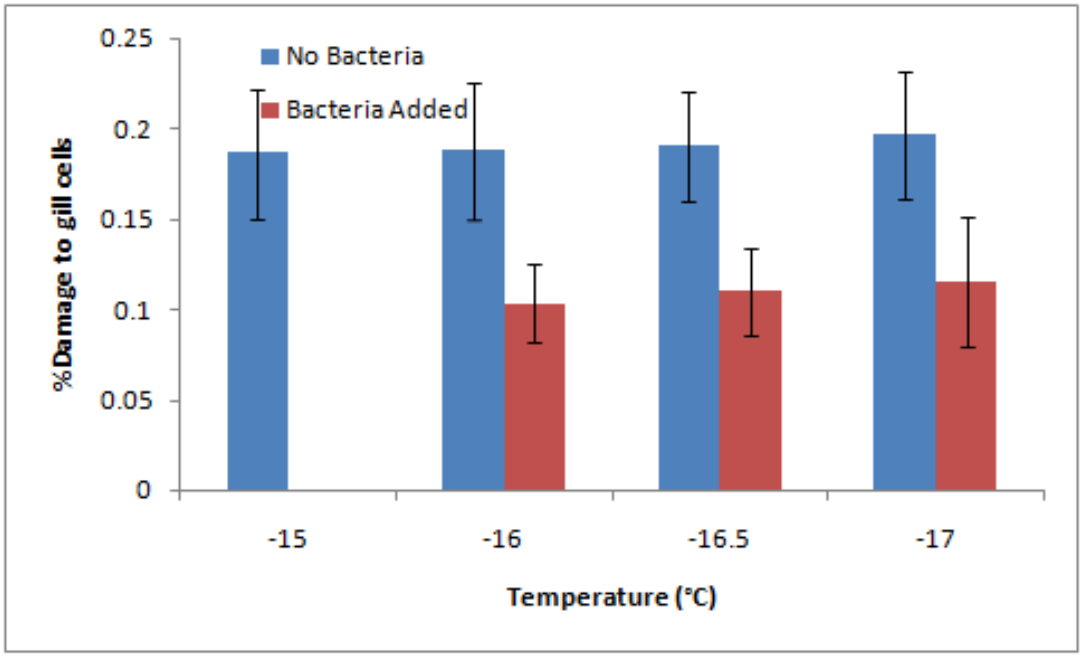

Figure 16. %Damage to *Geukensia* gill cells in winter-acclimatized specimens with and without a *Psychrobacter* sp. and seawater (28 ppt) solution injected.

4.0 Discussion

4.1 *Psychrobacter* sp.

The genus *Psychrobacter* has only recently become well known, though it was originally described in 1986 (Juni and Heym 1986). The bacteria are largely psychrophilic and grow best in salt water (Bowman 2006). They have been isolated from habitats ranging from seawater (*P. submarinus*, Romanenko et al. 2002) to the lungs of lambs (*P. pulmonis*, Vela et al. 2003), though the former is much more common. *Psychrobacter* are widespread, and have been isolated from Japanese coastal water (Venkatewaran et al. 1991) and salt marshes in South Carolina, where they appear to break down dimethylsulfoniopropionate, an algal osmoprotectant, (Ansede et al. 2001).

Psychrobacter sp., isolated in this study, displayed relatively high INA when grown at 5°C. Though no *Psychrobacter* has yet shown INA, the species is strongly associated with cold temperatures and ice. *P. aquimaris* (Yoon et al. 2005), *P. aquaticus* (Shivaji et al. 2005), *P. nivimaris* (Heuchert 2004) and *P. glacincola* (Bowman, Nichols and McMeeken 1997), all species that are closely related phylogenetically to *Psychrobacter* sp., were initially isolated from Antarctic sea ice and unidentified species of *Psychrobacter* with INA have also been isolated from Siberian permafrost (Ponder et al. 2005).

In addition to our phylogenetic results, the fact that *Psychrobacter* are gram-negative and occasionally motile is consistent with our observations. The antibiotic susceptibility of the genus is not well-studied (Bowman 2006), though some strains have been shown to be sensitive to streptomycin (Romanenko et al. 2002) in agreement with the results obtained in this study.

The phylogenetic results combined with the characteristics of the unidentified bacteria in this study make us confident that it belongs in the *Psychrobacter* genus. It is difficult to

comment on the INA of this strain with regards to others in the genus, but with regards to other INA bacteria, its activity is moderate. Consistent with other studies, *Psychrobacter* sp. loses its ice nucleating-activity as it is grown at higher temperatures.

Further research should be conducted into the ice-nucleating activity of *Psychrobacter*, as it is very likely that more strains also have these abilities and knowledge of their ecological role could forward our understanding of how the Ice^{+} phenotype became prevalent.

4.2 *Shewanella* sp.

Shewanella is a more thoroughly studied genus than *Psychrobacter*, having been identified as early as 1931 (Venkateswaran et al. 1999). They are often identified as causing food spoilage (Shewan 1977) and can also be pathogenic. The majority of identified strains are associated with aquatic environments, including Antarctic environments and Atlantic seawater (Bowman et al. 1977, Bozal et al. 2002, Venkateswaran et al. 1999).

Of the strains of the genus, *Shewanella frigidimarina* has been tested for INA on at least one occasion (Junge and Swanson 2007) and was found to have no INA. This study, discussed previously, was to test atmospheric ice-nucleation and therefore had no substrate, a factor that may have affected ice nucleation-activity. No species in the genus has yet displayed activity. Consistent with our study, most strains are gram-negative and non-motile rods and some are psychrophilic. Combined with our phylogenetic analysis, we can confidently label the bacteria isolated in this study as a member of the *Shewanella* genus.

Like *Psychrobacter* sp., there is little to be said about the ice-nucleating activity of *Shewanella* sp. It also decreased with warmer incubation, in accordance with other studies of INA bacteria. It did not display any unexpected ice nucleation qualities.

More procedures need to be conducted to confirm the identity of the bacteria isolated in this study, but it appears that they have not been previously identified. Their well-documented presence in cold Atlantic seawater and ice might explain the difference in freeze tolerance of *Geukensia* collected in the winter.

Both of the bacteria in this study are quite similar. *Psychrobacter* is more often associated with cold environments, but *Shewanella* also have connections to these environments. At present, their role in salt marshes is unknown.

One further procedure that deserves comment is our attempt to isolate the Ice^{+} genes of both strains using previously identified primers. Further attempts with different annealing temperatures could yield results, though the temperatures used in this study did not. Because the C and N sites are highly conserved in all genes so far identified, the primers designed by Watanabe and Sato (1998) and Keikhasaber et al. (2007) should work on other INA bacteria and were in fact used cross-species in each study.

4.3 *Geukensia* freezing tolerance and the effects of *Psychrobacter* sp.

The data collected in this study strongly support INA bacteria of multiple species as a mechanism for freeze tolerance in *Geukensia*. To date, three bacterial species isolated from the pallial fluid of *Geukensia* have been shown to display INA. This study has also shown that INA bacteria can significantly increase the survival of summer-acclimatized *Geukensia*, allowing them to survive to temperatures 2.5°C colder.

This study marks the first time the presence of INA bacteria has been shown to significantly increase the freezing tolerance of an animal. Winter-acclimatized *Geukensia* are still more tolerant of freezing than summer-acclimatized *Geukensia,* even with added ice

nucleators, probably due to the presence of INA bacteria in their palial fluid. The LT50 of winter-acclimatized *Geukensia* found in this study (-16.5°C) is lower than the value (-13.76°C) Murphy and Pierce obtained in 1977 for *Geukensia* acclimated to 0°C temperatures. It is unknown why the *Geukensia* we collected were able to survive to lower conditions than in a previous study, though it is probably be due to differing environmental conditions and climate. Winter-acclimatized *Geukensia* are able to tolerate colder temperatures than summer-acclimatized specimens. This result was expected due to the fact that *Geukensia* produce various cryoprotectants when exposed to cold temperatures (Storey and Churchill 1995).

The results of gill cell viability tests indicate that INA bacteria significantly protect the gills from damage, though our strobe lighting technique did not provide as much insight and simply served to show that upwards of 24 hours of freezing cause irreversible damage to gills. Gill survival is important due to their function in both feeding and respiration. We were unable to show that gills are the site of nucleation in *Geukensia*, but the fact that they are directly exposed to ice within the mantle cavity and yet still show significantly less damage when inoculated with INA bacteria lends further strength to this idea. If the gills were the site of nucleation, we might expect them to show very little damage when INA bacteria are present due to the fact that nucleation would be at the warmest temperature and therefore the slowest near the gills. This observation by no means proves this hypothesis, but shows that it merits further investigation.

Overall, the data collected in this study strongly suggest that INA bacteria are one of the primary mechanisms by which *Geukensia* tolerate freezing. It has been previously shown that *Geukensia* do not produce ice-nucleating agents themselves (Loomis and Zinser 2001). With this study, we see that INA bacteria are in fact capable of increasing whole-organism survival

and reducing gill damage in *Geukensia.* We propose that the presence of INA bacteria in salt marshes provided *Geukensia* with no need to evolve mechanisms to produce their own ice nucleators, like in the case of the similar bivalve *Mytilus edulis* (Aunaas 1982). This relationship is more likely than any sort of mutualistic relationship due to the facts that *Geukensia* gills filter bacteria, which are therefore only incidentally present in their palial fluid, and because bacteria are abundant in nutrient rich salt marsh ecosystems.

These findings are also important in that they reveal New England salt marshes as a wholly untapped resource in the field of INA bacteria research. At least three bacteria have been isolated from the palial fluid of *Geukensia,* and it is our belief that their function within this context is incidental and that they naturally are present in the water. The relative ease with which these three bacteria have been isolated from such an unlikely source suggests numerous research possibilities. Many more are likely present in salt marshes and their function within this complex ecosystem remains to be further explored.

Acknowledgements

I would first like to thank Dalin Chan ‘07 for her previous work in isolating the bacteria and Edward Cook ‘09 for his assistance in measuring their effects on *Geukensia* gills. I would

also like to thank Dr. Anne Bernhard for helping me to understand and complete many of the procedures involving bacterial identification. Dr. Stephen Loomis I thank for his constant advice and support, and for involving me in this subject in the first place.

Lastly, I am grateful to my family and friends for their help and encouragement during the writing process.

Literature Cited

Abe K, Watabe S, Emori Y, Watanabe M, Arai S. 1989. An ice nucleation active gene of *Erwinia ananas*. FEBS Lett 258:297–300.

Ansede JH, Friedman R, Yoch DC. 2001. Phylogeneticanalysis of culturable dimethyl sulfide-producing bacteria from a Spartina-dominated salt marsh and estuarine water. Appl. Environ. Microbiol. 67(3): 1210–1217.

Bowman JP, Nichols DS, McMeekin TA. 1997. *Psychrobacter glacincola* sp. nov., a halotolerant, psychrophilic bacterium isolated from Antarctic sea ice. System. Appl. Microbiol. 20: 209-215.

Bowman JP, McCammon SA, Nichols DS, Skerratt JH, Rea SM, Nichols PD, McMeekin TA. 1997. *Shewanella gelidimarina* sp. nov. and *Shewanella frigidimarina* sp. nov., Novel Antarctic Species with the Ability To Produce Eicosapentaenoic Acid (20:5ω3) and Grow Anaerobically by Dissimilatory Fe(III) Reduction. Int J Syst Bacteriol 47: 1040-1047.

Bowman JP. 2006. The genus *Psychrobacter*. Prokaryotes 6: 920-930.

Bozal N, Montes MJ, Tudela E, Jimenez F, Guinea J. 2002. *Shewanella frigidimarina* and *Shewanella livingstonensis* sp. nov. isolated from Antarctic coastal areas. Int J Syst Evol Microbiol 52: 195-205.

Burke MJ, Lindow SE. 1990. Surface properties and size of the ice nucleation site in ice nucleation active bacteria: theoretical considerations. Cryobiology 27: 80-84.

Buttner MP, Amy PS. 1989. Survival of ice nucleation-active and genetically engineered non-ice nucleating Pseudomonas syringae strains after freezing. Appl. Env. Microbiol. 55: 1690-1694.

Chilvers MA, O'Callaghan C. 2000. Analysis of ciliary beat pattern and beat frequency using digital high speed imaging: comparison with the photomultiplier and photodiode methods. Thorax 55: 314-317.

Christner BC, Morris CE, Foreman CM, Cai R, Sands DC. 2008. Ubiquity of biological ice nucleators in snowfall. Science 319: 1214.

Edward AR, Van Den Bussche RA, Wichman HA, Orser CS. 1994. Unusual pattern of bacterial ice nucleation gene evolution. Mol. Biol. Evol. 11(6): 911-920.

Goto M, Goto T, Inaba T. 1989. Identification of ice nucleation-active bacteria isolated from frost-damaged vegetable leaves. Ann. Phytopathol. Soc. Jpn. 55: 330-335.

Graether SP, Jia Z. 2001. Modeling Pseudomonas syringae Ice-Nucleation Protein as a beta-helical protein. Biophysical Journal 80: 1169-1173.

Green R, Warren G. 1985. Physical and functional repetition in a bacterial ice nucleation gene. Nature 317:645–648.

Gurian-Sherman D, Lindow SE. 1993. Bacterial ice nucleation: significance and molecular basis. The FASEB Journal 7: 1338-1343.

Hazra A, Saha M, De UK, Mukherjee J, Goswami K. 2004. Study of ice nucleating characteristics of *Pseudomonas aeruginosa*. Aerosol Science 35: 1405-1414.

Heuchert A, Glockner FO, Amann R, Fischer U. 2004. *Psychrobacter nivimaris* sp. nov., a Heterotrophic Bacterium Attached to Organic Particles Isolated from the South Atlantic (Antarctica). System. Appl. Microbiol. 27: 399-404.

Hirano SS, Upper CD. 1995. Ecology of ice nucleation-active bacteria. In: Lee RE, Warren GJ, Gusta LV (eds) Biological ice nucleation and its applications pp. 41-61.

Junge K, Swanson BD. 2007. High-resolution ice nucleation spectra of sea-ice bacteria: implications for cloud formation and life in frozen environments. Biogeosciences Discuss. 4: 4261-4282.

Juni E, Heym GE. 1986. *Psychrobacter immobilis* gen. nov., sp. nov.—genospecies composed of Gram-negative, aerobic, oxidase-positive coccobacilli. Int. J. Syst. Bacteriol. 36(3): 388–391.

Kajava AV, Lindow SE. 1993. A model of the three-dimensional structure of ice nucleation proteins. J. Mol. Biol. 232: 709-717.

Kajava AV. 1995. Molecular modeling of the three-dimensional structure of bacterial INA proteins. In: Lee RE, Warren GJ, Gusta LV (eds) Biological ice nucleation and its applications pp. 101-114.

Kaneda T. 1986. Seasonal population changes and characterization of ice-nucleating bacteria in farm fields of central Alberta. Appl. Environ. Microbiol. 52: 173-178.

Kawahara H. 2008. Cryoprotectants and ice-binding proteins. In: Margesin R, Schinner F, Marx JC, Gerday C (eds) Psychrophiles: from Biodiversity to Biotechnology pp. 229-246.

Keikhasaber M, Rahimian H, Jelodar NB, Moghaddam MB. 2007. Production of ice nucleation deficient (Ice^-) mutants of the epiphytic strains of *Erwinia herbicola*. Iranian Journal of Biotechnology 5: 153-157.

Kim HK, Orser C, Lindow Se, Sands DC. 1987. Xanthomonas campestris pv. Translucens strains actice in ice nucleation. Plant Dis. 71: 994-997.

Klement Z, Rozsnyay ZD, Balo E, Panczel M, Prileszky G. 1984. The effect of cold on development of canker in apricot trees infected with Pseudomonas syringae pv. Syringae. Physol. Plant Pathol. 24: 237-246.

Kobashigawa Y, Nishimiya Y, Miura K, Satoru O, Miura A, Tsuda S. 2005. A part of ice-nucleation protein exhibits the ice-binding activity. FEBS Letters 579: 1493-1497.

Kozloff LM, Turner MA, Arellano F, Lute M. 1991. Phosphatidylinositol, a Phospholipid of Ice-Nucleating Bacteria. Journal of Bacteriology 173: 2053-2060.

Kumaki Y, Kawano K, Hikichi K, Matsumoto T, Matsushima N. 2008. A circular loop of the 16-residue repeating unit in ice nucleation protein. Biochemical and Biophysical Research Communications 371: 5-9.

Lee RE, Strong-Gunderson JM, Lee MR, Grove KS, Riga TJ. 1991. Isolation of ice nucleating active bacteria from insects. The Journal of Experimental Zoology 257: 124-127.

Lee MR, Lee RE, Strong-Gunderson JM, Minges SR. 1995. Isolation of Ice-Nucleating Active Bacteria from the Freeze-Tolerant Frog, *Rana sylvatica*. Cryobiology 32: 358-365.

Lee RE, Costanzo JP. 1998. Biological ice nucleation and ice distribution in cold-hardy ectothermic animals. Annu. Rev. Physiol. 60: 55-72.

Lindow SE. 1983. The role of bacterial ice nucleation in frost injury to plants. Ann. Rev. Phytopathol. 21: 363-384.

Lindow SE, Arny DC, Upper CD. 1975. Increased frost sensitivity of maize in the presence of *Pseudomonas syringae*. Proc. Am. Phytopathol. Soc. 2: 158.

Lindow SE, Arny DC, Upper CD. 1978. Distribution of Ice Nucleation-Active Bacteria on Plants in Nature. Applied and Environmental Microbiology 36: 831-838.

Loomis SH. 1995. Freezing tolerance of marine invertebrates. Oceanography and Marine Biology: An Annual Review 33: 337-350.

Loomis SH, Zinser M. 2001. Isolation and identification of an ice-nucleating bacterium from the gills of the intertidal bivalve *Geukensia demissa.* Journal of Experimental Marine Biology and Ecology 261: 225-235.

Lucas JW. 1954. Subcooling and ice nucleation in lemons. Plant Physiology 29: 245-251.

Maki LR, Galyan EL, Chang-chien M, Caldwell DR. 1974. Ice nucleation induced by *Pseudomonas syringae*. Applied Microbiology 28: 456-459.

Michigami Y, Watabe S, Abe K, Obata H, Arai S. 1994. Cloning and sequencing of an ice nucleation active gene of *Erwinia uredovoa*. Biosci Biotechnol Biochem 58:762–764.

Michigami Y, Abe K, Obata H, Arai S. 1995. Significance of the C-Terminal domain of *Erwinia uredovora* ice nucleation-active protein (Ina U). J. Biochem 118: 1279-1284.

Mizuno H. 1989. Prediction of the conformation of ice-nucleation protein by conformational energy calculation. Proteins Struct. Funct. Genet. 5: 47-65.

Murphy DJ, Pierce SK. 1975. The physiological basis for changes in freezing tolerance of intertidal molluscs. Journal of Experimental Zoology 193: 313 1975.

Murphy DJ. 1977. Calcium-dependent mechanism responsible for increasing freezing tolerance of bivalve mollusk *Modiolus-demissus*. Journal of Experimental Biology 69: 13.

Nicolai A, Vernon P, Lee M, Ansart A, Charrier M. 2005. Supercooling ability in two populations of the land snail *Helix pomatia* (Gastropoda: Helicidae) and ice-nucleating activity of gut bacteria. Cryobiology 50: 48-57.

Obata H, Nakai T, Tanishita J, Tokuyama T. 1989. Identification of an ice-nucleating bacterium and its nucleation properties. J. Ferment. Bioeng. 67: 143-147.

Obata H, Takinami K, Tanishiti JI, Hasegawa Y, Kawate S, Tokujama T, Ueno T. 1990. Identification of a new ice nucleating bacterium and its ice nucleation properties. Agric. Biol. Chem. 54: 725-730.

Obata H, Muryoi N, Kawahara H, Yamade K, Nishikawa J. 1999. Identification of a novel ice-nucleating bacterium of antarctic origin and its ice nucleation properties. Cryobiology 38: 131-139.

Panagopoulos CG, Crosse JE. 1964. Frost injury as a predisposing factor in blossom blight of pear caused by P. syringae van Hall. Nature 202: 1352.

Ponder MA, Gilmour SJ, Berholz PW, Mindock CA, Hollingsworth R, Thomashow MF, Tiedje JM. 2005. Characterization of potential stress responses in ancient Siberian permafrost psychroactive bacteria. FEMS Microbiol Ecol 53: 103-115.

Romanenko LA, Schumann P, Rohde M, Lysenko AM, Mikhailov VV, Stackebrandt E. 2002. *Psychrobacter submarinus* sp. nov. and *Psychrobacter marincola* sp. nov., psychrophilic halophiles from marine environments. International Journal of Systematic and Evolutionary Microbiology 52: 1291-1297.

Schnell RC, Fall R, Nemecek-Marshall M, Sweeting K, LaDuca R. 1991. A new INA bacterium from high altitude equatorial vegetation. Int. Conf. on Biological Ice Nucleation, 5th. Madison, WI.

Shewan JM. 1977. The bacteriology of fresh and spoiling fish and the biochemical changes induced by bacterial action. In: Proceedings of the Conference on Handling, Processing and Marketing of Tropical Fish pp. 51-66. London: Tropical Products Institute.

Shivaji S, Reddy GSN, Suresh K, Gupta P, Chintalapati S, Schumann P, Stackebrandt E, Matsumoto GI. 2005. *Psychrobacter vallis* sp. nov. and *Psychrobacter aquaticus* sp. nov., from Antarctica. Int J Syst Evol Microbiol 55: 757-762.

Skirvin RM, Kohler E, Steiner H, Ayers D, Laughnan A, Norton MA, Warmund M. 2000. The use of genetically engineered bacteria to control frost on strawberries and potatoes. Whatever happened to all that research? Scientia Horticulturae 84: 179-189.

Storey KB, Storey JM. 1984. Biochemical adaptation for freezing tolerance in the wood frog, *Rana sylvatica*. J. Comp. Physiol. B 155: 29–36.

Tanaka K, Watanabe M. 2003. Transmission of ice-nucleating active bacteria from a prey reduces cold hardiness of a predator (Araneae: Theridiidae). Naturwissenschaften 90: 449-451.

Upper CD, Vali G. 1995. The discovery of bacterial ice nucleation and its role in the injury of plants by frost. In: Lee RE, Warren GJ, Gusta LV (eds) Biological ice nucleation and its applications pp. 29-39.

Vali G. 1995. Principles of ice nucleation. In: Lee RE, Warren GJ, Gusta LV (eds) Biological ice nucleation and its applications pp. 1-28

Vela AI, Collins MD, Latre MV, Mateos A, Moreno MA, Hutson R, Dominguez L, Fernández-Garayzábal JS. 2003. *Psychrobacter pulmonis* sp. nov., isolated from the lungs of lambs. Int J Syst Evol Microbiol 53: 415-419.

Venkateswaran K, Iwabuchi T, Matsui Y, Toki H, Hamada E, Tanaka H. 1991. Distribution and biodegradation potential of oil-degrading bacteria in north eastern Japanese coastal waters. FEMS Microbiol. Ecol. 86(2): 113–121.

Venkatewaran K, Moser DP, Dollhopf ME, Lies DP, Saffarini DP, MacGregor BJ, Ringelberg DB, White DC, Nishijima M, Sano H, Burghardt J, Stackebrandt E, Nealson KH. 1999. Polyphasic taxonomy of the genus *Shewanella* and description of *Shewanella oneidensis* sp. nov. International Journal of Systematic Bacteriology 49: 705-724.

Vigouroux A. 1989. Ingress and spread of Pseudomonas in stemsof peach and apricot promoted by frost-related water-soaking of tissues. Plant Dis. 73: 854-855.

Warren G, Corotto L, Wolber P. 1986. Conserved repeats in diverged ice nucleation structural genes from two species of *Pseudomonas*. Nucleic Acids Res 14:8047–8060.

Warren GJ, Corotto L. 1989. The consensus sequence of ice nucleation proteins from *Erwinia herbicola, Pseudomonas fluorescens,* and *Pseudomonas syringae*. Gene 85: 292-294.

Warren GJ. 1995. Identification and analysis of ina genes and proteins. In: Lee RE, Warren GJ, Gusta LV (eds) Biological ice nucleation and its applications pp. 85-99.

Watanabe K, Sato M. 1998. Detection of variation of the R-Domain structure of ice nucleation genes in *Erwinia herbicola*-group bacteria by PCR-RFLP analysis. Current Microbiology 37: 201-209.

Watanabe M, Kikawada T and Okuda T. 2002. Increase of internal ion concentration triggers trehalose synthesis associated with cryptobiosis in larvae of *Polypedilum vanderplanki*. The Journal of Experimental Biology 206, 2281-2286.

Worland MR, Block W. 1999. Ice-Nucleating Bacteria from the Guts of Two Sub-Antarctic Beetles, *Hydromedion sparsutum* and *Perimylops antarcticus* (Perimylopidae). Cryobiology 38: 60-67.

Yi SX, Lee RE Jr. 2003. Detecting freeze injury and seasonal cold-hardening of cells and tissues in the gall fly larvae, *Eurosta solidaginis* (Diptera: Tephritidae) using fluorescent vital dyes. J Insect Physiol 49: 999-1004.

Yoon J, Lee C, Yeo S, Oh T. 2005. *Psychrobacter aquimaris* sp. nov. and *Psychrobacter namhaensis* sp. nov., isolated from sea water of the South Sea in Korea. Int J Syst Evol Microbiol 55: 1007-1013.

Zhao J, Orser CS. 1990. Conserved repetition in the ice nucleation gene inaX from *Xanthomonas campestris* pv. *translucens*. Mol Gen Genet 223:163–166.