

**Bacterial low temperature survival, ice nucleation
proteins and ice-associating polymers**

by

Zhongqin Wu

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in Conformity with the Requirement for
the Degree of Doctor of Philosophy

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Abstract

Microorganisms have developed ways to preserve cellular functions under low temperature conditions using a variety of biochemical adaptations including the modification of ice formation. In order to conduct a limited survey of microbial ice-associating strategies, a bacterial community associated with frost-exposed leaves was assessed by the construction of a 16S rDNA library, followed by the characterization of some isolates. Fifteen different species were identified based on their 16S rDNA. Among these, *Pseudomonas syringae* J6 had ice nucleation activity (INA), which promoted ice formation close to 0°C, whereas *Erwinia billingiae*, *Flavobacterium sp.* and *Sphingobacterium kitahiroshimense* inhibited the recrystallization of small ice crystals at temperatures close to melting. The *Erwinia billingiae* isolate showed adhesive and swarming behaviour, which can be associated with biofilm formation. Visualization using negative staining, transmission electron microscopy and scanning electron microscopy confirmed the presence of flagella in addition to the presence of slimy biofilm architecture in these *Erwinia billingiae* cultures. Subsequent purification of the extracellular polymeric substance followed by mass spectrometry allowed the identification of a putative outer membrane protein A, which may be involved in the protection of this bacterium to freeze-thaw cycles.

To further explore bacterial ice nucleation activity, an ice nucleation protein was cloned from *Pseudomonas borealis*, a bacterium originating from tundra soil, using degenerative PCR and chromosome walking. The sequence of the putative ice nucleation protein gene (*inaPb*) was cloned and expressed in *Escherichia coli*, and its identification was confirmed in the recombinant cells. Although the INPPb was more divergent than other plant-related bacterial INPs, it retained the highly conserved, repetitive core region.

The protein may fold so that it has two flat faces, one for protein-protein interactions and the other for ice binding. Expression of the INPPb coding region fused to jelly fish green fluorescent protein showed a temperature-dependent polarized distribution of the recombinant protein in *E. coli*.

In summary, results from this thesis suggests that low temperature survival may be associated with a number of ice-associating adaptations including the presence of biofilm formation in *Erwina billingiae* amongst other bacteria, INA in *P. borealis* and INA-expressing recombinant *E. coli*.

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Co-Authorship

Chapter 2: This chapter represents a paper “in preparation” for submission, with authors Zhongqin Wu, Frederick W.K. Kan, Yi-Min She, and Virginia K. Walker. I designed and prepared the 16S rDNA library of culturable bacteria, prepared extracellular polymeric substance, performed freeze-thaw assays and did all the ice-association activities of the isolates. Dr. Frederick W.K. Kan performed the scanning electron microscopic examination of the isolates as shown in Fig. 2.3. Dr. Yi-Min She performed the MS of the main protein product as shown in Fig. 2.5. I also wrote the initial draft of this manuscript and participated in the subsequent editing.

Chapter 3: This paper was published in *Microbiology* in 2009 (155, 1164-1169). The authors are Zhongqin Wu, Lei Qin and Virginia Walker. I designed and prepared the all of the structures of pGEMcoreinp, PGEM5extension, pGEM3extension, pGEMT, pGEMeinp, pET24aINP, I also performed all of the experiments for the manuscript except for Fig. 3.3 which was produced by Dr. Lei Qin, who also performed the computer analysis of the ice nucleation protein sequences. I also wrote the initial draft of the manuscript and participated in subsequent editing.

Chapter 4: This chapter represents a paper that is in progress for eventual submission. The authors that have contributed to date are Denian Miao, Zhongqin Wu, and Virginia K Walker. This is a part of project on the characterization *inaPb*. I designed and prepared PGEMTINP and pGEM5extension plasmids, Dr. Denian Miao, a visiting researcher, designed and prepared the pGEMCINP, pGEMNINP, pGEMGFP, pGEMEINPGFP and pEINPGFP constructions, and most of the experiments were done together. Dr. Miao wrote the first draft of the Methods and Results, and I initiated a portion of the first draft of the Discussion and participated in the subsequent editing.

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List of Abbreviations

The standard abbreviations and symbols are used in this thesis listed as follows.

aa	amino acid
AHL	acyl homoserine lactones
AFP	antifreeze protein
AFGP	antifreeze glycoprotein
CfAFP	<i>Choristoneura fumiferana</i> antifreeze protein
HypAFP	hyperactive antifreeze protein
MpAFP	<i>M. primoryensis</i> antifreeze protein
LpAFP	<i>Lolium perenne</i> antifreeze protein
TmAFP	<i>Tenebrio molitor</i> antifreeze protein
BSA	bovine serum albumin
BCA	bicinchoninic acid assay
CFU	colony forming unit
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
DSC	differential scanning calorimeter
DSF	diffusible signal factor
EM	electron microscopy
EPS	extracellular polymeric substance
GFP	green fluorescent protein
Ih	hexagonal form of ice
IN	ice nucleation
INA (INA ⁺)	ice nucleation activity

INA ⁻	bacteria without ice nucleation activity
INP (INP ⁺)	ice nucleation protein
<i>inaPb</i>	<i>Pseudomonas borealis</i> ice nucleation protein gene
IR	ice recrystallization
IPTG	isopropyl-β-D-thiogalactopyranoside
LB	Luria-Burtani
MALDI	Matrix assisted laser desorption ionization
MS	mass spectrometry
OD	optical density
PB	phosphate buffer
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PGL	polyglycerol polymers
PTA	phosphotungstic acid
PVA	polyvinyl alcohol
PVC	polyvinyl chloride
QS	quorum sensing
SDS-PAGE	sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SEM	scanning electron microscopy
SA	LpAFP-GFP/INP
SM	snowmax (INP)
SG	glycerol/INP

SP	PEG/INP
SPG	glycerol/ PEG /INP
SPGA	LpAFP-GFP/ glycerol/ PEG /INP
TEM	transmission electron microscopy
TH	thermal hysteresis
THP	thermal hysteresis protein
TSB	tryptic soy broth

Chapter 1

Introduction and Literature Review

1.1 Introduction

Water is the basis of life on earth. Oxygen is more electronegative than hydrogen, and thus water is a polar molecular, with high adhesion and surface tension. Water has a high heat energy and heat of evaporation, which inhibits rapid temperature fluctuations and allows living organisms to survive and reproduce. As a main component of cells, water works as a solvent for biochemical reactions of proteins (including enzymes), sugars and lipids, important for cell functions including basic metabolism and cell division. Water is also a crucial component of energy production and consumption by such biological processes as photosynthesis ($12\text{H}_2\text{O} + 6\text{CO}_2 + \text{light} \rightarrow \text{C}_6\text{H}_{12}\text{O}_6 + 6\text{H}_2\text{O} + 6\text{O}_2$), and respiration ($\text{C}_6\text{H}_{12}\text{O}_6 + 6\text{O}_2 + n\text{ADP} \rightarrow 6\text{H}_2\text{O} + 6\text{CO}_2 + n\text{ATP}$).

Vapor, liquid and ice, three states of water, are in equilibrium. When water changes phase from vapor to liquid (condensation) or from liquid to solid (freezing), energy is either gained or lost as heat flow. Under different temperatures and pressures, water can be crystallized into 15 different types of ice, each with a specific density and optical character. However, on a practical level, the hexagonal form of ice (Ih) is the only form considered in this thesis as it is the most common stable phase and has a lower density (0.9167 g/cm) than water (0.9998 g/cm³) at the same temperature. This characteristic of ice allows it to float above water, thus allowing aquatic organisms to thrive below ice (Junge *et al.*, 2002; Krembs *et al.*, 2000; 2002; Bottos *et al.*, 2008).

Although external ice formation can be beneficial to some aquatic organisms, unscheduled ice formation can lead to severe consequences. Examples range from causes of human suffering including hail-mediated crop destruction and transport and emergency

service delays caused by snow storms, ice storms and freezing rain. Such weather conditions, slippery roads and extra ice gravity load are all factors that can cause significant accidents in the operation of automobiles, utility failures, and aircraft crashes (e.g. American Eagle Flight 4184, Fredrick, 1996). As well as inconveniences to humans, animals and plants living at high altitudes or seasonally, at even moderate latitudes, can be threatened by subzero temperatures. For humans, frozen foods such as ice cream are important commodities in daily life. Freezing and freeze-drying also are well known preservation methods for not only food but for biological products such as bacteria, virus, cell lines, bovine serum, cytokines, enzymes and monoclonal antibodies (Pegg, 2002). It is clear that research related to ice is an important and active area, covering various fields such as atmospheric physics, agriculture, chemistry and biology including microbiology. Ice-related research also has applications in conserving ecological samples, saving energy, improving crop production and promoting organ preservation and transplantation (Zobrist *et al.*, 2008; Chaplin 2006; Spoel *et al.*, 2008; Bagis *et al.*, 2008; Kami 2008; Costa *et al.*, 2008).

Studying microbes in extreme environments may expand our knowledge about adaptations for harsh conditions and the evolution of life on the planet. For example, certain Archaea as well as some Eubacteria live in extreme environments, such as high temperature (thermophiles), high salt concentrations (halophiles) and low pH (acidophiles). Archaea survive in these niches that are often characterized by low available energy by utilizing distinct biochemical pathways such as nitrification, methanogenesis and methane oxidation. Archaeal organisms are different from bacterial and eukaryotic cells since they use bilayer lipids which consist of esterified fatty acids to a glycerol moiety. Some Archaea use lipid monolayers which consist of isoprenoidal

alcohols that are ether-linked to glycerol. In this way, Archaeal membranes are less permeable to ions so they can survive in extreme environments (Valentine, 2007). With the discovery of ice on other planets and moons, and the realization of the abundance of microbes associated with permanent ice on our own planet (Thomas and Dieckmann, 2002), microbiologists are enthusiastic about the concept of exploring outer space life associated with ice (Tung *et al.*, 2005).

1.2 Thermal dynamics of ice nucleation and ice crystal inhibition

Freezing represents a phase change from a metastable state to a stable state. Freezing begins with a small cluster of low-energy water or an ice embryo. Below a critical size (nucleus) the ice embryo is unstable and will decay, but above a critical size, nucleation is favored and the ice crystal will grow. Small volumes of pure water will not spontaneously freeze until supercooled to as low as -40°C (Cwilong, 1945). Research in the mid 20th century showed that, in practice, the freezing temperature of ice is affected by impurities in the water, called the solute effect (Altberg, 1938). Different solutes can affect ice nucleation differently. Those agents that promote ice nucleation are called ice nuclei or ice nucleation agents, while those agents that decrease the probability of ice growing from ice embryo are said to have antifreeze activity. Such agents and activities can be microbiological in origin, and the following section focuses on these aspects.

1.2.1 Ice recrystallization inhibition and antifreeze proteins

Ice recrystallization (IR) may be associated with low temperature survival (Urrutia *et al.*, 1992, Carpenter and Hansen, 1992). At high subzero temperatures, small amounts of liquid water continue to grow into big crystals at the expense of small crystals, but the presence of ice active substances and antifreeze proteins may modify this ice crystal regrowth (Raymond and Knight, 2003; Carpenter and Hansen, 1992). IR inhibition assays

were first used to study antifreeze agents by Sjöström (1975). With the aid of a microscope, he found that skeletal muscle fibers treated with cryoprotective additives such as glycerol, dimethyl sulfoxide (DMSO), and sucrose eliminated ice crystal growth. About 10 years later, Knight *et al.* (1984) reported that antifreeze glycoprotein (AFGP) showed IR inhibition behaviour. Since that time several methods have been developed to study antifreeze proteins (AFPs) by monitoring the size or the size change of the ice crystals (knight *et al.*, 1988; Regand and Goff, 2002; Gilbert *et al.*, 2004; Tomczak *et al.*, 2003).

AFPs and AFGPs depress freezing by adsorbing to the ice surface and increasing the energy required for the addition of water molecules in a process known as the Kelvin effect (Raymond and DeVries, 1977; Mao and Bao, 2006). In this way, the melting point of the ice is less affected, resulting in a thermal hysteresis (TH) or a difference in the freezing and melting temperatures. AFPs have been well studied in fish (Type I AFPs, Type I-hyperactive (hyp) AFP, Type II AFPs, Type III AFPs, Type IV AFPs, and AFGPs) as well as in some arthropods such as the beetle *Tenebrio molitor* AFP (TmAFP), moth *Choristoneura fumiferana* AFP (CfAFP), and snow flea *Hypogastrura nivicola* AFP. Each of these AFPs has a distinct TH at a particular millimolar concentration. Those that have a high TH are known as hyperactive AFPs, such as CfAFP, TmAFP, Type I-hyp AFP and snow flea AFP (Graham *et al.*, 2005). Although a low TH activity is often a characteristic of plant AFPs such as the AFP from rye grass, *Lolium perenne* (LpAFP) or the bittersweet nightshade, *Solanum dulcamera*, thermal hysteresis protein (THP; Urrutia *et al.*, 1992), IR inhibition is still apparent. To date, a limited number of bacterial AFP genes have been cloned (Muryoi *et al* 2003; Raymond *et al.*, 2007; Garnham *et al.*, 2008), but bacterial IR inhibition has been reported in several species, such as *P. putida*, *P.*

fluorescens, *Colwellia sp.*, *Marinomonas primoryensis* and *Flavobacterium xanthum* (Sun *et al.*, 1995; Gilbert *et al.*, 2004; 2005; Muryoi *et al.*, 2003; Kawahara *et al.*, 2007). The first bacterial hyperactive AFP was reported from *M. primoryensis* (MpAFP), a Ca²⁺ dependent AFP (Garnham *et al.*, 2008). Structural studies show that region IV of this AFP is folded into a right-handed β -helix, with 11 Ca²⁺-binding loops, and each loop with a relatively flat XGTGND ice-binding face.

1.2.2 IR inhibition and extracellular polymeric substance

Some microbes including bacteria, yeast and molds can secrete a slime-like extracellular polymeric substance (EPS). EPS from different species can have different composition, structures and properties, and early work concentrated on the characterization of the polysaccharide and the establishment of methods for maximum industrial production. Two important EPS products from *Leuconostoc mesenteroides* and *Xanthomonas campestris* (Jeanes *et al.*, 1948a; Cadmus *et al.*, 1976) were developed after extensive selections of bacterial strains and identification of their polysaccharide constituents (Jeanes *et al.*, 1948b; Jeanes *et al.*, 1961; Jeanes *et al.*, 1962). The first application of EPS was as a blood volume extender (Jeanes *et al.*, 1954), and the second one, xanthan gum, is often used in salad dressings, sauces, ice creams and in the oil industry.

Bacterial EPS can be associated with IR inhibition and low temperature protection. xanthan gum, the EPS product from *Xanthomonas campestris* showed IR inhibition in an ice cream model (Regand and Goff, 2002; 2006). Further, EPS capsules were shown to increase microbial freeze-thaw survival and desiccation resistance (Hong and Marshall, 2001; Tamaru *et al.*, 2005). This association was strengthened by the demonstration that

purified EPS from *P. arctic* protected *E. coli* membranes from freeze-thaw damage (Kim and Yim 2007).

Low temperature protection mediated by EPS may be related to increased production, which in turn may be related to low temperature, salt stimulation and ice formation (Mancuso *et al.*, 2004; Nichols *et al.*, 2005a; 2005b; Marx *et al.*, 2009; Battin *et al.*, 2001; Riedel *et al.*, 2006; Wallner *et al.*, 1986; Roberson and Firestone, 1992). However, even these relationships are not clear since Yamashita *et al.* (2002) found that bacterial EPS (from *Bacillus thuringiensis* YY529) depressed bacterial INA. It is not clear how bacterial EPS would interact with bacterial INA, and thus the evidence and the mechanism of low temperature protection by EPS should be further studied.

1.2.3 EPS and biofilm

Biofilm formation may be an important adaptation to stressful environments. Biofilms appear to provide a protection for microbes to grow and survive in hostile conditions (Costerton, 1999). Different from their planktonic counterparts, biofilm-producing microbes adhere to inert surfaces or living organisms, producing a mass of heterogeneous EPS that glues together cells, released detritus, DNA and other macromolecules (Sutherland, 1982; 2001). This slippery coating and the microbial community together constitutes a biofilm. Using biofilms as a diffusive barrier, sessile bacteria embedded in biofilm architecture become more resistant to antibiotics, bactericides, radiation and other stresses (Ito *et al.*, 2009; Gilbert *et al.*, 1997). Slimy EPS provides the major matrix structure which can account for up to 50-90% of total organic carbon of the biofilm (Flemming *et al.*, 2000). EPS is also important for biofilm differentiation and antibiotic susceptibility (Danese *et al.*, 2000; Hentzer *et al.*, 2001).

Biofilms have well-defined matrix architecture as well as channels to transport water and nutrients. Biofilm research was initially started with studies of slimy bacterial cultures and their self-purification activity (Sanders, 1966; Wuhrmann, 1964). The first images of biofilms on epoxy resin discs were captured by Jones *et al.* (1969), using a high concentration of bacterial EPS, slime matrix and bacteria using thin section preparations and transmission electron microscopy. The advantage of biofilms reflects the benefits obtained by adhering to surfaces in a particular ecological niche (Costerton *et al.* 1978). Costerton and other teams expanded the biofilm concept from environmental microorganisms to clinical microbes, which cause catheter infections, oral infections, contact lens infections, emergencies associated with cystic fibrosis and several other diseases (Costerton *et al.*, 1999). With the introduction of confocal laser scanning electron microscope and computer assisted analysis (Lawrence *et al.*, 1991; Caldwell *et al.*, 1992), a three dimension biofilm image was reconstructed (Lawrence *et al.*, 1991). This non-invasive method showed water channels within the biofilm architecture. Microbial differentiation was demonstrated by a change in cell morphology and metabolic pattern associated with biofilm formation (James *et al.*, 1995; Costerton, 2002, Lawrence *et al.*, 1991; Stoodley *et al.*, 2002). Recently a freeze-fracture transmission electron microscope was used to study *Pseudomonas aeruginosa* and *Myxococcus xanthus* biofilms assisted with tomography (Hunter and Beveridge, 2005; Palsdottir *et al.*, 2009).

Research using proteome approaches to compare *Pseudomonas aeruginosa* planktonic cells with sessile cells has found that several groups of regulated proteins are upregulated in biofilm-producing cells including proteins that regulate metabolism, phospholipids, LPS-biosynthesis, membrane transport and secretions (Sauer *et al.*, 2002).

Some genes that are well studied include *alg C*, *algD*, and genes that encode quorum sensors and polyphosphate kinase (Clark *et al.*, 2007; Rashid *et al.*, 2000; Lau *et al.*, 2009; Stoodley *et al.*, 2002).

1.3 INA bacteria and their ecological impacts

Certain bacteria can promote ice nucleation at temperatures close to 0°C, which is a concern for plant pathology and ecology during frosts. Although different organic and inorganic ice nucleation agents have been reported, including CuI, AgI, AgBr, CaCO₃ and kaolinite (a special clay from Gaoling, Jingdezhen, China), egg albumin, snow molds (Snider *et al.*, 2000), the fungus *Fusarium acuminatum* (Humphreys *et al.*, 2001), and lichens (Kieft and Ruscetti, 1990), to date, most biological work on ice nucleation has concentrated on bacterial ice nuclei.

Pseudomonas syringae, a plant-associated bacterium, was the first microorganism reported with INA. It was discovered in the early 1970s by two different research groups (Maki *et al.*, 1974; Arny *et al.*, 1976). Both groups had distinct research interests, one group worked on plant pathology and the other group worked in atmospheric science. Interestingly, results from both groups led them to draw similar conclusions: *P. syringae* has high INA, and it can promote ice nucleation close to 0°C, which is even better than the best inorganic ice nucleation agent, AgI. This bacterial INA may be a factor for frost injury in some frost-sensitive plants (Lindow *et al.*, 1982a; 1982 b). This co-discovery opened new doors in microbiology, biochemistry and molecular biology and the identification of other INA positive (INA⁺) bacteria and the isolation of the genes responsible for INA has continued over the past decades.

So far, bacteria that have been reported to promote ice formation include: *P. syringae*, *P. fluorescens*, *P. viridiflava*, *P. chlororaphis*, *Erwinia herbicola*, *E. ananas*, *E.*

uredoovora, *Enterobacter agglomerans*, *Xanthomonas campestris* pv. *translucens*, *X. campestris*, *P. putida* and *P. antarctica* (Maki *et al.*, 1974; Arny *et al.*, 1976; Lindow *et al.*, 1978a; Lindow *et al.*, 1978b; Kaneda, 1986; Newton and Hayward, 1986; Kim *et al.*, 1987; Obata *et al.*, 1999; Kaneda, 1986). It is noted that not all of the strains from those species have INA, and even those INA⁺ bacterial strains have slightly different INA properties. The INA of studied bacteria was increased by phosphate starvation and low temperature incubation (Fall and Fall, 1998; Nemecek-Marshall M *et al.*, 1993). Further, some extracellular ice nuclei from *P. antarctica* and *E. herbicola* were isolated and examined using transmission electron microscopy (TEM; Muryoi *et al.*, 2003; Phelps *et al.*, 1986). Those ice nuclei had well-defined structures which were sensitive to phospholipase and proteinase K. Their INA remained after density gradient centrifugation in Percoll, but interestingly, this INA was destroyed by using sucrose in density gradient centrifugation (Phelps *et al.*, 1986). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed a band at 190 kDa, with the composition of lipids, protein and polysaccharides accounting for 55%, 33% and 12% of the total, respectively (Muryoi *et al.*, 2003).

Plants provide habitat to different bacteria. It was shown that INA⁺ bacteria could reside on the leaf surface in numbers corresponding to a population of 10⁶ CFU/g leaf tissues (Lindow *et al.*, 1978b). A variety of epiphytic communities were found including pigmented bacteria, *P. syringae*, *P. fluorescens*, *P. putida* and *E. herbicola* (Wilson *et al.*, 1999). In a field experiment, after two days of spraying the same concentration of different bacteria on bean leaves, more pathogenic *P. syringae* (3.6%) were recovered from the inside the leaves than non-pathogenic *P. syringae* (0.77%; Wilson *et al.*, 1999). By aggregating in the phyllosphere close to the trichomes and stomata, and promoting ice

formation close to 0°C, pathogenic bacteria may have an advantage by living in a less stressful environment and increasing their population by having access to additional nutritional resources (Edward *et al.*, 1994).

1.3.1 Classification of bacterial ice nuclei and their molecular properties

Bacteria with ice nucleation activity promote ice formation with different frequencies and at different temperatures. According to the temperature at which these bacteria nucleate ice, INA⁺ bacteria can be divided into three different classes (Yankofsky *et al.*, 1981): Type I INA bacteria promote ice nucleation above -5°C; type II INA bacteria nucleate at about -5 to -7°C; and type III INA bacteria freeze between -8 to -10°C. Bacterial INA can be reduced by enzymes such as proteinase K, phospholipase A, phospholipase C, and mannosidase, either by disruption of the outer membrane or by modification of membrane structure (Ruggles, 1991; Ruggles *et al.*, 1993). Thus, bacterial ice nuclei may be involved in glycol and lipid pathways. Some cross reaction was seen between *P. syringae*, *P. fluorescens* and *E. herbicola* by immunological analysis, suggesting a common epitopes for INAs (Deininger *et al.*, 1988). Later the genes encoding ice nucleation protein (INP) were isolated from *P. syringae*, *P. fluorescens*, *E. ananas* and *X. campestris pv. translucens* by selection of cosmids containing sequences encoding INA and subsequent subcloning and function assays in *E. coli* (Orser *et al.*, 1985; Corotto *et al.*, 1986; Abe *et al.*, 1989; Arai *et al.*, 1989; Hasegawa *et al.*, 1990; Zhao and Orser, 1990). Despite being derived from divergent species, the genes encoding INAs that are active at temperatures above -5°C are quite similar, and they encode highly repetitive membrane proteins.

So far, INP genes have been cloned from three different genera of bacteria: *Pseudomonas*, *Erwinia*, and *Xanthomonas*. It should be noted that most of these INA⁺

bacteria are epiphytes, and are often the cause of economic losses in frost-sensitive crops and fruit trees. INPs from all three genera share a homogenous core of 960 to 1296 amino acids (aa) that together form repetitive peptide modules, flanked by a nonrepetitive aminoterminal of 161 - 203 residues, and a nonrepetitive carboxyterminal portion of 41 - 68 residues (Edward *et al.*, 1994). In the repetitive region, each 16 aa stretch (aGYGSTxTAGxxSSLi) is a repeat, with three repeats making a 48 aa ice nucleation protein repeat unit (where x is any amino acid and lower case letters represent more frequently substituted aas in the consensus). The whole repetitive region is almost entirely composed of such repeats. These repeat units may form a coherent template, facilitate big ice crystal formation, and promote ice nucleation at high, subzero temperatures (Graether and Jia, 2001). The divergence of different species and the similarity of INPs among these epiphytes suggested a possible horizontal gene transfer due to their similar ecological niche on common plants (Edward *et al.*, 1994). Interestingly, when a two-repeat unit (96 aa) peptide from *P. syringae* was used experimentally, it modified ice crystal shape in a way similar to that of AFPs (Kobashigawa *et al.*, 2005), suggesting a mechanistic link between these two different protein families.

1.3.2 Control of ice nucleation

A variety of methods have been tried to utilize bacterial ice nucleation for practical or commercial reasons, including reducing the formation of frost on agricultural crops at high subzero temperatures or by promoting freezing at high temperatures to save energy. Recently, bacterial ice nuclei have been used in agriculture, the food industry, recreational and industrial applications. A preparation of *P. syringae* (commercial name “Snowmax”) has successfully been used in artificial snow making in ski areas, while *X. campestris* has been used in the freeze-concentration of lemon juice, milk, strawberry jam

and egg whites (Jung, 1990; Liao and Ng, 1990; Lindow, 1987; Goodnow *et al.*, 1990; Watannabe *et al.*, 1989; Kumeno *et al.*, 1994).

Reducing the population of INA⁺ bacteria may improve the production of some crops. INA⁺ bacteria were found to increase their population rapidly on flowers of fruit crops, corn and snap bean leaves, coincidental with the period of maximum likelihood of frost damage (Lindow *et al.*, 1982a; 1982b; Cody *et al.*, 1987). To decrease the probability of frost, several studies were tried to reduce the population of INA⁺ bacteria on crops (Okada *et al.*, 1990; Anderson and Ashworth, 1986; Menkissoglu and Lindow, 1991; Watababe *et al.*, 1990). Smoke was used to reduce INA⁺ bacteria (*P. syringae* and *E. herbicola*) *in vitro* and *in vivo*, and a decreased ice nucleation temperature was recorded accordingly (Zagory *et al.* 1983). The effect of soluble and complexed copper applications was compared after application to the surface of navel oranges and beans, and a decrease in the copper-sensitive *P. syringae* population as well as the related INA was observed (Menkissoglu and Lindow, 1991). Streptomycin treatment and UV irradiation also reduced ice nucleation frequency (Anderson *et al.*, 1986). Although antibiotic treatments were effective in reducing frost damage, the potential of introducing antibiotic resistance into the environment was an issue that restricted the use of bactericides. Introducing competitive INA minus (INA⁻) *P. syringae*, *P. fluorescens* and recombinant INA⁻ *P. syringae* to treat frost-sensitive crops was commercialized by Plant Health Technologies (Lindow, 1987; Lindow *et al.*, 1989). It took almost 10 years to have the public to accept it.

Other ways of using AFP or polymers to decrease ice nucleation frequency have been investigated as well. With traditional INA assays, AFGP was shown to decrease bacterial INA (Parody-Morreale *et al.*, 1988a), but when a nucleation spectrometer was

used to repeat these observations, Holt (2003a) found that different concentrations of AFP could affect INA agents differently. At low concentrations, Type III AFP decreased INA of both tap water and bacteria, but at high concentrations, Type III AFP increased bacterial INA (Holt, 2003a). Similarly, the addition of AFGP reduced tap water INA at low concentrations, but increased bacterial INA at high concentrations (Holt, 2003a). In contrast, polyvinyl alcohol (PVA) showed good inhibition to both tap water INA and bacterial INA (Holt, 2003a). Similar results were obtained by Wowk and Fahy (2002); they found that PVA was effective to both bacterial ice nuclei and 5/7 of organic ice nuclei, while polyglycerolpolymers (PGL) selectively inhibited bacterial INA. They also suggested that a combination of PGL and PVA could be useful for cryopreservation. Using a differential scanning calorimeter (DSC), ethylene glycol, glycerol, polyethylene glycol (PEG) 300, $\text{Ca}(\text{NO}_3)_2$ as well as other salts were compared in the presence of different ice nucleation agents and it was found that heterogeneous INA decreased with increasing solute concentrations (Zobrist *et al.*, 2008).

1.4 Premelt theory, Kelvin effect and pressure theory

To understand the interaction of AFPs with ice, it is important to briefly review some of the physical characteristics and theories of ice. The ice premelt theory was developed by Dash *et al.* (1995), which suggests that the slippery property of ice, glaciers and snow mountains are a result of an ice premelt before the bulk of the ice melts. Slightly below melting temperatures, pressures, heat or energy input (*e.g.* friction, or force) can cause a small part of ice premelt. The prevailing theory explaining TH is the Kelvin effect (Wilson, 1993). In this model, AFPs adsorbs to ice surfaces and makes the ice grow as a convex ice front, which produces more boundaries, grains and barriers and in turn, more energy is required for the ice to grow. Based on these two theories,

Kristiansen and Zachariassen (2005) developed a pressure theory. They proposed a reversible AFP binding to ice occurring below the melting temperature as opposed to an irreversible AFP binding to ice occurring between the melting temperature and the freezing temperature. According to this theory, the border of water and ice is a fluid-like layer, creating a higher pressure environment to produce convex ice crystal fronts, and this higher pressure is required to decrease the freezing temperature.

1.5 Methods used in ice-related research

Ice nucleation or IR inhibition is related to solutes in water, but freezing speed, pressure, volume and other factors also affect ice nucleation. Some simple methods are used in ice nucleation assays, IR inhibition and TH assays as follows:

1.5.1 Ice nucleation assays

1.5.1.1 Drop-freezing assays: Vali and Stansbury (1966) described some examples of this method, which is a traditional way to assay bacterial INA using several microliter drops on plate, with the plate is cooled by the circulation of antifreeze liquid or liquid nitrogen. Frozen drops can be detected utilizing light that has passed through cross polarized filters and recorded using video images interfaced to a computer. The temperature of the plate is taken as the temperature of the drops. When 90% of the drops are frozen, the temperature is recorded as the ice nuclei temperature. It is customary, however, to plot data as ice nuclei/cell, do sample dilutions and calibrate the concentration of the viable cells as well (Lindow *et al.*, 1978; Vali, 1971).

1.5.1.2 Large volume assays (thermal analyzer): A thermal analyzer or a calorimeter can also be used in the study of phase changes. When ice begins to nucleate, heat will be produced and the temperature of the system will change at the same time. The large volume assays (1-1.4 ml) used in this thesis are based on controlling the cooling and

heating speed process. Instead of measuring the probability of freezing at a particular temperature, calorimetry measures the actual temperature or the temperature difference between the samples and a reference with the same volume of ethylene glycol. Bacteria with INA can be detected using this assay (Walker *et al.*, 2008).

1.5.1.3 Differential scanning calorimeter (DSC) assays: Differential scanning calorimeter is a newer version of a calorimeter which is more often used for cryobiology and food polymer science as well as in analytical chemistry laboratories (Sacha and Nail, 2009; Clausse *et al.*, 2005; Slade and Levine, 1991). DSC has integrated software to process all of the data and draw graphs. Some small sample assays (μL) were reported in several studies. For example, bacterial INA was measured with this method by Parody-Morreale *et al.* (1988b). By using DSC, Zobrist *et al.* (2003; 2008) compared water activity in salts with different molar masses, as well as polyethylene glycol in homogenous ice nucleation and heterogeneous ice nucleation systems, and Kimizuka *et al.* (2008) measured the homogenous nucleation depression and equilibrium melting depression of different molecules of polymers (PEG, PVP and dextran), and concluded that non equilibrium freezing depends on solution properties.

1.5.2 Antifreeze activity

1.5.2.1 Thermal Hysteresis (TH) assay: Traditionally, TH assays are used to measure the activity of AFPs. Nanoliters of sample material are loaded onto a cold stage, precoated with immersion oil. With the aid of microscopy, the temperature difference of a single ice crystal burst and melt is recorded as TH.

1.5.2.2 Ice Recrystallization (IR) assays: These assays have been used to assess AFP activity in several studies (Walker *et al.*, 2006; Wilson *et al.*, 2006; Knight *et al.*, 1988), but it is important to recall that not all samples testing positive in IR assays are AFPs or

have TH activity. Briefly, samples are loaded in microcapillaries and frozen at -50°C before being transferred to -6°C. Ice crystal sizes are monitored by capturing images immediately and after annealing overnight at -6°C.

1.5.2.3 Differential scanning calorimeter (DSC) assays: DSC assays were described under ice nucleation assays, but they may also be used to study the effects of AFPs as well (Hansen and Baust, 1988; Hansen and Carpenter, 1993; Amornwittawat *et al.*, 2008; 2009). Several agents that may enhance AFP activities were compared on the temperature difference between holding temperature and freezing temperature using DSC (Amornwittawat *et al.*, 2008; 2009).

1.6 Thesis goals and objectivities

Previous work has shown that biofilm formation confers some resistance to antibiotics, radiation and desiccation. However, limited work has been done on the association of biofilms and low temperature survival. It is not known, for example, how bacterial EPS affects freeze-thaw and how different microbes interact with each other during cold stress. Recently, ice-related microbial research has shifted from identifying bacteria with INA to bacteria with AFPs (Gilbert *et al.*, 2004; Obata *et al.*, 1999; Kawahara *et al.*, 2007), or to ecological studies of different species living in the Arctic, Antarctic, glaciers and other cold environments. Despite the rich history of research in this area (Steven *et al.*, 2007; 2008; Karl *et al.*, 1999; Mountfort *et al.*, 1997; Koch *et al.*, 2009; Groudieva *et al.*, 2004; Pearce, 2008; Yergeau *et al.*, 2007), several questions still need to be answered.

1.6.1 What happens when epiphytes on perennial plants are subject to frost damage? Can EPS interact with other ice-associating properties? Since most previous work has been done with single isolates, a survey of the epiphytic community and their low temperature

adaptations are of interest. What kinds of bacteria exist in this community, and do they have special cryoprotective strategies?

1.6.2 Do soil bacteria or non plant pathogens have INA? Since INPs have been traditionally associated with plant pathogens, this is of interest. If non epiphytes with INP exist, are their amino acid sequences similar to INP from those epiphytic bacteria?

The overall objective of this thesis is to examine low temperature survival and/or ice-related activity in lesser known microbes. It is known that microbial products can adsorb to ice and either facilitate heterogeneous nucleation or inhibit ice growth. Although these activities appear to be in opposition, it is my hypothesis that they will be in fact related. To test my hypothesis and the above mentioned questions my specific goals include:

1. Investigate some properties of an epiphytic bacterial community and their ice associated activities.
2. Characterize the INA from a soil bacterium, *P. borealis*, isolated by ice incorporation.
3. Examine the recombinant expression of the INP gene isolated from *P. borealis* by marking it with jelly fish green fluorescent protein (GFP).

1.7 References

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Preamble 1

In the Introduction, a brief review of the literature with respect to the survival of microorganisms at low temperature. Some of the challenges posed by ice formation were reviewed and some classical studies in microbiology were mentioned. During mid winter, I noticed that some plants in my garden were still green, and I was inspired by this observation to think about the epiphyte community on the leaves. Although other studies, including those previously cited, have investigated individual isolates from such consortia and characterized bacteria with INA, relatively little work has been done with bacteria with other ice-associating properties. Thus, the forthcoming chapter works was an initial attempt to describe some of the culturable microorganisms and some of their properties.

Chapter 2

Biofilms, ice recrystallization inhibition and freeze-thaw protection in an epiphyte community

2.1 Statement of co-authorship

This chapter represents a paper “in preparation” for submission, with authors Zhongqin Wu, Frederick W.K. Kan, Yi-Min She, and Virginia K. Walker.

I designed and prepared the 16S rDNA library of culturable bacteria, prepared extracellular polymeric substance, performed freeze-thaw assays and did all the ice-association activities of the isolates. Dr. Frederick W.K. Kan performed the scanning electron microscopic examination of the isolates as shown in Fig. 2.3. Dr. Yi-Min She performed the MS of the main protein product as shown in Fig. 2.5. I also wrote the initial draft of this manuscript and participated in the subsequent editing.

2.2 Abstract

A microbial community derived from frost-exposed leaves was used to construct a microbial 16S rDNA library. Subsequent DNA sequencing as well as ice nucleation, ice recrystallization (IR) inhibition and freeze-thaw resistant assays were used to characterize individual isolates. Amongst the bacterial species identified, *Pseudomonas syringae* J6 promoted ice nucleation close to 0°C, and cultures of *Sphingobacterium kitahiroshimense* Y2, *Flavobacterium sp* Yin and *Erwinia billingiae* J10 showed IR inhibition as well as significant freeze-thaw resistance. Adhesion assays and scanning electron micrographs provided evidence of biofilm formation in two of these strains, *S. kitahiroshimense* and *E. billingiae*. Purification of the *E. billingiae* extracellular polymeric substance (EPS) showed that the preparation could help confer freeze-thaw protection to more susceptible

bacteria, which was at least partially due to a protein component of the EPS. Subsequently, a 35 kDa major protein found in the *E. billingiae* EPS preparation was putatively identified as an outer membrane protein A. Despite the experimentally-dictated limited survey, this report nevertheless underscores the wealth of low temperature adaptations and the complexity found in a single epiphyte community.

2.3 Introduction

Winter survival in temperate latitudes has necessitated the acquisition of low temperature survival strategies in many organisms. Microorganisms have been particularly successful in this regard and can thrive in such cold environments as glaciers, polar soils, high latitude salt springs, and sea ice (Cavicchioli, 2002; Shivaji *et al.*, 1989; Stallwood *et al.*, 2005; Kaartokallio, 2005; Sullivan and Palmisano, 1984; Edgcomb *et al.*, 2009; Zierenberg *et al.*, 2000). Depending on the species, microorganisms can respond to low temperatures by changing their membrane lipid constitution to maintain fluidity, producing cold-shock proteins, chaperones and cold-acclimation proteins, as well as the synthesizing cold-adapted enzymes (Cavicchioli, 2002; Cao-Hoang *et al.*, 2008; Villeret *et al.*, 2008; Huston *et al.*, 2004). A few microbes produce specialized products, such as ice nucleation proteins, which allow for rapid freezing at temperatures close to 0°C (Lindow *et al.*, 1982; Orser *et al.*, 1985), and other species inhibit ice recrystallization (IR), which may be associated with the synthesis of antifreeze proteins (*e.g.* Sun *et al.*, 1995; Gilbert *et al.*, 2004; Muryoi *et al.*, 2004; Kawahara *et al.*, 2007), or the production of extracellular polymeric substance (EPS; Regand and Goff, 2002; Marx *et al.*, 2009; Krembs *et al.*, 2002). For example, EPS production is known to enhance low temperature survival of marine bacteria with ~30% of a sea ice bacterial community attached to living organisms or other substrates using EPS matrix structures, likely facilitating mutualistic

interactions (Sullivan and Palmisano, 1984). Together, sessile bacteria and EPS may form biofilms, which are well known to confer stress resistance to a variety of toxic challenges including antibiotics, osmotic changes, nutrition limitations, phagocytes and radiation exposure (Sutherland 1982; Gilbert *et al.*, 1997).

Recently, we have developed a laboratory-based selection regime to identify microbes from temperate climates with low temperature adaptations. Although previous studies are not extensive, in at least one case, a soil bacterium showing high freeze-thaw resistance, as well as IR inhibition properties, was shown to facilitate the survival of a more susceptible species, suggesting that low temperature adaptations may be of community benefit (Walker *et al.*, 2006). However, there is no way to determine if the particular bacteria identified were originally associated in these composted soil collections. Here we have surveyed an epiphyte community from frost-exposed perennial leaves in order to first characterize some of their low temperature adaptations, and secondly to investigate if these adaptations could benefit other members of this spatially restricted community.

2.4 Materials and methods

2.4.1 Samples: Leaves of *Chrysanthemum indicum* (cultivar “Arizona Sun”) were collected in Kingston, Ontario, in mid January 2008, after 81 days at temperatures that had dipped below 0°C (http://www.weatheroffice.gc.ca/canada_e.html, Environment Canada). After washing with sterilized water, the leaves were homogenized with 0.01 M phosphate buffered saline (PBS, PH 7.4), and stored in -20°C until processing.

2.4.2 Culturable microbial community identification: Serially-diluted leaf homogenates were inoculated on 10% tryptic soy broth agar (TSB; Difco, MI, USA) and 50 mL sterilized 10% TSB. After incubation overnight at 22°C, the liquid cultures were used to

construct microbial community libraries (see below). Individual colonies growing on agar were examined to identify those with distinct morphologies, which were then streaked on fresh TSB agar plates in order to isolate single colonies.

DNA from the community liquid cultures, as well as from single colonies, was isolated using a standard phenol-chloroform method (Sambrook *et al.*, 1989). Bacterial 16S rRNA genes were amplified from the purified genomic DNA with a forward primer F8 [5'-AGAGTTTGATCCTGGCTCAG] and a reverse primer R1406 [5'-ACGGGCGGTGTGTAC] (Telang *et al.*, 1997). Polymerase chain reactions (PCR) were performed as 94°C for 5 min, followed by 94°C for 0.5 min, 55°C for 0.5 min, 72°C for 2 min for 35 cycles, and finished with an extension at 72°C for 30 min. PCR products were electrophoretically separated on 1% agarose gels, purified using standard methods (Nucleotide removal kit, Qiagen, ON, Canada), and then cloned into pGEMT to create 16s rRNA gene libraries (Promega, CA, USA). Individual plasmids from the microbial community library (48) and derived from single colonies (12) were sequenced and subsequently analyzed using BLAST (National Center for Biotechnology Information web site and the Genbank database; www.ncbi.nlm.nih.gov).

2.4.3 Ice-association assays: Ice nucleation activity (INA) of different isolates was tested as previously described (Wu *et al.*, 2009) and compared to *Pseudomonas syringae* B728a, , which was used as a positive control. Briefly, samples (1 mL) were cooled from 6°C to -20°C at 0.2°C/min and the heat of crystallization recorded automatically. Small volume (2 µL) assays were conducted analogously but data were collected using crossed-polarized images of the frozen samples. IR inhibition assays were performed as previously described (Walker, *et al.*, 2006) by sampling leaf homogenates, *P. syringae* B728a, and selected isolates (see Results) in microcapillaries and freezing at -50°C before

being transferred to -6°C. Sizes of crystals were monitored by capturing images immediately and after annealing overnight at -6°C, as indicated (Knight and Devries, 1988). Ice association assays were routinely done three or more times.

2.4.4 Freeze-thaw resistant assays: Freeze-thaw resistance was assessed using a cryocycler (Walker *et al.*, 2006). Isolates were normally assayed in triplicate, but experiments using extract preparations were done in duplicate. For the extract experiments, EPS preparations (see below) were UV-sterilized for 30 min prior to their addition to cultures (0.8 mL) of individual tested isolates or control *E. coli* TG2. Extracts containing digestive enzymes were prepared by treating the EPS preparations (0.2 mL) with proteinase K (1 mg/mL) or phospholipase C (40 U/mL) at 37°C for 60 min and subsequently inactivating the enzymes at 65°C for 30 min. Control experiments contained EPS preparations without enzyme addition, and as well, 10% TSB. Bacterial viability was assessed before subjecting the cultures to freeze-thaw cycles and after 12 and 24 freeze-thaw cycles (each cycle was 1 h at -18°C and 1 h at 5°C). Numbers of viable bacteria were determined as colony forming units (CFU) on 10% TSB agar, incubated at 22°C for 144 h. Comparisons between strains was facilitated by using stationary phase cultures (at $\sim 10^8$ cells/mL), assessing cell number and subsequently normalizing to 1×10^8 cells/mL.

2.4.5 Adhesion, motility and biofilm determinations: Selected isolates (see Results) and *E. coli* TG2 controls were assessed for their adhesion to polyvinyl chloride (PVC) microtitration plate as previously described (Balestrino *et al.*, 2008; Rashid *et al.*, 2000). Bacterial cultures in stationary phase were inoculated at 1% concentration to PVC microtitration plates using 10% TSB medium and cultured at 22°C for 48 h and 72 h. After adding crystal violet (0.5% final concentration) to the wells and incubating for 15 min, the medium and unattached bacteria were decanted and the wells were rinsed 5 times

with distilled water. Adhering dye was subsequently washed from the wells with 95% ethanol (200 μ L) and the absorbance of the ethanol was determined spectrophotometrically at 595 nm.

Biofilms were visualized by scanning electron microscopy (SEM). First, selected isolates (see Results) and control *E. coli* TG2 cultures were streaked on 10% TSB agar and incubated at 22°C for 2 days, prior to incubation at 4°C for 5 days. The cells, with their substrates were fixed with 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), overnight and then washed three times (5 min each) with 0.01 M phosphate buffered saline (PBS; pH 7.4). Each sample was retrieved by cutting 4 or 5 agar-cube plugs (2 \times 5 mm) from regions containing cells. The plugs from each investigated strain were then soaked in vials containing 1% osmium tetroxide in 0.1 M cacodylate buffer, gently shaken for 2 h, and subsequently washed three times for 5 min each with 0.01 M PBS. The samples were then dehydrated with a graded series of ethanol (30% for 5 min, 50% for 5 min, 75% for 5 min, 95% for 5 min, and finally 100% for 3 X 10 min) and afterward dried with ethanol/CO₂ in a critical drying apparatus. The dried samples were mounted on stubs, coated with a thin layer of gold and examined under a S-450 scanning electron microscope (Hitachi, Tokyo, Japan) operated at 20 kV. Images were examined and recorded at magnifications of approximately 5,000 X, 10,000 X and 20,000 X.

Selected bacterial strains (see Results) were examined under transmission electron microscopy (TEM) after negative staining with phosphotungstic acid (PTA). Briefly, bacterial cells, which had been cultured in 10% TSB with gentle shaking overnight at 22°C (~to 1 x 10⁸ cells/mL), were centrifuged at 10,000 g for 10 min. The pelleted cells were resuspended in 0.01 M PBS (1 mL, pH 7.4), and recentrifuged three more times, resuspending the second time in 0.01 M PBS as described, and twice more in

50 mM MgSO₄ (pH 7.4; 1 mL, followed by 50 µL). The washed cells were then negatively stained by mixing 10 µL of the bacterial suspension with 2 µL of 2% PTA (pH 7.0) for 1 min. Subsequently, the treated cells were transferred onto Formvar-coated gold grids and after gently absorbing excess liquid with Whatman filter paper. Finally, the grids were air dried the cells were viewed on a Hitachi H-700 electron microscope operating at 75 kV (VanDyke *et al.*, 2008; EI-Mestrah *et al.*, 2002). Micrographs of representative images were captured at magnifications of 15,000 X and 9,000 X.

Bacterial motility was assessed by preparing modified semi-solid Azotobacter agar (0.2 g/L KH₂PO₄, 0.8 g/L K₂HPO₄, 0.2 g/L MgSO₄, 0.1 g/L CaSO₄, 0.5 g/L yeast extract, 2 g/L mannitol, and a trace of Na₂MnO₄ and FeCl₃ in different agar or agarose concentrations, as indicated). Swimming assays were conducted on 0.3% agarose, swarming assays on 0.5% agar and twitching assays on 1.5% agar. All motility assays were initiated by the transfer of colonies grown on 10% TSB to the appropriate Azotobacter agar with sterilized tooth picks as described (Rashid *et al.*, 2000). All Petri dishes were sealed in parafilm (Alcan Packaging, WI, USA) to prevent evaporation.

2.4.6 Extracellular polymeric substance testing: A single large, wet colony of *E. billingiae* J10 was isolated and used to inoculate Azotobacter broth medium (10 mL). After 24 h, these initial cultures were used to inoculate 4 L of medium in shake flasks. After 5 days at 22°C, the cultures were transferred to 4°C for 1 week. After centrifugation at 8,000 g for 60 min, the cell pellet was discarded and the supernatant was added to two volumes of 95% ethyl alcohol or, alternatively, 60% saturated ammonium sulfate. After precipitation overnight at 4°C, the suspension was centrifuged at 8,000 g for 90 min. The precipitate was suspended in distilled water and dialyzed against 6 changes of distilled water over 3 days. The dialyzate was then lyophilized in plastic centrifuge tubes (50 mL) and

subsequently resuspended in distilled water (2 mL). Proteins were separated on a 12.5% SDS polyacrylamide gel and visualized by staining with Coomassie Blue G250 stain (Sigma, QC, Canada). Protein concentration was determined using a bicinchoninic acid protein assay (BCA; ThermoScientific, IL, USA) with a bovine serum albumin standard curve. The polysaccharide concentration was determined using a phenol-sulfuric acid assay using glucose as reference (Marx *et al.*, 2009). The EPS preparation was also used to investigate its potential for freeze-thaw protection, and its susceptibility to proteinase K and phospholipase C as previously described (see 2.3.4).

Further analysis of the EPS preparation was undertaken by excising the most prominent band (~35 kDa) from the SDS-PAGE gels (see above). After crushing into small pieces, the gel was destained with 25 mM ammonium bicarbonate in 50% acetonitrile solution, and subsequently treated with 10 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate at 56°C for 1 h. After subsequent incubation with 55 mM iodoacetamide at 24°C for 45 min, the protein band was dried using a SpeedVac centrifuge and digested overnight in 25 mM ammonium bicarbonate containing 1 ng/μL sequencing grade trypsin (Roche, QC, Canada). Proteolytic peptide products were sequentially extracted using 0.1% formic acid, followed by 60% acetonitrile/0.1% formic acid, and finally pure acetonitrile. The collected fractions were dried using a SpeedVac centrifuge, and desalted using C18 Zip tips (Millipore, MA, USA) and used for subsequent analysis.

2.4.7 MALDI QqTOF mass spectrometry: Matrix assisted laser desorption ionization (MALDI) of the purified EPS protein samples was performed in an Applied Biosystems / MDS Sciex QStar XL Quadrupole Time-of-Flight (QqTOF) mass spectrometer (Department of Chemistry, Queen's University), equipped with a MALDI II source and a

nitrogen laser operating at 337 nm. The samples were prepared at a ratio of 1:1 (v/v) of the peptide digest to matrix (*i.e.* 2, 5-dihydroxybenzoic acid), and subsequently dried on a stainless steel MALDI plate. After MALDI MS mapping, the peptide sequences were identified by tandem mass spectrometry (MS/MS) measurements using argon as the collision gas. The obtained peptide fingerprint masses were searched using the MS-Fit program against the NCBI database with ProteinProspector at the UCSF web site (<http://prospector.ucsf.edu>) and the search for the MS/MS ions on each tandem mass spectrum used the Mascot search engine (MatrixScience; <http://www.matrixscience.com>). The search parameters were set for two missed trypsin cleavage sites, and the common modifications of methionine oxidation, carbamidomethylation, asparagine and glutamine deamidation to aspartic acid and glutamic acid, N-terminal pyroglutamation. The mass tolerance between calculated and observed masses in database search was in the range of ± 100 ppm for the MS peaks and ± 0.2 Da for the MS/MS fragment ions.

2.5 Results

2.5.1 The microbial community, ice associating activities and freeze-thaw survival: After culture and 16S rDNA sequencing, 15 different bacterial isolates (Table 2.1) were assigned to genera, or genera and species (using $\geq 97\%$ identity with sequence in the databases for species identification) as: *Pseudomonas sp.* L14, *P. syringae* J6, *Massilia aurea* J9, *Erwinia billingiae* J10, *Rahnella sp.* W11, *Duganella zooglooides* Y11, *Flavobacterium sp.* Yin, *Sphigomonas sp.* J2, *Sphingobacterium kitahiroshimense* Y2, *Geobacterium sp.* J6, *Chryseobacterium sp.* J3, *Janthinobacterium sp.* L1, *P. fluorescens* L37, *P. trivialis* L48 and *P. veronii* L32 (Table 2.1). These specific strain designations are assumed for the balance of this report. Of these, the *P. syringae* J6 isolate had INA (Table 2.1) showing activity as high as the type I INA in the *P. syringae* B728a controls. IR

inhibition, as evidenced by the retention of small size ice crystals after overnight annealing at -6°C , was observed in homogenates of whole leaves (not shown), as well as in 6 of the isolates including *Rahnella sp.*, *Duganella zoogloeooides*, *Chryseobacterium sp.*, *E. billingiae*, *Flavobacterium sp.* and *S. kitahiroshimense* (Table 2.1). In all, almost 50% of the recovered isolates, for which sequence had been obtained, had ice-associating activities.

Approximately half of the isolates showing ice-associating activities, including *P. syringae*, *E. billingiae*, *Flavobacterium sp.* and *S. kitahiroshimense*, were chosen to investigate their resistance to multiple freeze-thaw cycles, with the result that these epiphytes showed much higher levels of freeze-thaw survival than *E. coli* TG2 controls (Fig. 2.1A). *S. kitahiroshimense* had the highest levels of freeze-thaw survival, losing little viability over the entire course of the experiment, and *E. billingiae* and *Flavobacterium sp.* showed only ~ 3 orders of magnitude decline in viability after 48 cycles (Fig. 2.1A). In contrast, the *P. syringae* isolate, as well as the *E. coli* controls, were more freeze-thaw susceptible and these cultures lost almost all viability after 48 freeze-thaw cycles (not shown).

2.5.2 Biofilm, adhesion and motility analyses: Crystal violet staining for the presence of biofilm showed that *E. billingiae* had a mean of 44% more biofilm production than control *E. coli* cells (Appendix 2). Of the recovered, tested bacteria, *Flavobacterium sp.* showed the least adhesion, with putative biofilm accumulation at levels that were a mean of 15% less than that produced by *E. coli* under the same conditions. *E. billingiae* showed significant motility on semi-solid Azotobacter agar, with characteristic swimming behavior and on swarming agar; *E. billingiae* colonies continued to grow, with distinct swarming rings between different growth stages, branching into new colonies when the

colony grew to 2-3 cm diameter (Appendix 3). Such dendritic swarming and inconsistent consolidation appeared similar to descriptions of *Proteus mirabilis* (Allison and Hughes, 1991), a flagellated biofilm producer. In contrast, swarming motility was not observed in *S. kitahiroshimense* (not shown), but this isolate showed higher adhesion than *E. coli* and *Flavobacterium sp.* (Appendix 2).

In order to verify these macroscopic observations, *S. kitahiroshimense*, *Flavobacterium sp.* and *E. billingiae* cultures were examined using electron microscopy. Fimbriae-like structures but no clear flagella were seen after negative staining of *S. kitahiroshimense* (Appendix 3) and as well, no flagella were seen after examination of *Flavobacterium sp.* (Appendix 4). Electron micrographs of negative-stained bacteria with TEM, however, corroborated the motility observations for *E. billingiae* since a flagellum was visualized on single cells, and two flagella were associated with dividing cells (Fig. 2.2). SEM examination of *S. kitahiroshimense* showed filaments protruding out of some of their rough surfaces, suggestive of biofilm formation (Fig. 2.3), but there was no obvious extracellular structure associated with *Flavobacterium sp.* (Fig. 2.3). A small amount of matrix was visualized with the *E. coli* controls, which had thin filament-like structures (Fig. 2.3). In contrast, SEM examination of the *E. billingiae* showed complex structures, which appeared to be composed of biomass matrix material, void space and with ‘slimy’ structures adhering to the bacteria cells (Fig. 2.3). When *E. billingiae* was subjected to preliminary characterization of this matrix, the *E. billingiae* EPS preparation appeared to be typical of biofilm material, primarily composed of polysaccharides, at an average concentration of 2.4 mg/mL as determined using phenol-sulfuric acid assays, and a mean 1.5 mg/mL protein concentration.

Since two of the three isolates showing the highest freeze-thaw survival appeared to be associated with ice-associating activities as well as biofilm production, we investigated the possibility that the presence of biofilm could increase low temperature survival of the other community members. After 24 freeze-thaw cycles, the viability of the more freeze-sensitive co-epiphyte, *P. syringae*, was increased about 1000-fold with the addition of the *E. billingiae* EPS preparations (Fig. 2.1b). *E. coli* cultures without additives were completely inviable after 24 freeze-thaw cycles, but viability was increased by an estimated 4 orders of magnitude with the addition of the EPS preparation. To investigate the component of the preparation that conferred such a benefit, the EPS was subjected to limited digestion with phospholipase C and proteinase K prior to addition. Phospholipase digestion reduced the apparent protection about 2-fold but proteinase K digestion resulted in a 10 fold reduction of *E. coli* viability after 24 cycles of freeze-thaw treatment, compared to experiments using the untreated *E. billingiae* EPS (Table 2.2).

Since the protein component of the EPS preparation appeared important for freeze-thaw protection, SDS-PAGE analysis was used to visualize the major proteins in the *E. billingiae* EPS preparations (Fig. 2.4). After recovery of the major 35 kDa protein from the gel and subsequent digestion with trypsin, several distinct peaks were seen using MALDI QqTOF mass spectrometric analysis (Fig. 2.5). After matching the peptide fingerprint using the Mascot algorithm and with reference to the peptide databases, a best match was obtained with the outer membrane protein A (OmpA) from *E. tasmaniensis* Et1/99 (representing about 14.7% of the OmpA sequence), *E. coli* ED1a and outer membrane protein F (representing about 7.7% of the OmpF sequence), a porin from *Pantoea sp.* At-9b (Fig. 2.6).

2.6 Discussion

Epiphyte communities on the overwintering leaves of perennial plants in temperate latitudes are exposed to freezing conditions, water stress and UV-radiation (Wilson *et al.*, 1999). As a consequence, they have had to develop strategies to adapt to these stresses. Although individual isolates may not be equipped for survival, the consortium as a whole may allow the opportunity for bacteria of different genera to share extracellular macromolecules. Here we found that almost 50% of the identified 15 cultured isolates from frost-exposed perennial leaves showed some ice-associating activity, either INA as represented in the *P. syringae* strain or IR inhibition as shown by *S. kitahiroshimense*, *Flavobacterium sp.*, *E. billingiae*, *Chryseobacterium sp.*, *Rahnella sp.*, and *Duganella zoogloeoide*. Both these ice-associating activities could help low temperature survival either by reducing bacterial membrane damage caused by growing ice crystals or by ensuring freezing at temperatures close to 0°C and the generation crystallization heat.

Since a *Chryseobacterium* isolate with high freeze-thaw resistance and IR inhibition had been previously described (Walker *et al.*, 2006), four other isolates from the present leaf collection were selected for their ability to survive repeated freeze-thaw stress. Perhaps not surprisingly, those showing IR inhibition were amongst the most freeze-thaw hardy. *S. kitahiroshimense* lost little viability even after 48 freeze-thaw cycles, which may be related to its fatty acid composition (Matsuyama *et al.*, 2008) or its ability to restrict ice crystal growth and its association with biofilm formation as shown here (Fig. 2.3). *E. billingiae* also showed moderately high freeze-thaw resistance (Fig. 2.1). Further investigation of *E. billingiae* showed that it was motile, with flagellum and macro colony morphology. Motility and biofilms are related in certain bacteria such as *E.*

carotovora where flagella-associated motility is believed to be important for biofilm establishment and morphology (Mijan and Shinji, 2006). Subsequent characterization, both with adhesion assays and by SEM, demonstrated that the *E. billingiae* isolate produced a biofilm that appeared to be dominated by a thick matrix filled with some spaces. It is possible that this biofilm could act as a hydrocolloid-like stabilizer that would prevent the migration of forming ice crystals and also act to resist melting, thus reducing the probability of recrystallization (Regand and Goff, 2006). Indeed, it could act like xanthan gum that is associated with biofilm production in the *Xanthomonas* plant pathogen, and which is used by the food industry as a thickener and to inhibit ice recrystallization (Regand and Goff, 2002).

By themselves, individual isolates showed a range of freeze-thaw resistance properties (Fig. 2.1), but as a group of epiphytes they had presumably survived months of freeze-thaw stress events. Complex biofilms can accommodate multiple bacteria on a substrate (Rogers *et al.*, 1994), and thus we considered that at least some of the community members identified on the perennial leaves might be associated together in a biofilm and thus gain mutualistic low temperature stress resistance. The soil bacterium, *Chryseobacterium sp.* C14, with IR inhibition activity conferred freeze-thaw tolerance to more cold susceptible bacteria (Walker *et al.*, 2006), but this phenotype was attributed to a putative antifreeze protein without investigation of any biofilm properties. Thus having established the relative freeze resistance of *E. billingiae*, with only a 3 order of magnitude in decline in viability after 48 freeze-thaw cycles, as well as its biofilm properties, it was of interest to determine if *E. billingiae* EPS could contribute to consortium resistance. When the EPS preparations were added to isolates that had shown low freeze-thaw resistance in the laboratory, the viability of the recipient cells increased over 3 and 4

orders of magnitude for *P. syringae* and control *E. coli*, respectively (Fig. 2.1b), indicating that *E. billingiae* could be important for the overall low temperature stress tolerance of the community. Independent experiments using a relatively freeze-susceptible *P. borealis* DL7 isolate (Wu *et al.*, 2009) and the addition of the biofilm-producing bacteria *E. billingiae* and *S. kitahiroshimense*, also resulted in the recovery of about 5 times more viable *P. borealis* cells after 12 freeze-thaw cycles (Appendix 5 and 6). Together these experiments show that the ability to confer freeze-thaw resistance is not host- or recipient- strain specific. Previous studies of microorganisms isolated from sea ice and from various Arctic regions have shown the association of bacterial EPS and ice (Marx *et al.*, 2009; Kim and Yim 2007; Nichols *et al.*, 2005), and links have been established between biofilms and resistance to cold and osmotic stress (Wallner *et al.*, 1986; Roberson and Firestone, 1992; Marx *et al.*, 2009; Olien *et al.*, 1965; Regand and Goff, 2002; 2006). Here we have further strengthened this association using EPS preparations and laboratory experiments.

Polysaccharides represent the bulk of the biofilm mass and for the *E. billingiae* EPS preparation, they represented 1.6 times the concentration (mg/mL) of the protein component. Although proteins are less abundant, other studies have shown that protein concentrations increase as biofilms mature and that these proteins are important for biofilm dissolution and subsequent pathogen invasion (Stoodley *et al.*, 2002). Now we show here that at least some of the conferred freeze-thaw protection appears to be associated with the EPS protein component since limited proteolysis reduced the capacity of the extract to increase the survival of susceptible *E. coli* approximately 10 times. We believe that the EPS of the *E. billingiae* strain is not unique because initial characterization of the protein component produced a 35 kDa OmpA with peptide segment identity to the

OmpA of *E. tasmaniensis* and *E. coli*.

OmpA is a highly conserved and abundant membrane protein, which in other species is known to increase in quantity during biofilm formation and is important for biofilm integrity (Orme *et al.*, 2006; Smith *et al.*, 2007; Boles and Horswill, 2008). OmpA likely regulates its channel size under different conditions (Zakharian and Reusch, 2003), is vital for osmotic stress and its importance to the host has been clearly established by noting that strains with a disruption in the *OmpA* gene have a thinner biofilm, and are more sensitive to detergent, salt and antimicrobial peptide exposure (Choi *et al.*, 2008; Namba *et al.*, 2008; Wexler *et al.*, 2009; Llobet *et al.*, 2009). It remains to be determined if the high freeze-thaw viability in our *E. billingae* isolate and the conferred protection to other bacteria, mediated by co-culture or the EPS preparations, can be at least partially attributed to role of OmpA in biofilm integrity and adhesion. Certainly it has been previously shown that epiphytes are associated with biofilm production (Morries *et al.*, 1997), with studies demonstrating the importance of biofilms or EPS in stress resistance (Donlan, 2000; Ito *et al.*, 2009; Elasri and Miller, 1999; Doolittle *et al.*, 1996), and ice-associating activities such as INA and IR inhibition linked to low temperature survival. Certainly, our study underscores the complexity of the multiple mechanisms involved in the overwintering of an epiphyte community, even for that proportion of the consortium that is culturable (*ie*, the freeze-thaw resistance of *Flavobacterium sp* is not related to biofilm since it showed resistance as a single isolate). It further suggests that specific adaptations not only benefit a particular strain but serve to advantage the overall freeze-thaw resistance of microbes in the same niche.

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2.7 Figure legends:

Fig. 2.1. (A) Freeze-thaw resistance of some of the isolates that were derived from frost-exposed perennial leaves, cultured and subsequently subjected to 12, 24, 36, 48 freeze-thaw cycles and then monitored for viability (CFU/mL). Coloured lines represent different isolates including *Erwinia billingiae* J10 (blue), *Flavobacterium sp.* Yin (red) and *Sphingobacterium kitahiroshimense* Y2 (yellow). *E. coli* TG2 (purple) was used as a control. Starting CFUs/mL approximated 10^8 and was normalized to 1×10^8 .

(B) Comparison of cell viability of *E. coli* TG2 and *P. syringae* J6 when tested alone or with the addition of 20% EPS preparation from *E. billingiae* J10 (see Materials and Methods) after 12 and 24 freeze-thaw cycles. Viability was monitored as in Fig. 2.1 A. Control *E. coli* TG2 as well as *P. syringae* J6 are shown by the blue and yellow bars, respectively. Strains with the addition of the EPS preparation are shown as purple bars for *E. coli* and light blue bars for *P. syringae*.

Fig. 2.2. Representative electron micrographs of *Erwinia billingiae* J10 after negative staining with phosphotungstic acid. Note that a monotrichous polar flagellum is easily visualized (marked with arrows). The bar — represents 0.5 μm , with image A and B showing individual cells with single flagellum, C and D showing dividing cells with two flagella, and E showing apparently aggregated cells with multiple flagella.

Fig. 2.3. Scanning electron micrographs of selected epiphytes including *Flavobacterium sp.* Yin (top left), *Erwinia billingiae* J10 (bottom left) and *Sphingobacterium*

kitahiroshimense Y2 (bottom right), along with control *E. coli* TG2 (top right). A thick matrix appearing to represent biofilm is most obvious in the *E. billingiae* preparations, and somewhat less so in *S. kitahiroshimense*. Arrows show representative extracellular matrix structures. The magnification bars of the micrographs represent sizes of 10 μm , 5 μm and 2 μm (or 1 μm), respectively.

Fig. 2.4. Representative SDS-PAGE analysis of the partially purified EPS preparations from *E. billingiae* J10. After electrophoresis the 12.5% gel was stained with Coomassie Blue to visualize the abundant proteins as described in Materials and Methods. A main protein band (lane 1) at 35 kDa is indicated with an arrow. Protein markers were loaded in the lane marked M.

Fig. 2.5. Fingerprint of the trypsin-digested peptides from the *E. billingiae* J10 EPS preparation. Protein derived from the 35 kDa band, cut from 12.5% SDS acrylamide gel and destained for protein identification (as in Fig. 2.4) was subjected to MALDI QqTOF mass spectrometric analysis as described in Materials and Methods. Numbers at each of the depicted peaks indicate the molecular mass/ions of the noted peptide fragment.

Fig. 2.6. Alignment of MALDI-QqTOF-MS/MS-sequenced tryptic peptides of the 35 KD protein from the *Erwinia billingiae* J10 extracellular polymeric substance. MALDI-QqTOF-MS/MS analysis revealed 5 peptides unique to OmpA1 from *E. tasmaniensis* and OmpA2 from *E. coli* ED1a; and 2 peptides similar to OmpF, a porin from *Pantoea sp* At-9b. A motif typical of bacterial outer membrane proteins is outlined with dots and identical amino-acid residues are indicated with an asterisk. The corresponding NCBI

protein accession numbers are as follows: OmpA1 (gi_188534230), OmpA2 (gi_218688793) and OmpF (gi_258636144).

Table 2.1 Isolated bacteria from perennial plants and their ice associated activities.

Cultured Isolate	Closest BLAST Match	Identity	Ice- associating Activity ¹
<i>P. syringae</i> J6	CP000075.1	99%	INA ⁺
<i>Erwinia billingiae</i> J10	Y13249.1	98%	IR inhibition ⁺
<i>Flavobacterium</i> sp. Yin	AM177621.1	99%	IR inhibition ⁺
<i>Sphingobacterium kitahiroshimense</i> Y2	AB361248.1	98%	IR inhibition ⁺
<i>Chryseobacterium</i> sp. J3	DQ530158.1	99%	IR inhibition ⁺
<i>Rahnella</i> sp. W11	AB476622.1	100%	IR inhibition ⁺
<i>Duganella zoogloeoide</i> Y11	NR_025833.1	99%	IR inhibition ⁺
<i>Geobacterium</i> sp. J6	AM712156.1	100%	NA
<i>Massilia aurea</i> J9	AM231588.2	99%	NA
<i>Sphingomonas</i> sp. J2	AY661593.1	100%	NA
<i>Janthinobacterium</i> sp. L1	GQ179711.1	99%	NA
<i>P. sp.</i> L14	GQ179727.1	99%	NA
<i>P. fluorescens</i> L37	AM933520.1	99%	NA
<i>P. trivialis</i> L48	FJ179366.1	99%	NA
<i>P. veronii</i> L32	AY179328.1	99%	NA

¹ Ice-associating activity is classified as ice nucleating activity (INA⁺), ice recrystallization (IR) inhibition, or not active in these assays (NA)

Table 2.2 The mean viability of *E. coli* with the addition of *E. billingiae* EPS preparations

Cycle number	EPS addition ¹	EPS digested with proteinase K ¹	EPS digested phospholipase C ¹	No EPS added ¹
0	1 x 10 ⁸	1 x 10 ⁸	1 x 10 ⁸	1 x 10 ⁸
24	3.1 x 10 ⁴	3.1 x 10 ³	1.3 x 10 ⁴	0

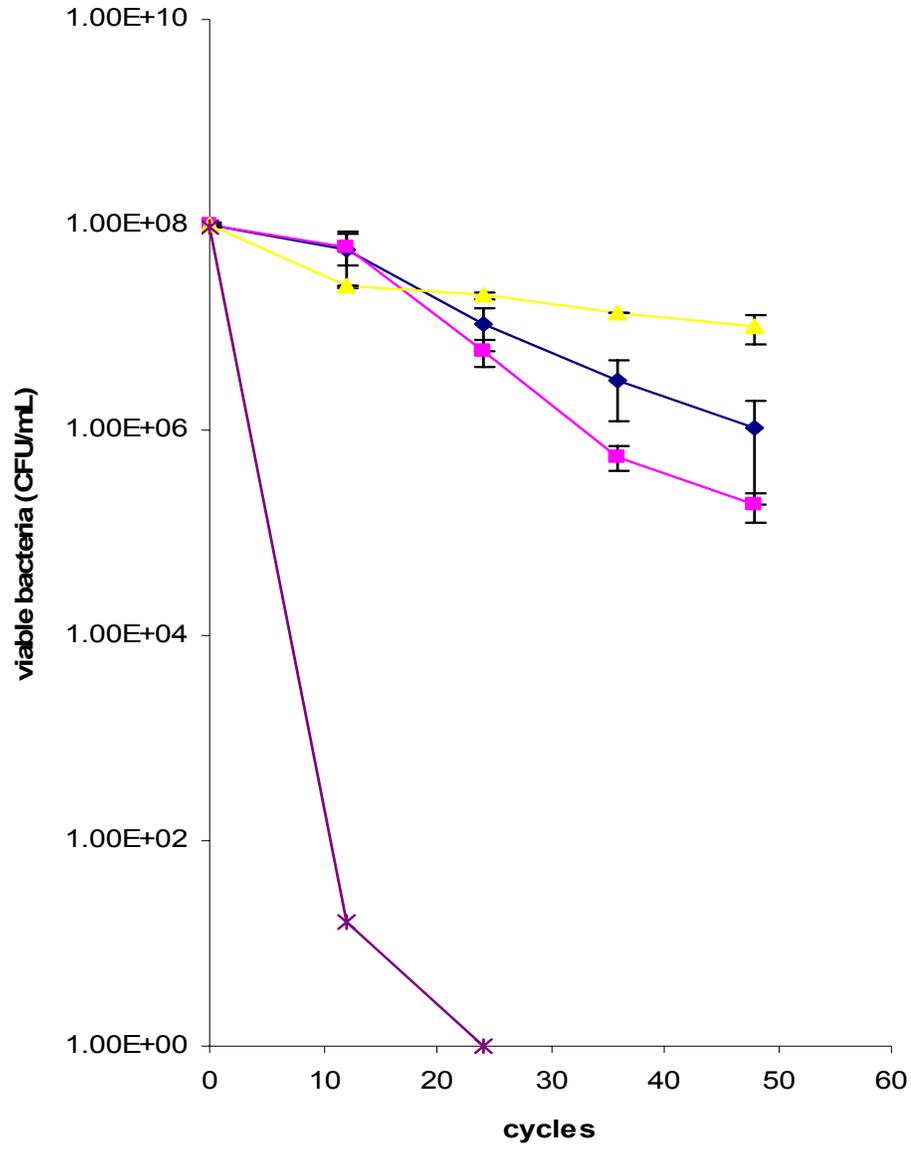


Fig. 2.1. (A)

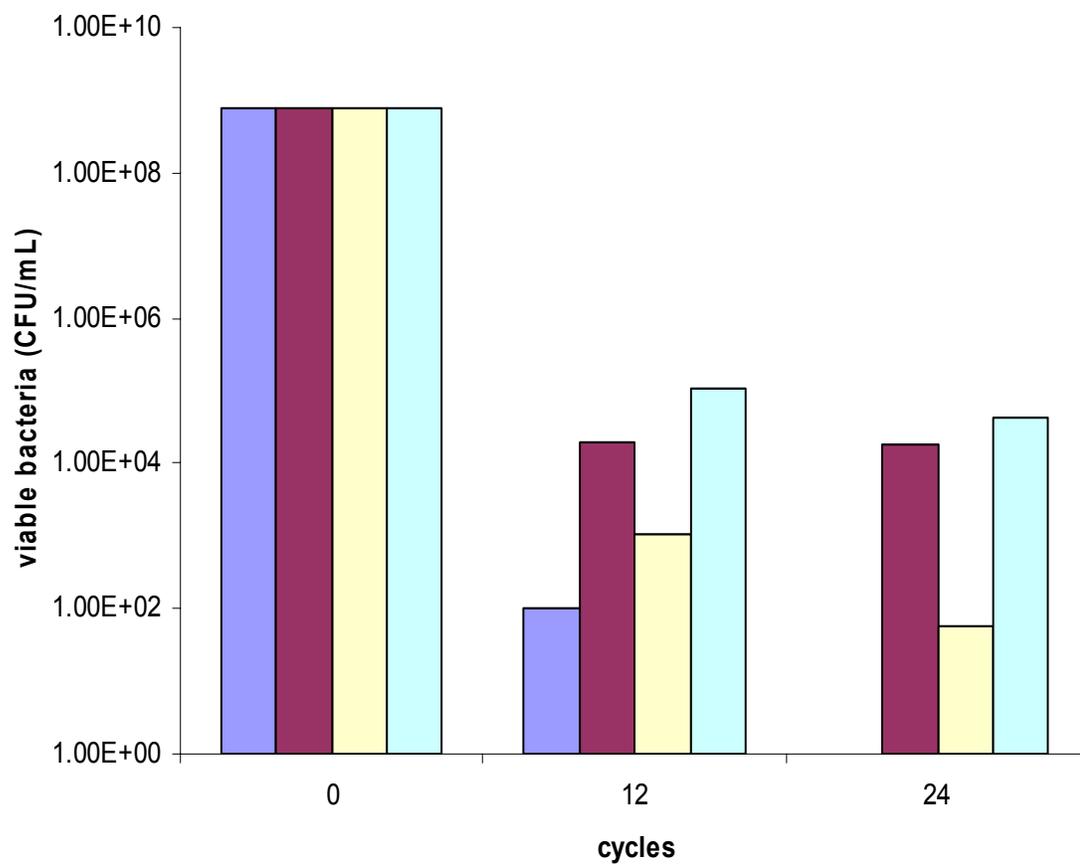
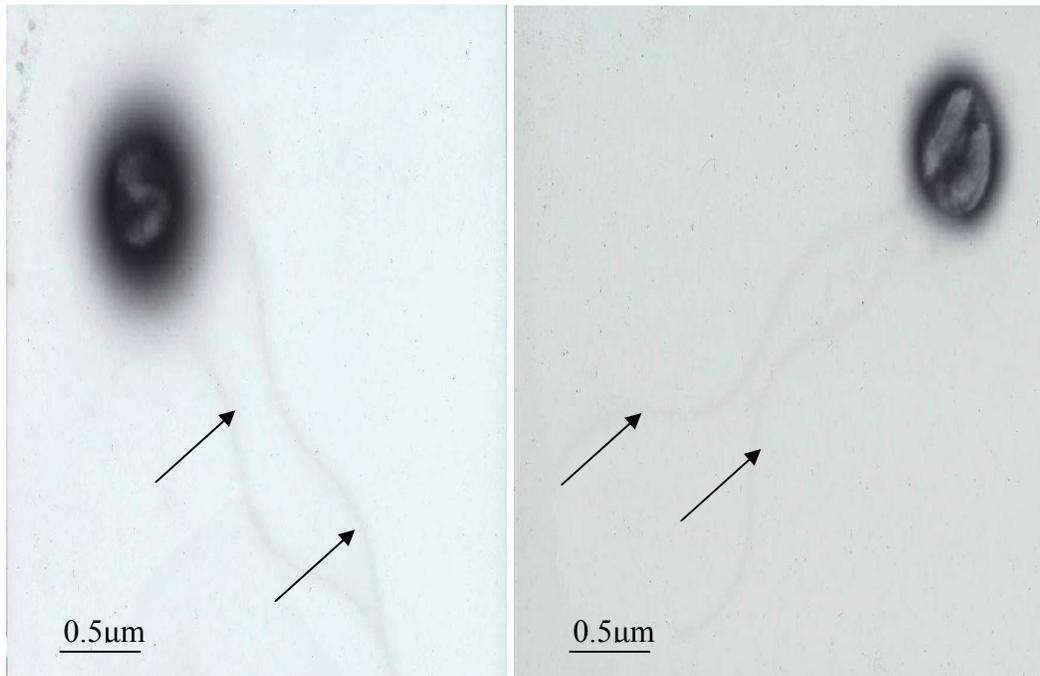


Fig. 2.1. (B)



A

B



C

D

Fig. 2.2



E

Fig. 2.2

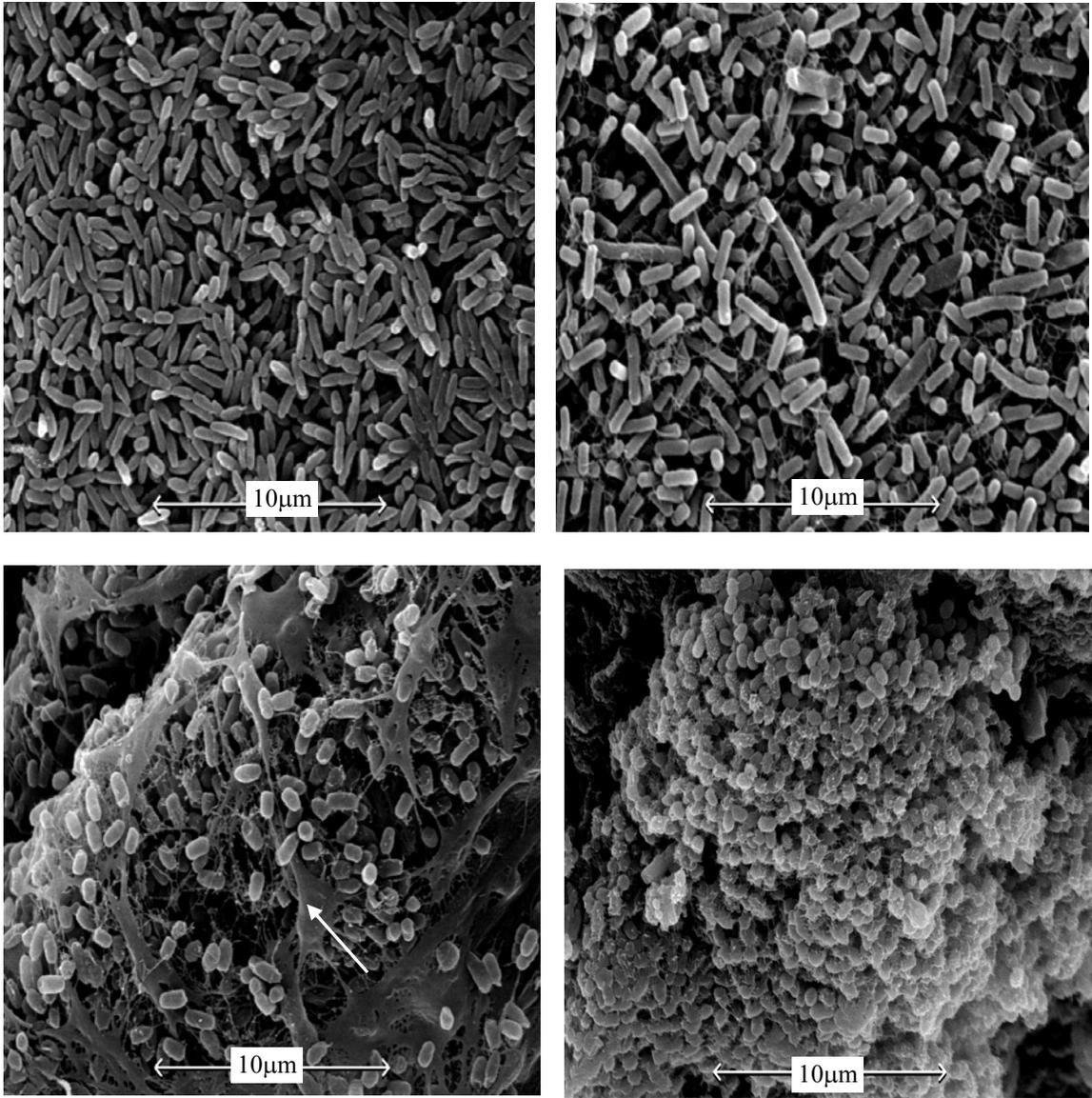


Fig. 2.3

A

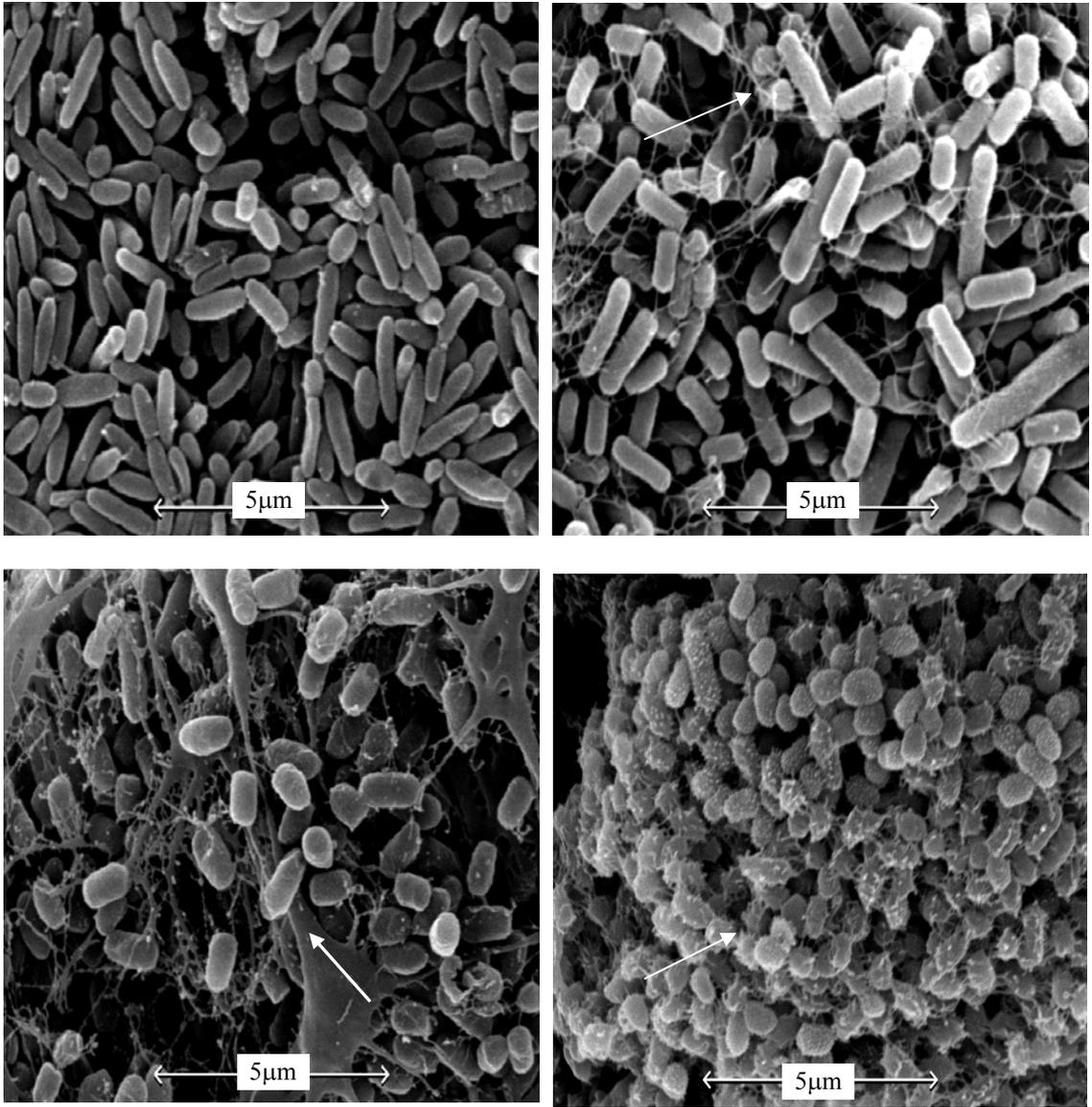
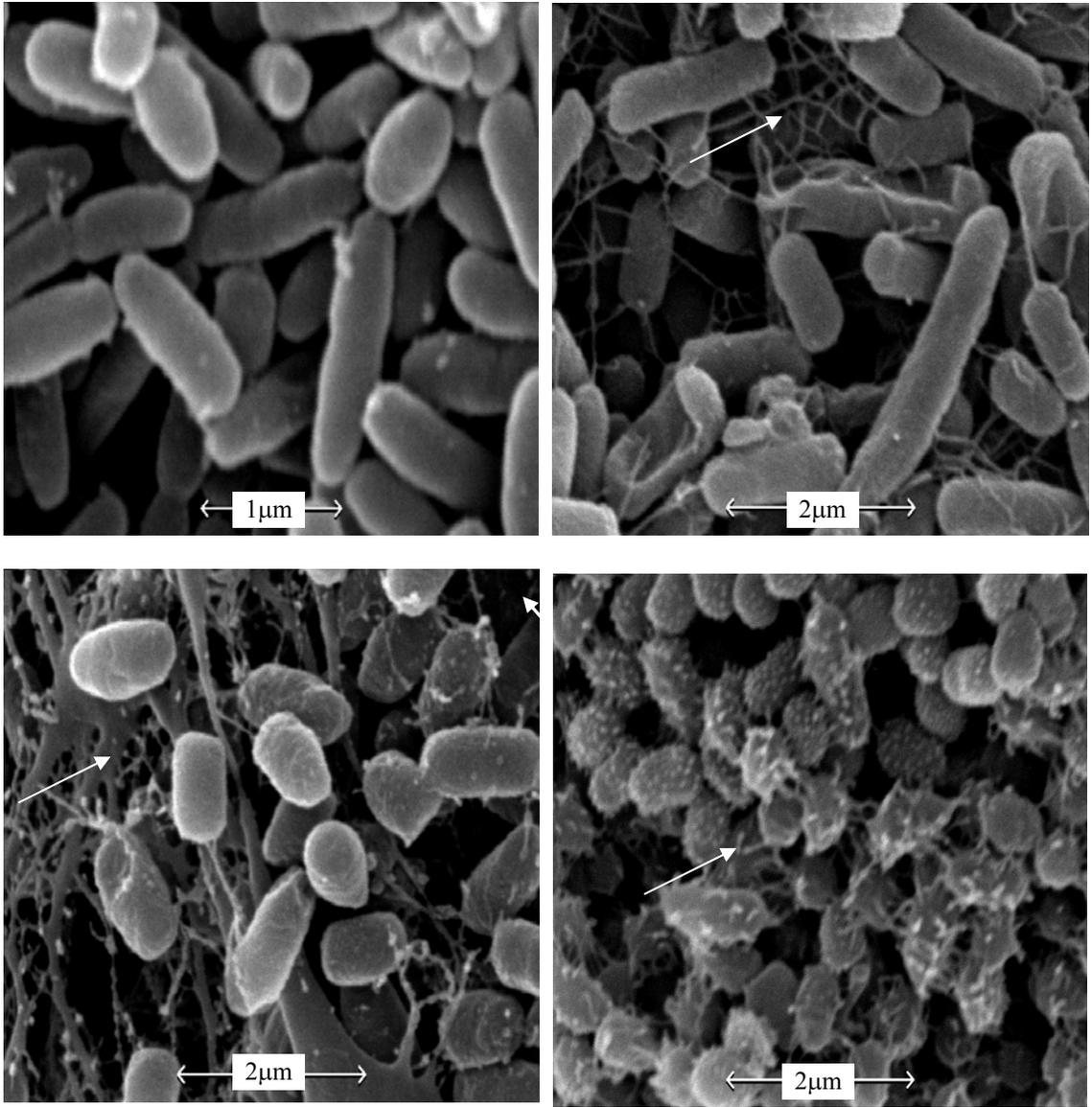


Fig. 2.3

B



C

Fig. 2.3

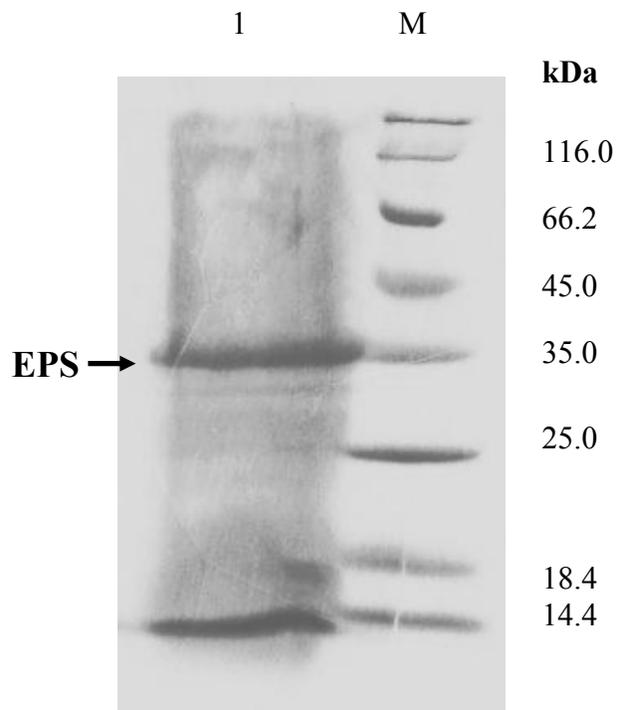


Fig. 2.4

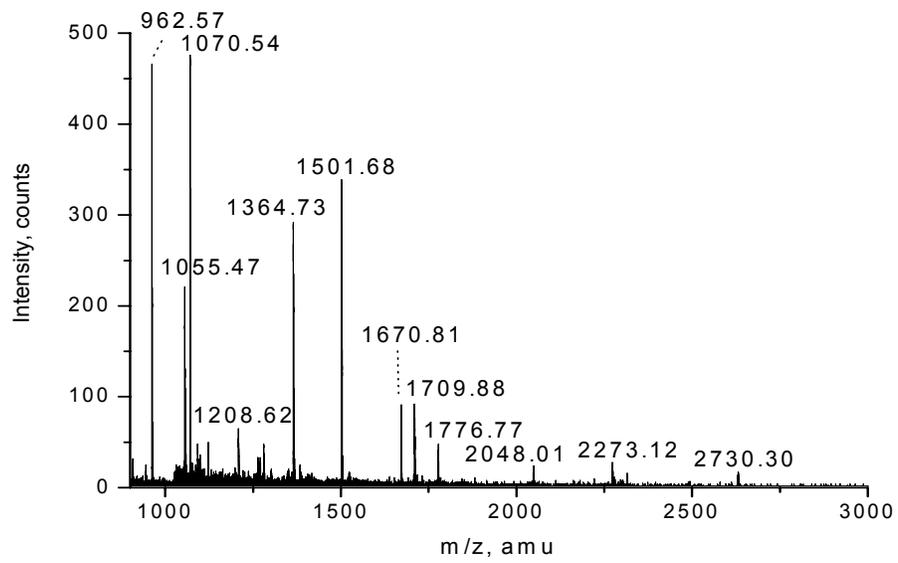


Fig. 2.5

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OmpA1      -MKKTAIAIAVALAGFATVAQAAPKDDTWTYGGKLGWSQYHDTGYYGNGYQNNNGPTHE 59
OmpA2      -MKKTAIAIAVALAGFATVAQAAPKDNWTY GAKLGWSQYHDT----GFIDNNGPTHE 54
OmpF       MMKRNI LAVVI PALLAAGAANA AEIYN--KDGNKLDLYGKVDGLHYFSDDSSNDGDQSYV 58
           *: . : * : : . * . : * : : * : * : * : * : * : * : * : * : * : *
           DNTWYTGAK (1055)

OmpA1      QLGAGAF LGYQANQYLGFELGYDWLGRMPNKGSVTNGAFKAQGVQLAAKLSYPIADDDLV 119
OmpA2      QLGAGAF GGYQVNPYVGFEMGYDWLGRMPYKGSVENGAYKAQGVQLTAKLGYPIITDDLDV 114
OmpF       RFGFKGETQITDQLTG YGQWEYNIQANNSEGGSDAQSGNKT R--LGFAGLKFADAGSFDY 116
           : * . : : : * : . . * : . . * : : * * : . : : * :
           LSYPLTDDLDV

OmpA1      YTRLGGMVWRADSTQTNPTYGRISNHDTGVSPLAAVGVEYALTK--NWA TRLDYQWVNN 176
OmpA2      YTRLGGMVWRADTKSN--VYG--KNHDTGVS PVFAGGVEYAITP--E IATRLEYQWTNN 167
OmpF       GRNYGVVYDAI GWT DMLPEYGGDSGYSDNFMAGRSTGLATYRNTNF FGLVDGLNFALQYQ 176
           . * : . . . * : . . . . . : * : . . * : : :
           YTR (1670)

OmpA1      IGDAGTVGTRPDNS-MLSVGVSYRFGQDEAA PVVAPAPAPA PVVETKRFT LKSDVLF TFN 235
OmpA2      IGDAHTIGTRPDNG-MLSLGVSYRFGQGEVAPVVAPAPAPA PEVQTKHFT LKSDVLF TFN 226
OmpF       GKNDRDTPRRANGDGGWGTSTVSYTSPIG-VGIVGSYASSDRINAQNMNYGNGDKAQQWA 235
           : * . * * . . . * : * : . . . : : * :
           SDVLF TFN

OmpA1      KASLKPEGQQA LDQLYTQLSN---LDPKDGSVVVLGYTDRI GSEQYNQKLS EKRAQS--- 289
OmpA2      KATLKPEGQAALDQLYSQLSN---LDPKDGSVVVLGYTDRI GSDAYNQALSERRAQS--- 280
OmpF       TALKYDANNVYLAAMYSETRNATYIANSNTS SVQNGFTGFANKTQVMEFVAQYQDFGLR 295
           . * . : * : * : * : * : * : * : * : * : * : * : * : * :
           K (1070) RAQS

OmpA1      -VVDYLVSKGI PSSKI SARGE GKS NPVTGNT CDSVKAR---KALIDCLGPD RRVE IEVKG 345
OmpA2      -VVDYLISKGI PADKI SARGMGESNPVTGNT CDNVKQR---AALIDCLAPDRRVE IEVKG 336
OmpF       PSVAYVQSKGKDIEGI GDADLYKYAEVGATY YFNKNMSTYVDYKINLLNDNPLGLNTDD 355
           * * : * * . * . . : * . . . : * : * * . : : : .
           VVDYLVSK (1364, 1208) YFEVGATYFENK(1501) GINTSD

OmpA1      IKDVVTQPQA 355
OmpA2      IKDVVTQPQA 346
OmpF       IVAVGLVYQF 365
           * * *
           IVALGLVYQF (1709)

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Fig. 2.6

2.8 References

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Preamble 2

In Chapter 2, a community associated with overwintering plants was investigated and isolates were characterized with respect to their ice-associating properties. Similarly, the analysis of a soil community from an exposed esker at the low Arctic Daring Lake research station in the North West Territories was also initiated (Wilson *et al.*, 2006; unpublished). Analogous to the ice nucleation activity (INA) of the epiphyte, *Pseudomonas syringae*, on overwintering leaves (Chapter 2), *P. borealis*, with some INA was identified after screening. However, since *P. borealis* is a beneficial species that associates with plant roots, INA was not expected. I wondered if in this species the INA phenotype was associated with an ice nucleation protein (INP), and wished to investigate growth and survival of this *Pseudomonas* species at low temperatures. Thus, in the forthcoming chapter *P. borealis* was characterized with respect to its growth characteristics, INA, and the gene encoding its INP was cloned.

Chapter 3

Characterization and recombinant expression of a divergent ice nucleation protein from *Pseudomonas borealis*

3.1 Statement of Co-authorship

This paper was published in Microbiology in 2009 (155, 1164-1169). The authors are Zhongqin Wu, Lei Qin and Virginia Walker. I designed and prepared the all of the structures of pGEMcoreinp, PGEM5extension, pGEM3extension, pGEMT, pGEMeinp, pET24aINP, I also performed all of the experiments for the manuscript except for Fig. 3.3 which was produced by Dr. Lei Qin, who also performed the computer analysis of the ice nucleation protein sequences. I also wrote the initial draft of the manuscript and participated in subsequent editing.

3.2 Summary

Isolates of *Pseudomonas borealis* were recovered after ice-affinity selection of summer-collected soils. *P. borealis* DL7 was further characterized and shown to have ice nucleation activity (INA), a property that allows the crystallization of ice at temperatures close to the melting point, effectively preventing the supercooling of water. INA was optimally detected after culturing at temperatures consistent with psychrophilic growth. The sequence encoding the *P. borealis* ice nucleation protein (INP) was obtained using both PCR and chromosome walking. When expressed in *E. coli*, the resulting *inaPb* recombinants had INA. The *P. borealis* sequence, dubbed *inaPb*, is clearly related to previously cloned INP genes, but it shows greater divergence. Sequence analysis suggests that there are two opposite flat surfaces, one relatively hydrophobic that likely serves as

an ice template, and the other that could function as a complementary face to facilitate interprotein interaction for ice step formation.

3.3 Introduction

It is only the presence of ice nucleators that ensure the crystallization of water at temperatures close to 0°C; without them water would supercool. Apart from ice itself, the most active known ice nucleators are found in bacteria. Several decades ago the plant epiphyte, *Pseudomonas syringae*, was discovered to have ice nucleation activity (INA) (Maki et al., 1974). It was speculated that INA contributed to its pathogenicity since freezing and subsequent wounding of the leaves and stems would allow these strains ready access to plant nutrients (Lindow et al., 1978). More recently, INA has been reported in *P. fluorescens* and *Erwinia herbicola* showing similar serological properties to *P. syringae* (Deininger et al., 1988). Isolation the genes that encodes of ice nucleation proteins (INP) sequences from these as well as *Xanthomonas campestris* show that that they are similar despite the evolutionary divergence of the host bacteria (Abe et al., 1989; Warren and Corotto, 1989). It has been speculated that the INP gene was horizontally transferred by transduction events between plant epiphytes (Edwards et al. 1994). These INP have a conserved amino terminal and a highly repeated middle region of 960-1296 amino acids consisting of three 16-residue repeats (yielding a 48 amino acid unit). This internally-periodic middle region accounts for 80% of the protein (Warren and Corotto, 1989) and is followed by a 41-68 residue carboxyl-terminal sequence.

INA is not necessarily correlated with high freeze-thaw resistance. Indeed, in experiments to select for microbes with this phenotype from soil samples, no microbes with previously-known INPs were recovered (Walker et al., 2006). However, when bacteria from several temperate soils were adsorbed to growing multicrystalline ice,

several isolates including two *P. borealis* strains (DL7 and YIC), identified on the basis of their 16S rRNA sequences, were recovered in the melt water (Wilson et al., 2006). They were subsequently shown to have INA. Since INA had not been previously observed in these bacteria, the phenotype was examined further to determine if this activity could explain ice association and the gene sequence was isolated to compare it to better known species with this activity.

3.4 Methods

3.4.1 Bacterial strains

P. borealis DL7 originated from soil samples collected from the Tundra Ecosystem Research Station at Daring Lake in the North West Territories, Canada (64°52'N, 111°35'W). This isolate was recovered after ice affinity selection as previously described (Wilson et al., 2006). *Chryseobacterium* sp. C14 was originally isolated by freeze thaw selection (Walker et al., 2006) and has no INA. Other strains used were *P. syringae* B728a, *E. coli* TG2 and *E. coli* BL21 (Novagen, USA).

3.4.2 Characterization of *P. borealis* DL7

A single colony of *P. borealis* DL7 was inoculated in 10% tryptic soy broth (TSB), cultured at 22°C for 24 h, stained with Gram dyes according to standard protocols, and examined microscopically at X1000. The antibiotic resistant phenotype was determined on 10% TSB plates supplemented with different antibiotics and agents, and verified by liquid cultures. The different agents and their concentrations were: ampicillin (100 mg/mL), kanamycin (50 mg/mL), tetracycline (15 mg/mL), streptomycin (100 mg/mL), and CuSO₄ (0.8 mM).

The optimal growth temperature of the isolate was investigated by inoculating a single colony into 10% TSB, shaking, and monitoring growth at 22°C. Once the cultures

reached stationary phase, new cultures were initiated by inoculating 1% of the stationary phase cells into fresh 10% TSB and maintaining the culture temperature at 4°C, 8°C, 22°C or 37°C. Growth was monitored using optical density as well as by viable cell counts (CFU/mL).

3.4.3 Ice nucleation assays and ice affinity

Ice nucleation was assessed using three different sample volumes. A thermal analyzer was used to determine the freezing point, as assessed by the heat of fusion of first-order freezing (Borchardt and Daniels, 1957). A thermister was placed in 1.4 ml samples that were chilled from 5°C to -15°C at 0.2°C/min, and temperatures were automatically recorded on a spread sheet and subsequently plotted (Walker et al., 2008). Smaller volume samples were assayed essentially as previously described (Vali, 1971; Maki et al., 1974; Kozloff et al., 1983; Wilson et al., 2006). Freezing was monitored visually by a fiber optic light source directed through crossed polarized filters as the temperature was lowered from -1°C to -15°C (at 0.2°C/min) and expressed as the logarithm of the number of ice nuclei/cell (Nemecek-Marshall et al., 1993).

Ice affinity was determined as previously described (Wilson et al., 2006). Briefly, the temperature of a hollow brass finger was set at -1°C to establish a thin ice coat, and then it was lowered into a beaker containing the bacteria of interest (1.0×10^4 CFU/ml). By keeping the ice finger at -1°C, an ice hemisphere could be slowly formed. After two days ~50% of the liquid fraction was frozen, during which time the temperature of the ice finger was lowered incrementally. Subsequently, the hemisphere was rinsed and the ice was melted and poured onto 10% TSB plates. Identification of the bacteria as well as the numbers of viable cells (CFU/mL) was then determined by using plates without additives

or containing the appropriate discriminating antibiotics (tetracycline at 15 mg/mL, ampicillin at 100 mg/mL and streptomycin at 100 mg/mL).

3.4.4 Ice nucleation protein sequence determination

Genes encoding known INPs were aligned and degenerate primers were designed to amplify the conserved, repetitive repeats. After initial trials, two primers INPup (5'-AACATGGCCGATCAYTGCGG) and INPdown (5'-GTAVCKYTTSCCGTCCCAG) were used. DNA from *P. borealis* DL7 was isolated using standard phenol-chloroform methods in PCR experiments under the following conditions: 94°C for 5 min, followed by 94°C for 0.5 min, 55°C degree for 0.5 min, 72°C for 4 min for 35 cycles, and finished with an extension at 72°C for 30 min. The products were separated on agarose gels, purified using standard methods (Nucleotide removal kit, Qiagen, CA), and the recovered PCR products were ligated into pGEMT vector (Promega USA). A plasmid with the appropriately sized insert was sequenced by ACGT Corporation and named pGEMcoreinp.

Based on the known sequence, 4 more primers were used to extend the 5' terminal sequence of the putative *P. borealis* INP gene. The template for the first reverse PCR was *P. borealis* genomic DNA which was cut by Sall and self ligated overnight, then purified as previously indicated. The PCR product (1 µl) of the first two primers (INPR887: 5'-ATAGAATTCTGCCGTAGCCCCGAGTGAGAT and INPL1549: 5'- CCTCGGGTTA TGGCAGTTCCC) was used as template for a second PCR reaction with two new primers (INPR188: 5'-TCAGCCTGCATATTCAAATGCG and INPL2573: 5'-ACCCAGACTGCCAGGAAGA). A similar method was used for 3' terminal extensions. The product (1 µl) from the first PCR amplification (using primers INPL3396: 5'-ATAGAATTCCGGGCGACCGAAGCAAACACTATTG and INPR3350: 5'-CGGCCAT

CTGGATGCTGTCGGC) were used in a second amplification (with INPL3581: 5'-CTGACGGGCGGGCGAGAACTCG and INPR2768: 5'-TGCGTACTTCCATAACCTGC GACCAGT). Both PCR products were ligated into pGEMT (Promega, USA) and positive colonies were designated pGEM5extension and pGEM3extension, respectively.

After sequencing, alignments were made using BioEdit for pGEMcoreinp, pGEM5extension and pGEM3extension. Similarities between the putative *P. borealis* INP sequence and all 13 known INPs in the data base were determined by Blast (Altschul et al., 1997) and consensus sequence was produced using the statistical tool, hidden Markov models (HMM) according to Schuster-Boeckler (2004). Alignments with multiple sequences were made with the coding regions of INP genes from the following microbes: *P. borealis*, *P. fluorescens (inaW)*, *P. syringae B728a*, *P. syringae (inaV)*, *Pantoea ananatis (inaA)*, *Pantoea agglomerans*, *Xanthomonas campestris (inaX)*, and *Xanthomonas translucens*. Separate alignments were assembled for the repetitive region, as well as the amino and carboxyl terminal regions using BioEdit. The maximum likelihood model was employed using ProML (Felsenstein, 2004), which does not assume a molecular clock, and this was used for the phylogenetic trees.

3.4.5 Expression of recombinant *P. borealis* INP

PCR was performed to introduce a NotI site to pGEM3extension with primers EC32: 5'- GCGAATTCTCATTATTCCCCGGATTTATTCACG and EL2351: 5'- GGATCCATGGCAGGCTTCCAAAGCACATTGAT, and the product was ligated to pGEMT and named pGEM2351. Subsequently, pGEM2351 was cut with SalI and ligated to pGEMcoreinp which had been digested by the same enzyme, and the recovered plasmid was designated pGEM3core. Another PCR was performed to introduce EcoRI restriction enzyme site at the 5' terminal site of pGEM5extension using primers EN11: 5'-

GGGAATTCGGGATGAACGATGACAAAG and EN22: 5'- CGTAGATCTATCGCC AGCGGTTTTCGGTA. The resulting amplified product was ligated to pGEMT and designated pGEM1122, which was then digested using XhoI and ligated to pGEM3core, which had been digested with same restriction enzyme. The sequence of pGEMeinp was then obtained. Finally the putative INP gene was introduced to pET-24a(+) (Novagen, USA) at the EcoRI and NotI sites by digesting both the vector and pGEMeinp. This last plasmid was designated pET24aINP.

A single colony of *E. coli* BL21 (DE3) harboring pET24aINP was used to inoculate 10 ml of LB containing 30 µg/ml kanamycin. After culture at 37°C, isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to 1 mM when the OD₆₀₀ reached 0.6. Subsequently the culture was shifted to 4°C for 3 h. INA was assessed in the transformed cells as well as control cultures treated as the transformed cells and as previously stated.

3.5 Results

3.5.1 Growth and characterization of *P. borealis* DL7

P. borealis DL7, which had been previously isolated by ice affinity (Wilson et al., 2006) was microscopically visualized as Gram negative cells, ~0.5 x 3 µm (not shown). Cultures grew well on 10% TSB, with a maximum cell density at 10⁹ CFU /mL, corresponding to an OD₆₀₀ at ~1.1. Colonies on 10% TSB plates were white with round and smooth margins. *P. borealis* DL7 can be broadly classified as psychrotrophic since there was growth at 22°C (doubling time of 3.3 h), but cultures also grew well at 8°C and 4°C (with a doubling time of 12.6 h and 13.6 h, respectively). Even after one year of storage at temperatures at 0°C, with ice floating on the surface of the medium, the cultures remained viable with no diminution of INA (see below).

When characterized in the presence of selected additives (see Materials and methods) *P. borealis* DL7 continued to divide in the presence of ampicillin, but growth was inhibited by kanamycin, tetracycline and streptomycin, as well as CuSO₄, amongst others (not shown). Thus the resistance phenotype was distinct from that of *E. coli* TG2 cells, which were inhibited by ampicillin but not by tetracycline.

3.5.2 Ice nucleation activity and ice affinity

INA in *P. borealis* DL7 was shown using several different methods. As demonstrated in other studies (Vali 1995), absolute ice nucleation temperatures not only depend on cell number, but also on sample size. Thermal profiles of *P. borealis* (~10⁹ CFU/mL; 1.4 ml volume) cultured at 22°C and held at 4°C for two days, showed a mean freeze temperature of -2.8°C, compared to a mean freeze temperature of -2.4°C for *P. syringae* cultures under the same conditions (Fig. 3.1 insert). Thermal profiles for the two *Pseudomonas* species were significantly different than those for *E. coli* TG2 and *Chryseobacterium* C14, with mean freeze temperatures of -6.8°C and -7.1°C (P < 0.03). Ice nucleation spectra show the same trend although at temperatures consistent with the small volumes used to assess large numbers of samples, and confirmed that *P. borealis* had levels of INA (expressed as the logarithm of the number of ice nuclei/cell) that were slightly less than that of *P. syringae* (Fig. 3.2).

Although INA appeared to be independent of growth phase (not shown), it was affected by culture temperature. There was little INA (log ice nuclei/cell between -6 and -7) in *P. borealis* cultures at 22°C, but substantially more activity (log ice nuclei/cell between -2 and -4) at 4°C or 8°C. Likewise, when cells cultured at 22°C reached stationary phase and were transferred to 4°C for 2 or 4 days, a substantial increase in INA was observed (log ice nuclei/cell increased from about -7 to -4 and -2, respectively).

There was no INA and no growth at 37°C.

P. borealis DL7 was originally isolated after incorporation into growing ice and therefore its relative ice affinity was compared to *E. coli*. Identification of the two bacteria was facilitated by their sensitivity to different antibiotics. Although it was not possible to accurately estimate *P. borealis* cell numbers in the unfrozen liquid fraction because it is a psychrotroph, there was an average five-fold higher recovery of *P. borealis* in the ice fraction compared to *E. coli* TG2 (not shown). This higher recovery could only be partially attributed to the three-fold higher survival of *P. borealis* compared to *E. coli* in the ice fraction ($P < 0.001$).

3.5.3 Isolation, sequence and analysis of *P. borealis* ice nucleation protein gene

Previously reported INP gene sequences (Wolber et al., 1986) allowed the design of ‘consensus’ DNA primers for the amplification of a DNA fragment from *P. borealis*. Eventually, degenerate primers (see Materials and methods) were used to successfully amplify a 3.6-kb product (not shown). After cloning in pGEMT, subsequent sequencing allowed the synthesis of ‘exact match’ primers which were used in a reverse PCR strategy to extend the sequence in both directions.

After assembly of the sequenced fragments, the resulting putative *P. borealis* INP DNA sequence conceptually encoded 1244 residues. The amino terminal region extended for about 163 residues and accounted for 13% of the gene with the carboxyl terminal region making up 41 residues. The remainder (~84%) of the sequence was highly repetitive with 65 typical 16 amino acid repeats (Genbank accession EU573998). The pattern was generally of two relatively hydrophilic repeats followed by a more hydrophobic 16 residue repeat for each 48 amino acid repeat unit. Comparison of the *P. borealis* DNA sequence with known Inas indicated that there was no homolog amongst

previously known INP genes in first 500 bp. Overall there was ~66% identity with *P. syringae* INPs. Phylogenetic analysis (Fig. 3.3A) showed that *P. borealis* INP was divergent from the better known INPs but overall had more similarity to the *P. fluorescens* protein. Not unexpectedly, the repeat sequences showed the highest identity with other INPs. *P. borealis* INP (e.g. position 164-1076) had a consensus sequence of AGYGSTxTAGxxSxLi/t (Fig. 3.3B), compared to the consensus, AGYGSTQTsGseSsLT repeat in *P. syringae* InaZ (Kajava and Lindow, 1993)

3.5.4 Recombinant *P. borealis* INP is functional

To confirm that the PCR-amplified sequence encoded the INP, separate DNA fragments were amplified using primers containing judiciously chosen restriction sites. Subsequently, digestion with the appropriate restriction enzymes allowed the fragments to be assembled by ligation such that the entire 3735-bp sequence was introduced into pET24a. After inducing the *E. coli* host and transferring to 4°C, the expressed recombinant INP showed modest but consistent INA: 1.4 ml samples froze at a mean of -5°C (Fig. 3.1) compared to a mean of -7°C for control cells (nontransformed or *E. coli* transformed with an 'empty' pET24a plasmid). Ice nucleation spectra confirmed that transformed *E. coli* had modest levels of INA (log ice nuclei/cell between -4.2 and -6.3), which surpassed that of the controls (Fig. 3.2). Based on these functional assays, the gene sequence has been designated *inaPb*.

3.6 Discussion

Survival in a surface tundra community demands adaptations to long cold winters. Reports list more than a dozen microbes that appear to dominate low temperature consortia and amongst these are *Pseudomonas* species (Wynn-Williams, 1983; Shivaji et al., 1989). Some *Pseudomonas* strains are known to degrade hydrocarbon spills

particularly in the Arctic (Stallwood et al., 2005), valuable ‘cold enzymes’ have been isolated from others (Villeret et al., 1997; Cavicchioli, 2002), and membranes of some strains have enhanced fluidity at low temperatures (Kumar et al., 2002). Strains of *P. syringae* are well known for their INPs, and this phenotype might also be considered a low temperature adaptation. *P. borealis* DL7 originated from tundra soils and was recovered by ice affinity selection, the isolation that appears to have been partially due to its ability to retain high viability after freezing and its modest ice affinity. Indeed, because this strain is easily cultivated at temperatures just a few degrees above 0°C and did not divide at 37°C, it can be classified as a psychrotroph. *P. borealis* DL7 showed typical pseudomonad properties including its cell and colony morphology but in addition it had INA. This activity is above -5°C (Fig. 3.1 and 3.2), allowing the *P. borealis* INP to be classified as a high activity, type I nucleator (Phelps et al., 1986).

INA has been described in certain strains of several evolutionary-diverse plant pathogens and epiphytic bacteria, and the genes encoding the INPs are homologous (Abe et al., 1989; Warren and Corotto, 1989; Edwards et al., 1994). Since *P. borealis* is considered a beneficial soil bacterium (Raaijmakers et al., 2002), it was of interest to determine the sequence of its INP. Cloning the corresponding gene was challenging due to the large number of internal repeats (Genbank accession EU573998; Fig. 3.3) with alternative cloning strategies yielding disappointing results, but eventually the sequence was isolated using PCR and chromosome walking techniques. To ensure that the INP had been correctly identified, the entire gene was assembled from amplified fragments and used to transform *E. coli*. The resulting recombinant protein showed modest, but consistent INA (Fig. 3.1 and 3.2). Similarly, when INP genes from other bacteria are

expressed in *E. coli*, the resulting INA is not as high as in the originator cells (Wolber et al., 1986).

Although we can only speculate that INP might confer some freeze protection to *P. borealis*, the identification of the sequence in this soil bacterium and its divergence has prompted us to consider its structure and function. The *P. borealis* INP is composed of a repetitive core and is flanked by nonrepetitive hydrophobic amino and hydrophilic carboxyl terminal regions, similar to other INPs. The INP genes from *Pantoea ananatis*, *P. syringae*, *P. fluorescens*, and *X. campestris* are highly conserved with the main difference being the number of 16 -residue repeats. The hydrophobic amino terminal portion of the *P. borealis* shows no significant identity to other sequence in the data base and the carboxyl terminal was also divergent with more polar and less hydrophilic residues than other INPs, but with similar numbers of charged residues to *P. fluorescens*. When the *InaPb* sequence with its overall average 66% amino acid identity to other INPs was compared in phylogenetic trees, again it was more divergent than sequences from the plant epiphytes but appeared more closely related to *P. fluorescens*, another soil microbe (Fig. 3.3a). It is not known if *inaPb* is a result of horizontal gene transfer from an as yet unknown donor or if the sequence differences in *P. borealis* and *P. fluorescens* reflect an adaptation to a soil habitat.

Because of the divergence, *inaPb* should be useful for the discovery of functional residues in INPs. Previously, *P. syringae* INP has been modeled as a β -structure, with 16 residues per turn forming an ice-like surface (Wolber and Warren, 1989; Kajava and Lindow, 1993; Graether and Jia, 2001). Experimental evidence for this model has been provided by structural characterization of a 6-turn, truncated polypeptide (Kobashigawa et al., 2005). It is notable that the *P. borealis* sequence can also be modeled with similar

folds and similarly has a reiterated TxT (Fig. 3.3B), a motif which has been identified as the flat, ice adsorption face in antifreeze proteins (AFPs) from moths and beetles (Tyshenko et al., 1997; Graham et al., 1997). A second flat surface on the opposite side of the β -fold is predicted to derive from xLi/t motifs, where x is frequently Ser and the Leu residues project inward. Although a model for a grass AFP shows two flat, putative ice adsorption faces on opposite sides of the β -roll (Kuiper et al., 2001), we do not believe that this additional motif would have primarily evolved as a second ice binding surface in INPs. Rather, we speculate that this second face functions to provide a complementary surface for the “interdigitation” of the INPs (Kajava and Lindow, 1993). Since INPs are displayed on the membrane surface, their two flat surfaces on either side of the β -folds would allow close inter-protein association along the faces, thus permitting INPs to assemble as three-dimensional “stairs” consisting of overlapping INP molecules. Since less energy is required for the addition of water molecules to steps (Pruppacher and Klett, 1997), the propagation of ice along these discontinuities would be greatly facilitated, resulting in proteins that are second only to ice itself in the heterogeneous nucleation of water.

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We thank Dr. G. Palmer for assistance with the thermal analyzer and to Ms. S. Wilson for the *P. borealis* strain. Drs. D. Gutterman and G. Voordouw are acknowledged for the *P. syringae* B728a and *E. coli* TG2 strains. Drs. Z. Jia, P. Davies, S. Lougheed and Mr. C. Garnham as well as an anonymous reviewer are thanked for their encouragement. Financial support from a Queen’s University Research Chair and NSERC (Canada) is gratefully acknowledged.

3.7 Figure legends:

Fig. 3.1. Ice nucleation activity of *P. borealis* DL7. Typical thermal profiles showing the release of the heat of crystallization for *P. borealis* (◇; -1.9°C) and *E. coli* BL21 (□; -7.9°C), transformed *E. coli* BL21 contained either pET24a (-; -6°C) or PET24aINP (▲; -5°C) containing the *P. borealis inaPb* coding region. The insert shows further control experiments with *P. borealis* DL7 (A; -2.5°C) compared to *P. syringae* B728a strain (B; -1.8°C) cultures as well as control samples *E. coli* TG2 (C; -7.9°C) and *Chryseobacterium sp.* C14 (D; -7.7°C). Typical thermal profiles are shown; experiments were done three to five times using independent cultures.

Fig. 3.2. Ice nucleation activity expressed as the logarithm of the cumulative number of ice nuclei per microbial cell (ice nuclei/cell; 10 µl samples) for decreasing temperatures. Samples tested include *P. borealis* DL7 (◇), *P. syringae* B728a strain (■), *E. coli* TG2 (▲) and pET24aINP (×). Cultures were grown at 22°C and transferred to 4°C for 2-4 days. Typical data are shown with the experiments done in triplicate.

Fig. 3.3. The ice nucleation protein in *P. borealis* DL7 (A) Phylogenetic tree of the ice nucleation protein from *P. borealis* with other representative INPs (BioEdit).

(B) The 16 amino acid (aa) repeat (positions 164-1076) in *P. borealis* is depicted showing the frequency of each amino acid indicated by the relative size of the letters. The 16-residue unit is repeated three times to make a 48 residue unit, which itself is repeated 19 times (along with 8 more 16 aa repeats).

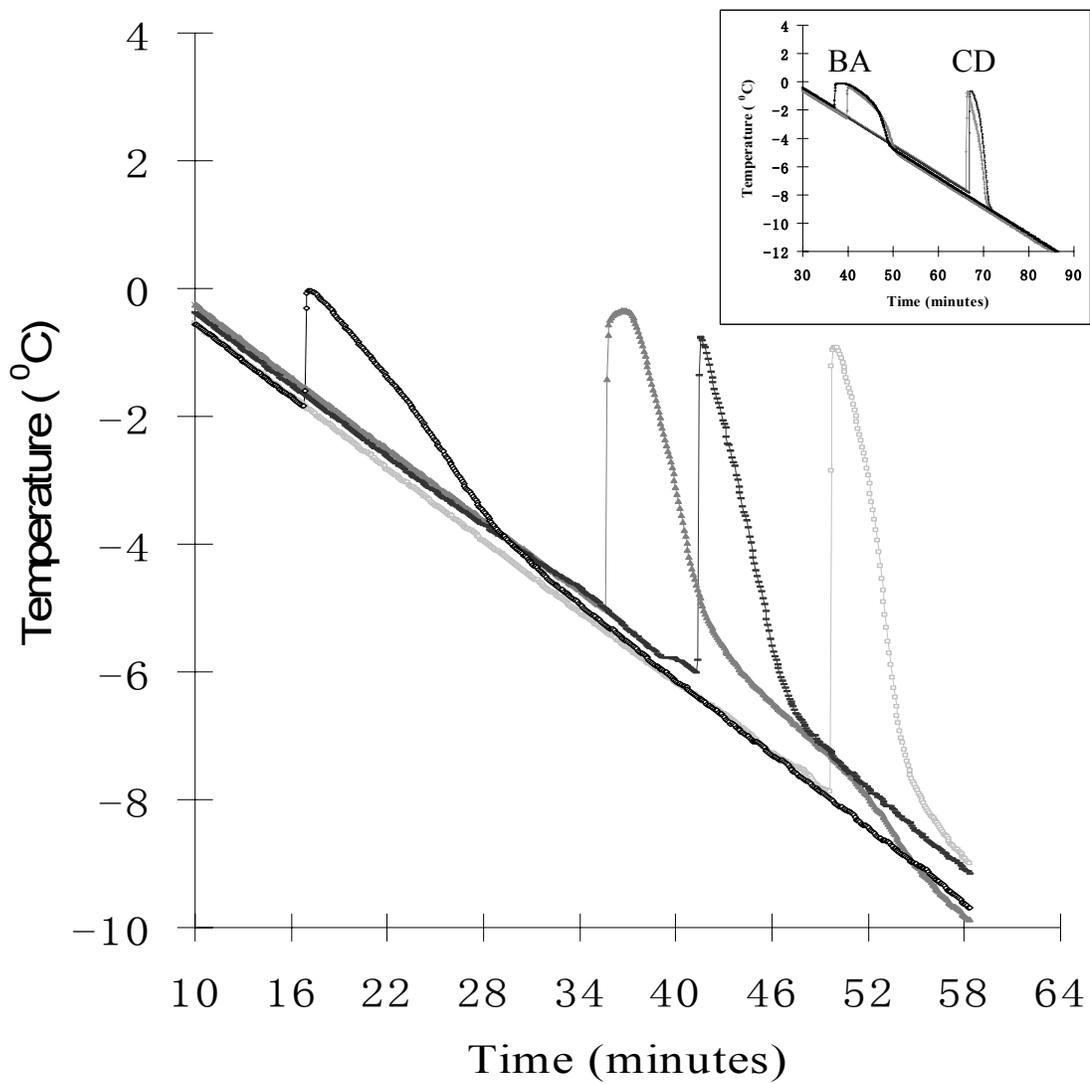


Fig. 3.1

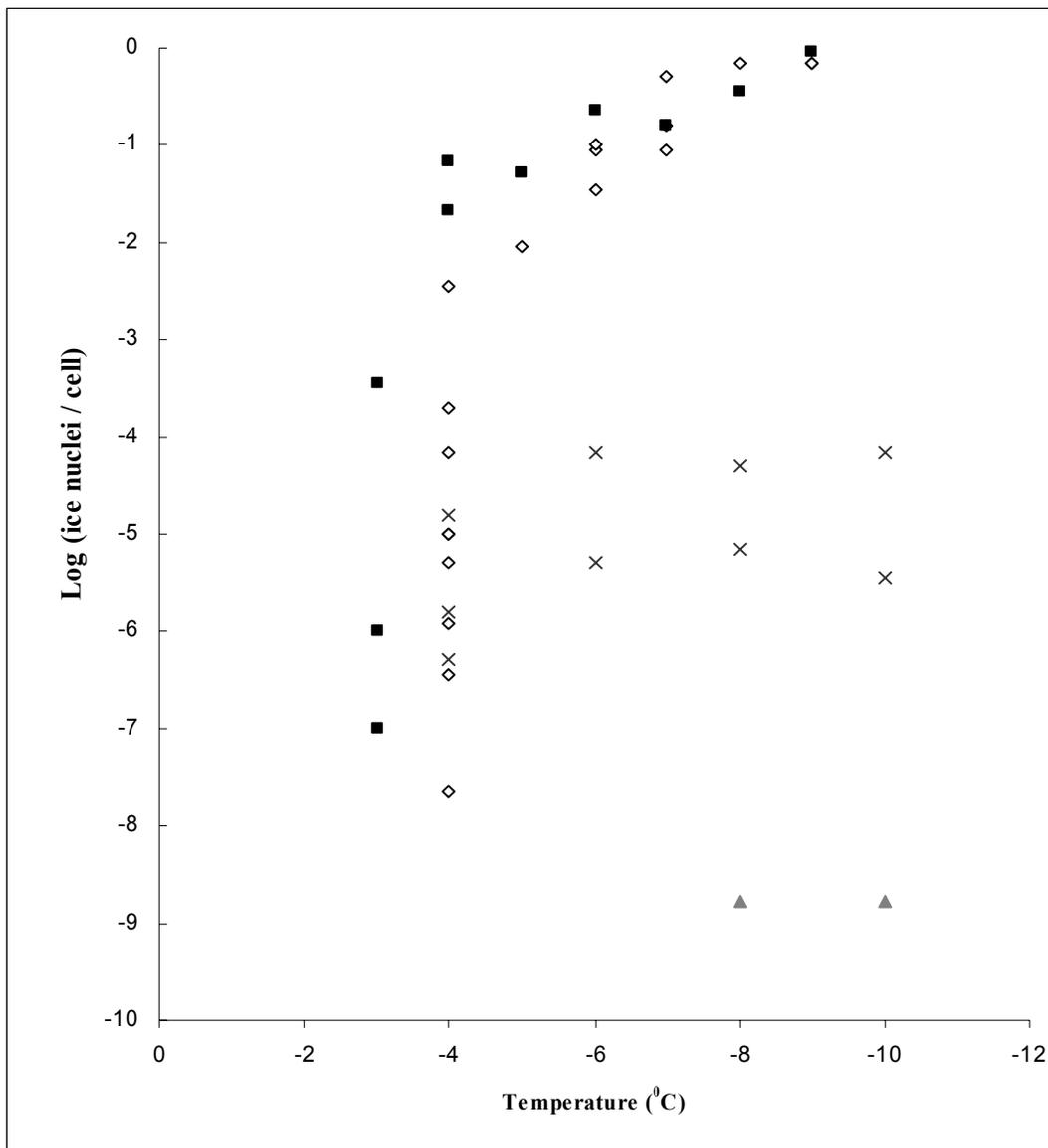
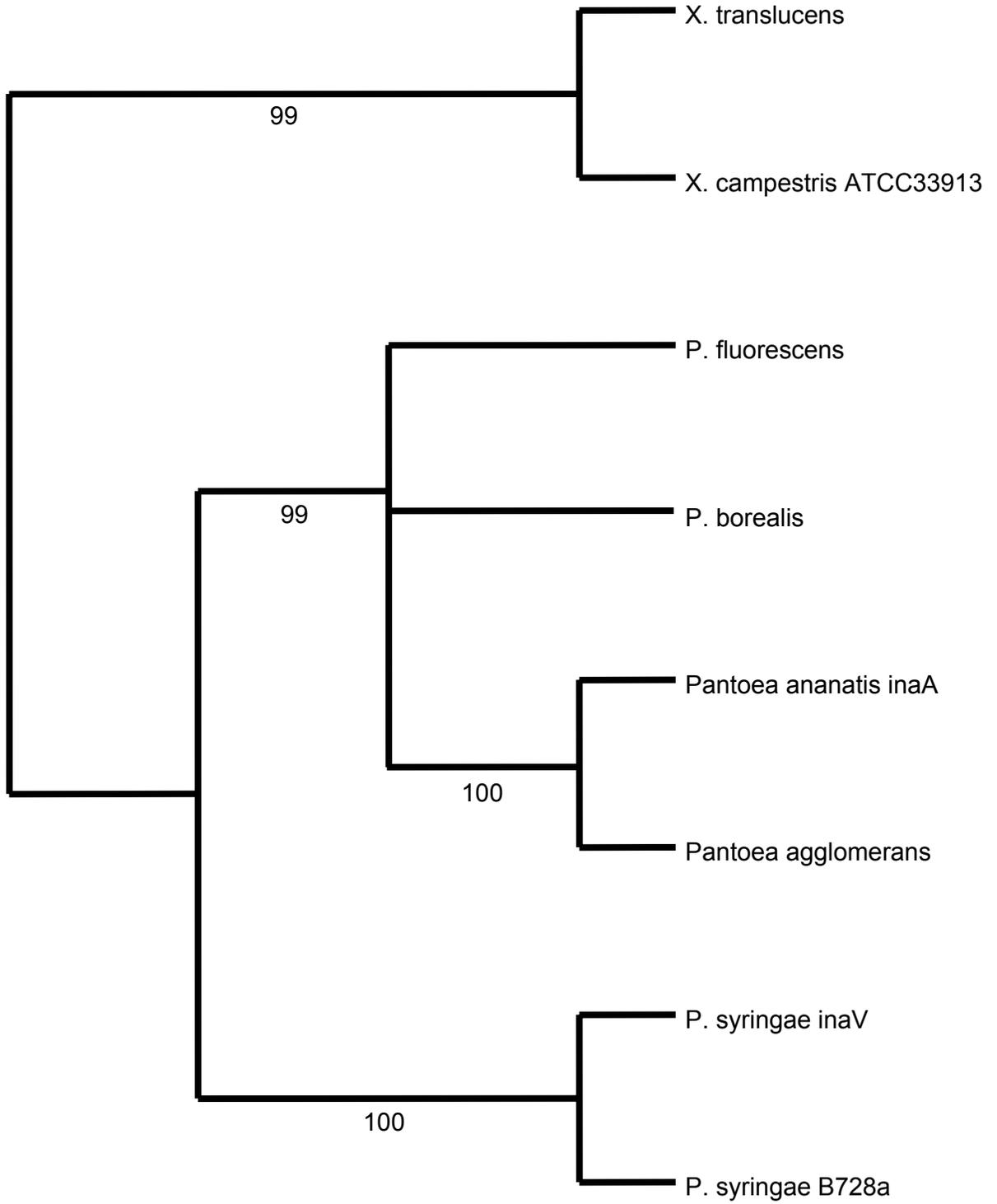


Fig. 3.2

A



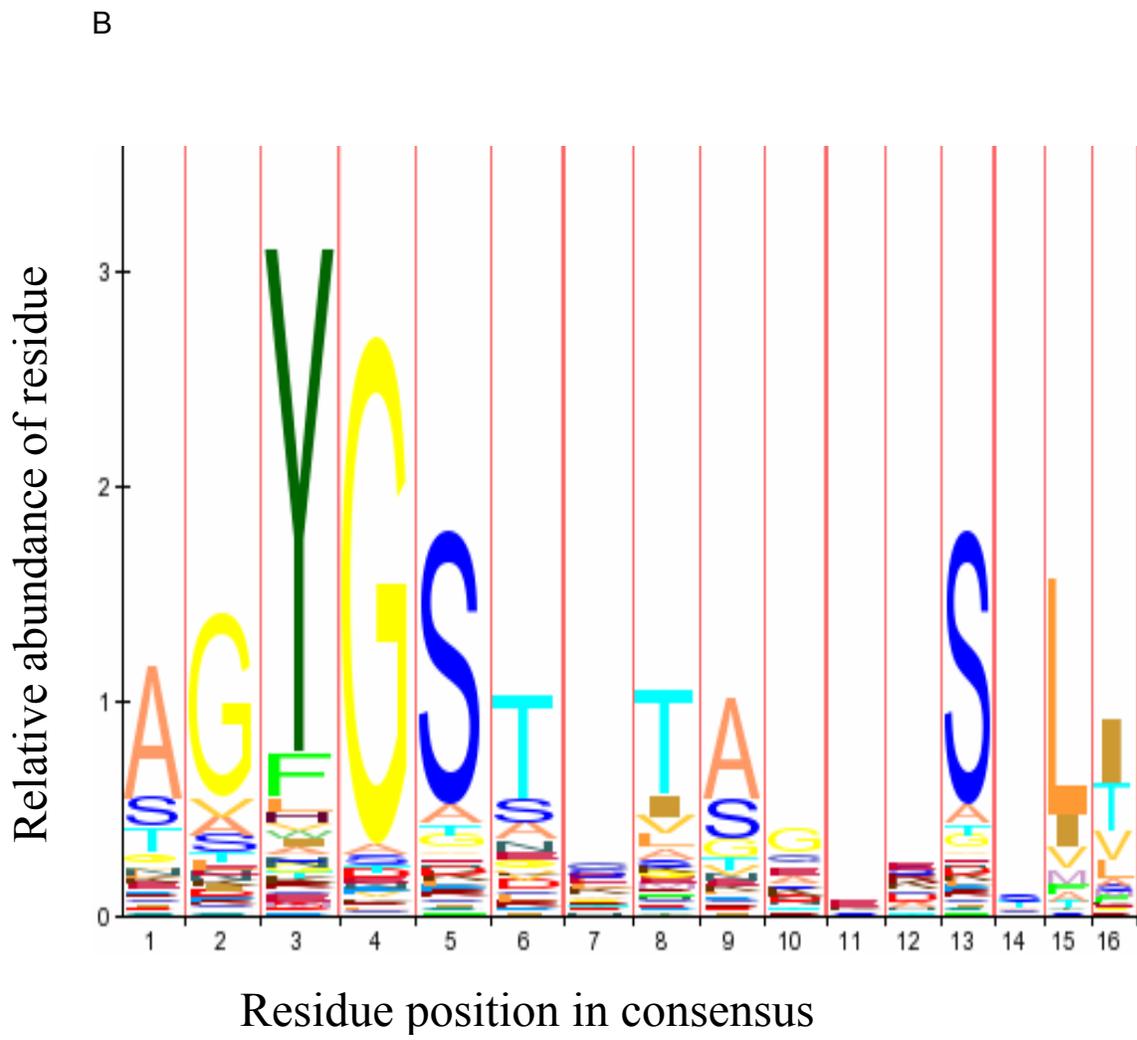


Fig. 3.3.

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Preamble 3

Antifreeze proteins and ice nucleation proteins adsorb to ice crystals. Protein structure simulations done by Chris Garnham in the Davies lab (unpublished) showed that the *Pseudomonas borealis* INP may be a left handed, β -helix structure, similar to the model that has been reported for the hyperactive spruce budworm antifreeze protein (sbwAFP) (see Chapter 1). Previously, the tagging of sbwAFP with a fluorescent protein sequence (jelly fish green fluorescent protein; GFP) was shown to be helpful in understanding the interaction of this protein with the basal and prism planes of single ice crystals (Pertaya *et al.*, 2008). In an analogous manner, it was hoped that the addition of a GFP tag to the *P. borealis* INP would enable the visualization of the protein on ice. Thus, the construction and subsequent expression of sequences encoding an INP-GFP was attempted. In the end, the work did not progress to the point of examining the interaction of the protein with ice, but the following chapter represents a stage in this process.

Chapter 4

***Pseudomonas borealis* ice nucleation protein: visualizing expression**

4.1 Statement of the co-authorship

This chapter represents a paper that is in progress for eventual submission. The authors that have contributed to date are Denian Miao, Zhongqin Wu, and Virginia K Walker. This is a part of project on the characterization *inaPb*. I designed and prepared PGEMTINP and pGEM5extension plasmids, Dr. Denian Miao, a visiting researcher, designed and prepared the pGEMCINP, pGEMNINP, pGEMGFP, pGEMEINPGFP and pEINPGFP constructions, and most of the experiments were done together. Dr. Miao wrote the first draft of the Methods and Results, and I initiated a portion of the first draft of the Discussion and participated in the subsequent editing.

4.2 Abstract

A highly active ice nucleation protein (INP) from *Pseudomonas borealis* has been cloned and shown to encode a protein with high sequence similarity to INPs from a number of bacteria including *P. syringae*. The ligation of gene sequences encoding the *P. borealis* INP and jelly fish green fluorescent protein (GFP) into an expression plasmid produced a recombinant fusion protein of ~ 200 kDa. This INP-GFP retained ice nucleation activity (INA) and as well, the resulting fluorescent phenotype of the *E. coli* host bacteria indicated that both protein domains were appropriately folded. When recombinant *E. coli* cells were shifted to low temperatures similar to those used for the expression of *P. borealis* INP, the distribution of the INP-GFP fusion protein changed. From an initial rather diffuse location throughout the cells, the protein subsequently appeared to be more apparent at the bacterial poles, suggesting a possible mechanism for

the assembly of large protein aggregates that are necessary in order to act as efficient templates for ice growth.

4.3 Introduction

Ice nucleating proteins (INPs) prevent supercooling, ensuring that water freezes at temperatures close to 0°C. Genes encoding these proteins have been cloned from several epiphytes including *Pseudomonas syringae*, *P. fluorescens*, *P. viridiflava*, *Erwinia herbicola*, *E. ananas*, and *Xanthomonas campestris* (Orser *et al.*, 1985; Corotto *et al.*, 1986; Abe *et al.*, 1989; Arai *et al.*, 1989; Hasegawa *et al.*, 1990; Zhao and Orser, 1990). The sequences of INPs found in these diverse bacteria are clearly related and it has been suggested that these genes have been laterally transferred between genera and species on common host plants (Warren and Wolber, 1991; Edward *et al.*, 1994), possibly by conjugation or transduction mechanisms (Kidambi *et al.*, 1994). Presumably these DNA sequences were retained by the new hosts since INPs could confer a selective advantage by giving access to plant nutrients after light frost damage (Lindow *et al.*, 1978). Indeed, the pioneering work by plant pathologists, Lindow and Panopoulos (Lindow, 1987; Lindow *et al.*, 1989), resulted in a genetically-engineered *P. syringae* INP deletion strain, which inhibited ice formation and thus frost damage to crops. Over 25 years ago, “Frostban” or the “Ice minus strain” was the first formulation of a genetically-modified organism given field approval and subsequently tested, albeit in difficult circumstances (Allender-Hagedorn, 2006).

Recently, the sequence encoding an INP was cloned from a non-pathogenic bacterium, *P. borealis*, selected by ice-affinity from soil consortia (Wu *et al.*, 2009; Chapter 3). The coding region shows 66% identity with the *P. syringae* INP and samples taken from stationary cultures initiate freezing at temperatures just below those shown by

the plant pathogen, typically in the range of -2 to -3°C. All INPs are relatively large proteins and the *P. borealis* INP is no exception with 1244 residues (123 kDa) comprising three distinct domains. The central repetitive (R) domain consists of ~20 repeats of a 48-residue high-fidelity consensus sequence that like the protein from *P. syringae*, probably forms a β -fold (Graether and Jia, 2001), mimicking a flat, ice-like surface and thereby allowing ice nucleation and subsequent freezing. This repetitive region is flanked by a 163-residue N-terminal sequence and a 41-residue C-terminal region. Generally, these INPs are known to be post-translationally modified (Warren and Wolber, 1987), transported across the inner membrane and the periplasm, with glycosyl-phosphatidylinositol anchors used to display the assembled lipoglycoprotein aggregates on the outer membrane (Kozloff *et al.*, 1991). Theories of heterogenous ice nucleation predict that large assemblies of properly positioned ice nucleation templates, such as those found on ice nucleation active bacteria, will have a higher nucleation temperature than smaller groupings (Govindarajan and Lindow, 1988). In an effort to further understand the production of these aggregations and their potential interaction with ice, the *P. borealis* INP coding sequence was tagged with jelly fish green fluorescent protein (GFP) and cells expressing the recombinant protein were examined.

4.4 Materials and Methods

4.4.1 Plasmids, strains, and culture conditions

The pET-24a(+) vector (Novagen, WI, USA) was used for expression of INP-GFP fusion protein. pGEM-T Easy (Promega, WI, USA) was utilized after polymerase chain reaction (PCR) cloning, with *E. coli* Top10 and BL21(DE3) (Novagen, WI, USA) employed as hosts for recombinant cloning and expression plasmids as detailed below. *E. coli* strains housing the various plasmids were grown on Luria-Burtani (LB) plates or LB

broth, with ampicillin or kanamycin as the selective antibiotics. These strains as well as *P. borealis* DL7 and *P. syringe* (B728a) were grown on 10% tryptic soy broth and growth was monitored by both OD₆₀₀ and viable cell counts (colony forming units, CFU; Table 4.1).

4.4.2 INP-GFP fusion protein construction and expression in *E. coli*

All of the used plasmids and sequence of the *P. borealis* INP gene, *inaPb*, are indicated in Table 4.2. Briefly, a gene fusion between the INP coding region and GFP was accomplished by assembling four plasmids into one construct including pGEMINP5N (with the 5' terminus of the *inaPb*), pGEMTINP (containing the repetitive (R) core sequence region of *inaPb*), pGEMINPC3 (with the 3' terminus of *inaPb*) and pGEMGFP (encoding GFP). To accomplish this, oligonucleotide primers were synthesized (ACGT Corp, Toronto, Canada) as indicated in Table 4.3. INPN, INPC and GFP gene sequences were generated by conventional PCR using the GeneAmp 2400 system (Perkin Elmer, CA, USA) and template plasmids pGEM5extension, pGEMTINP (see Chapter 2, Table 4.3) or pEGFP (U76561; Clontech, CA, USA). Amplified products were inserted into the pGEM-T vector in order to generate pGEMINP5N, pGEMINPC3 as well as pGEMGFP, and subsequently sequenced to confirm veracity. After digesting pGEMGFP with BamHI and NcoI, the GFP fragment was recovered based on its predicted size and inserted into pGEMINPC3, which had been digested with BglII and NcoI. After ligation, pGEMINPCGFP was generated. This plasmid was then digested with BglII and NcoI and the appropriate restriction fragment was inserted into pGEMINP5N that had previously been digested with the same two restriction enzymes, to give pGEMNCGFP. The region encoding the repetitive region of INP was obtained by digestion of pGEMTINP with XhoI and AfeI. This region was then inserted into

pGEMNCGFP digested with the same enzymes, with the result that pGEMINPGFP was generated that has the N-terminal, R domain and C-terminal of the INP, tagged with GFP.

To express the INP-GFP fusion protein under control of the T7 promoter, a T7-INP-GFP fusion gene (with T7 tagged at the N-terminus) was generated by subcloning pGEMINPGFP into the pET-24a(+) vector by conventional cloning methods (with NdeI and HindIII used to digest pET-24a(+) so that the T7 tag could be removed for the insertion of the INP-GFP fusion directly into the ATG start codon downstream of the T7 promoter). The construct was then used to transform *E. coli* Top10 cells, using CaCl₂ transformation. Standard methods of plasmid preparation and restriction analysis were used for the analysis of the transformants. Subsequently, the final plasmid construct containing the INP-GFP fusion gene sequence (pEINPGFP) was used to transform *E. coli* BL21(DE3) cells, as above.

DNA fragments were routinely sized on 0.8% agarose gels relative to a DNA ladder (GeneRuler™ 1kb DNA Ladder Plus; Fermentas, ON, Canada). For single colony PCR, individual colonies transfected with ligation reactions were isolated using a sterilized pipette tip and diluted into 30 µl sterilized water. The PCR reaction mixture contained 5.0 µl 10 X Taq Buffer with KCl [100 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl, 0.8% (v/v) Nonidet P40], 3.0 µl 25 mM MgCl₂, 1.0 µl 10 mM dNTP mixture, and 20.0 pM each primer. For the analysis of pEINPGFP, a pair of GFP primers were used (upstream Gw1: 5' GGAATTCAGATCTGTGAGCAAGGGCGAGGAGCTGTTC; downstream G12: 5' CGAAGCTTTCATTACTTGTACAGCTCGTCCAT GCCGAGAG). Diluted cells (3.0 µL) and 2.5 U Taq polymerase (Fermentas, ON, Canada) were added to the 50 µL PCR reaction mixture and the DNA was amplified according to the following

program: one cycle of 95°C for 5 min, 30 cycles of 94°C for 30 s, 50°C for 45 s, 72°C for 45 s, and then incubation at 72°C for 10 min, with a final hold at 4°C.

To express the INP-GFP fusion protein in the recombinant BL21 cells, single colonies harboring the recombinant plasmid pEINPGFP, and separate control cells containing only the null vector pET-24a(+), were used to inoculate LB broth containing 50 µg/mL kanamycin. They were cultured overnight at 37°C. Aliquots (400 µL) were then transferred fresh LB broth with the same concentration of kanamycin (10 mL) and grown (shaken at 200 rpm at 37°C) until the optical density at 600 nm reached 0.6. After induction with isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration 0.5 mM, the temperature was shifted to 22°C for 24 h.

4.4.3 Protein gel electrophoresis and microscopy

Induced cells were harvested by centrifugation at 10,000 g for 1 min and the recovered pellet was washed with 0.85% NaCl and then suspended in sterile water. Washed cell suspensions were mixed with equal volumes of 2 X gel loading buffer (100 mM Tris-HCl, pH 6.8; 4% SDS; 20% glycerol; 0.2% bromophenol blue and 2% 2-mercaptoethanol). The mixture was heated at 95°C for 10 min, immediately chilled on ice for 5 min, and digested by DNase I at 37°C for 10 min before recentrifugation (10,000 g for 5 min). The supernatant (20 µL) was analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a 7.5% (w/v) separating gel and a 5% stacking gel, and electrophoresed until the marker dye had migrated to the bottom of the gel. The gels were stained 0.05% Coomassie Brilliant blue G-250 (Sigma-Aldrich, ON, Canada) dissolved in 50% methanol and 10% acetic acid and the masses of the

visualized proteins were estimated using co-electrophoresed molecular weight protein markers.

Fluorescence was detected by microscopic examination using cells bearing the INP-GFP fusion protein 48 h after induction with IPTG (at 22°C for 48 h or 22°C for 24 h followed by a shift to 4°C for 24 h). In some cases, cells were examined after keeping the cells that had been shifted to 4°C for an additional 24 h (see Results). Fluorescence microscopy was conducted by placing cell suspensions (5 µL) on a cleaned microscope slide and topping with a cover glass. Cells were examined using a microscope (Zeiss Axioplan 2), equipped with fluorescence illumination (543 nm average) and with a confocal microscope (Leica TCS SP2 Multi Photon).

4.4.4 Ice nucleation activity assays

For convenience, large volume assays were conducted on bacterial cultures (10^9 CFU/mL; OD₆₀₀ of 1) treated as described, which were placed into vials (1 mL) and stored at 4°C for > 1 h. These were then submerged in a 50% ethylene glycol bath at 4°C, and the temperature of the circulating bath was decreased at 0.2°C/min from 4°C to -15°C. The mean freezing temperature for the cultures or controls (LB and *E. coli* BL21 harboring pET-24a(+)) was used to estimate the ice nucleation temperature.

4.5 Results

4.5.1 Expression plasmid assembly

The assembly of pEINPGFP, although involving multiple cloning steps, was successful. Following transformation, positive recombinants were resistant to kanamycin as predicted and were subsequently verified by PCR, and restriction enzyme digestion. To further confirm that the plasmid vectors did contain INPGFP inserts, single Hind III

digestions and double digestions with both NdeI and HindIII were done. After electrophoresis, the double digest yielded two fragments (one at 5.2 kb as predicted by the parent vector pET-24a(+)) and the other at 4.5 kb in agreement with target insert INPGFP), and the single digest generated one fragment (at 10 kb as predicted; not shown). The expression vector was 9707 bp. Limited nucleotide sequencing was performed to ensure that the codons were in frame and that PCR had not introduced mutations (not shown). A map of the pEINPGFP construct is found in Appendix 7.

4.5.2 Expression of the INP-GFP fusion protein

The constructed plasmid, pEINPGFP, encoded an INP-GFP hybrid protein consisting of the entire cloned *P. borealis* INP sequence fused in frame to GFP, and was expressed from the IPTG-inducible T7 promoter. After induction, SDS-PAGE analysis of the cell lysates showed a faint band larger than the 200 kDa molecular weight marker protein, as well as a lower molecular weight band, which was not present in the pET-24a(+) control preparation (not shown). The induced cells showed modest, but consistent INA (Fig. 4.1). Aliquots (1 mL) froze at -6 to -7°C compared to -9°C for control cells (non-transformed or *E. coli*-transformed with the pET-24a(+)) null plasmid).

Fluorescence microscopy of the cells showed that the majority of recombinant *E. coli* cells harboring pEINPGFP fluoresced green under UV light (Fig. 4.2 and not shown), indicating the GFP protein was still able to fold correctly and to form its chromophore when fused to the INP. A time course, followed with confocal fluorescence microscopy, showed that fluorescence seemed relatively evenly distributed throughout the cytoplasm at 24 h after induction at 22°C (Fig. 4.2, left image). In contrast, if the cells were kept for 24 h at 4°C after IPTG induction, fluorescence was noticeably bright at the poles of the bacterial cells with a weaker signal in the centre of the cell (Fig. 4.2, middle image). If

cells treated in this manner were left at 4°C for an additional 24 h, fluorescence appeared to be all localized to the poles in many of the cells (Fig. 4.2, right image). Fluorescence was not quantified and data on the percentage of fluorescent cells, although collected, were not provided.

4.6 Discussion

It has been appreciated for some time that INA is determined by the size of the nucleating structures (Fletcher, 1963). Although large as judged by recombinant protein measures, ~150 kDa bacterial INP by themselves are insufficient to nucleate ice above about -12°C (Govindarajan and Lindow, 1988); multiple INP copies must aggregate into larger templates to initiate ice growth at higher temperatures (Southworth *et al.*, 1988; Ruggles *et al.*, 1993). At -3°C, for example, it is estimated that at least 60 INP are needed to form a sufficiently large extracellular aggregate to nucleate ice growth (Hew and Yang, 1992). Although *P. borealis* showed high INA, when this INP gene sequence was expressed in *E. coli*, the observed low ice nucleating temperatures of -5 to -6°C (Fig. 4.1) was only two degrees above control cultures. These results are similar to previous observations on the lower activity of INP-expressing recombinant *E. coli* where active ice nuclei were observed to accumulate both on the inner and outer cell membrane (Wolber *et al.*, 1986; Watanabe *et al.*, 1990).

INA expression is associated with bacteria at low temperatures (Yankofsky *et al.*, 1983; Nemecek-Marshall *et al.*, 1993; Fall and Fall, 1998; Wu *et al.*, 2009). Indeed, it has been suggested that the genes cannot be induced unless the cells are in stationary phase, but then, if shifted to warmer temperatures after induction, proteolytic degradation and poor representation of INP aggregates on the fluid membranes result in the rapid loss of INA (Deininger *et al.*, 1988; Watanabe *et al.*, 1990; Nemecek-Marshall *et al.*, 1993). Here

we observed that after the transfer of *E. coli* cells to lower temperatures there was a progressive localization of INP-GFPs to the poles. Previously, researchers utilizing fluorescently-labeled antibodies or fusion proteins, both using only the N-terminal region of the INP, have reported visualizing the protein over the entire membrane surface (*e.g.* Wu *et al.*, 2006). In contrast, our construct consisted of the entire protein as well as the fluorescent marker, and thus this larger molecule at > 200 kDa (Fig. 4.2) may be more efficiently folded and transported across the periplasm and the outer membrane in the polar regions of rod-shaped bacteria.

Polarized structures such as flagella and pili are common in bacteria. Certain proteins involved in DNA replication are localized there and the poles are also the sites of the 90-200 kDa autotransporter secreted proteins, Type II and Type IV secretion systems, the chemotaxis receptor protein and even polysaccharide export (Shapiro *et al.*, 2002; Thanbichler and Shapiro, 2006; Bowman *et al.*, 2009; Jain *et al.*, 2006; Scott *et al.*, 2001; Judd *et al.*, 2005; Ping *et al.*, 2008; McNulty *et al.*, 2006). In the case of the polar localization of chemotaxis proteins, it is thought that these sites facilitate the clustering necessary for the proper functioning of the signal response (Ping *et al.*, 2008)

The mobility of cell surface proteins is restricted at the poles due to a relatively stable peptidoglycan (de Pedro *et al.*, 2004). Thus, Jain *et al.* (2006) have suggested that polar regions may provide a more stable scaffold for the translocation and folding of large proteins. It should be remembered, too, that in non-psychrophiles such as *E. coli*, the fluidity of the entire membrane is decreased at low temperatures (Phadtare, 2004), and thus it is likely that proteins would tend to remain at the sites of transport. It is known too that the structure of several ice-associating β -helix proteins is also only stable at lower temperatures (Middleton *et al.*, 2009; Scotter *et al.*, 2006). We thus hypothesize that low

temperature induced β -folded INPs are preferentially transported to the outer membrane at the poles. The density of the proteins produced at such localization would promote interprotein interactions, possibly on the flat sides opposite the ice-binding residues in the repeats (Wu *et al.*, 2009), which in turn, would facilitate the formation of even larger aggregates, effectively stabilizing ice templates. The decreased fluidity of the *E. coli* membrane would prevent the migration of these anchored assemblies across the entire outer membrane, likely resulting in lowered INA relative to that found in the natural host psychrophile.

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Table 4.1. Bacteria and their media used in pEINPGFP constructions and functions.

	<i>E. coli</i>	<i>P. borealis</i>	<i>P. syringae</i>
Bacterial strains	Top10, BL21(DE3)	DL7	B728a
medium	LB	10% TSB	10% TSB

Table 4.2. Plasmids used in the pEINPGFP construction, the PCR template DNAs and the sequenced regions derived from pbINP gene.

Plasmid	Template	Sequence (bp)
pGEMINPC	pGEMcoreinp	3576-3732
pGEMGFP	pEGFP	
pGEMCGFP		3576-3732
pGEMINPN	pGEM5extension	4-123
pGEMNCGFP		Δ123-3576
PGEMTINP		121-3631
pGEMINPGFP		4-3732
pEINPGFP	pET24a	1-3732

Table 4.3. Sequences of the primers use for PCR amplification in plasmid constructions

Name	Sequences	Length of PCR products
INPC	upstream primer 5' TTAGATCTACGCTGACGGGCGGCGAGAACT 3'	172
	downstream primer 5' GCGGATCCTTCCCCGGATTTATTCACGAT 3'	
GFP	upstream primer 5' GGAATTCAGATCTGTGAGCAAGGGCGAGGAGCTGTT C 3'	741
	downstream primer 5'CGAAGCTTTCATTACTTGTACAGCTCGTCCATGCCGAGA G 3'	
INPN	upstream primer 5' GGGAATTCATATGAACGATGACAAAG 3'	145
	downstream primer 5' TAGGATCCCTCGAGCTTTCGGGTG 3'	

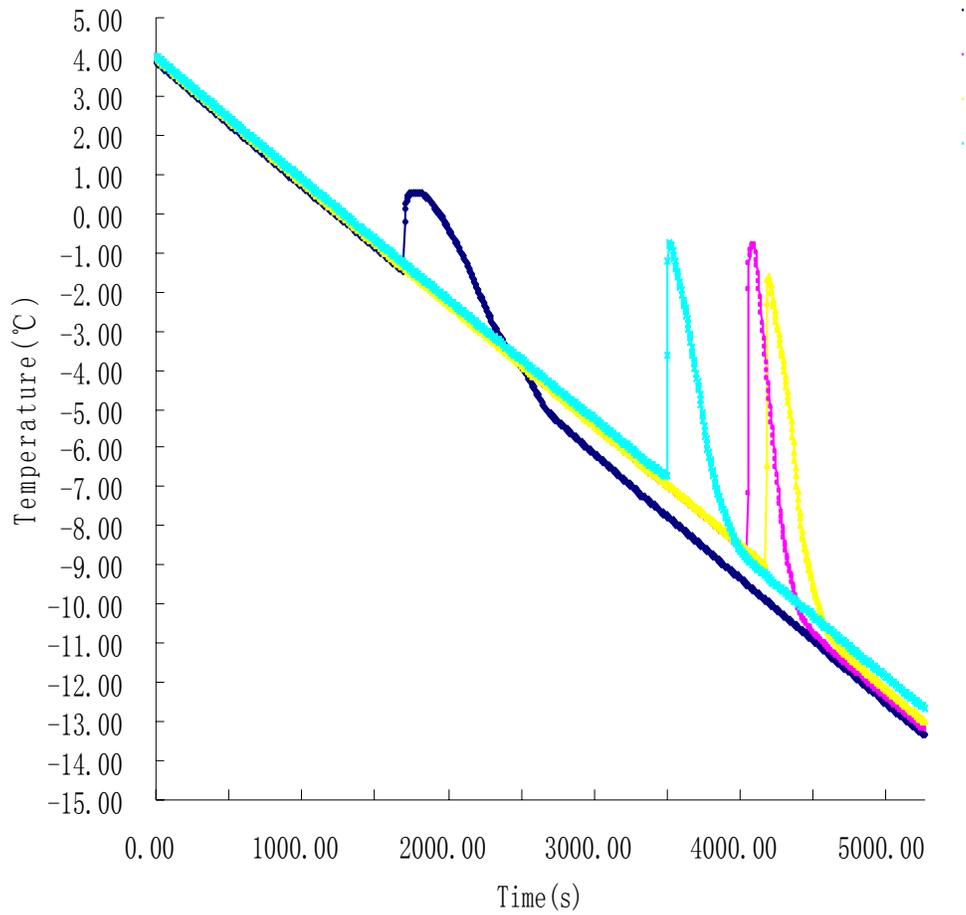


Fig. 4.1 Representative thermal profiles of *P. borealis* DL7 (dark blue) and recombinant *E. coli* containing the pEINPGFP construct (turquoise), which nucleated at -1.5°C and -6.7°C, respectively. *E. coli* pET-24a(+) (yellow) and culture medium controls (pink), nucleated at approximately -9.0°C. The Y-axis shows the temperature (°C) and the X-axis shows time (sec).

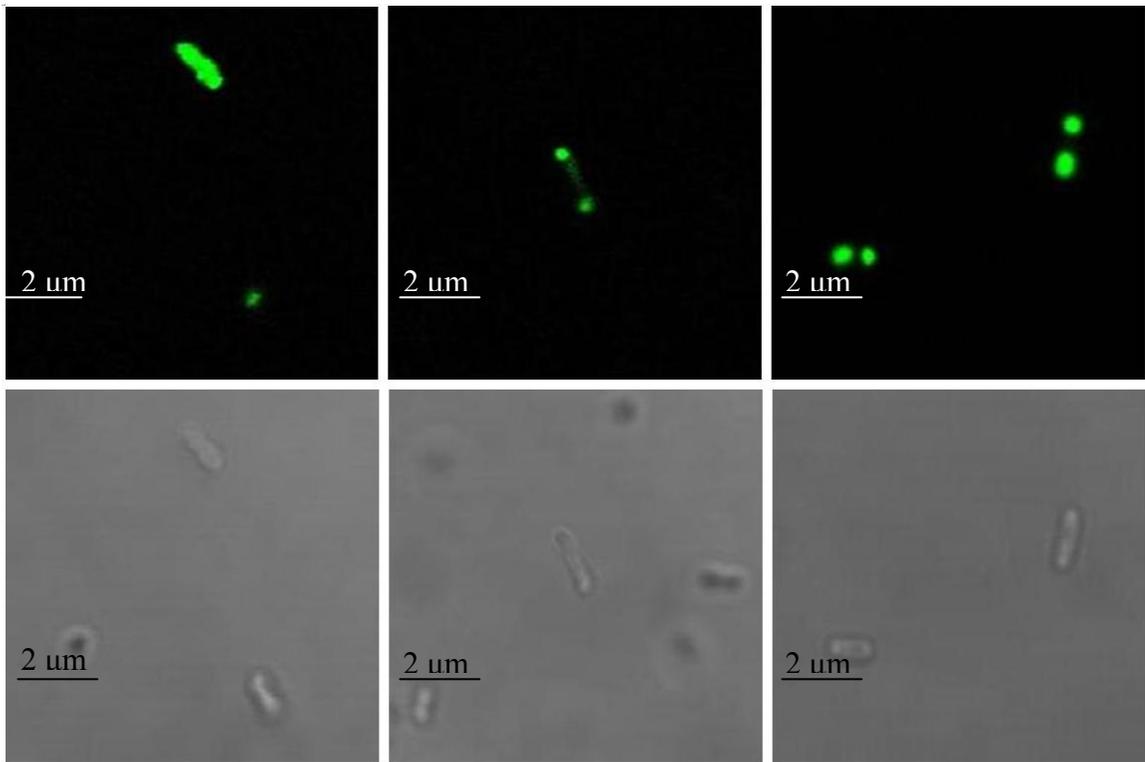


Fig. 4.2 Recombinant *E. coli* cells induced to express the INP-GFP fusion protein were visualized by confocal microscopy. Representative images show recombinant *E. coli* cells induced with IPTG after 24 h at 22°C (left), after storing those cultures at 4°C for 24 h (middle), and after storing these at 4°C for an additional 24 h (right). Each bar — represents 2 μm. The upper images are visualized with fluorescent illumination (see Materials and Methods) with the same lower images viewed using phase contrast microscopy.

4.7 References

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Chapter 5

General Discussion and Future Perspectives

Prokaryotes including Eubacteria and Archaeobacteria are the most successful extant organisms. Since appearing about 3.5 billion years ago (Schopf and Packer, 1987), and representing some of the oldest life forms, they remain the most dominant life on earth. The field of microbiology, and especially bacteriology, developed quickly subsequent to the invention of microscopy (including the light microscope, scanning electron microscope, and transmission electron microscope), and the simultaneous development of other technologies and disciplines including biochemistry, immunology, molecular biology (PCR, DNA sequencing, DNA cloning and protein expression systems etc.), and the more recent advances in genomics and bioinformatics technologies. Currently, more than 800 full length bacterial genomic DNA sequences have been registered in the microbial genome database (<http://mbgd.genome.ad.jp/>) to facilitate comparative genomic analysis. Bacterial genomic maps can be viewed and analyzed in the BacMap Atlas database (<http://wishart.biology.ualberta.ca/BacMap/>). As well, functional information on bacterial genes and metabolic pathways of genome-sequenced bacteria can be found in the KEGG database (<http://www.genome.jp/kegg/kegg2.html>). With additional bacterial genome sequencing and annotation projects currently ongoing in multiple genomic centers such as the Pasteur Institute and Sanger Institute, the number of bacterial genomes and the concomitant functional information is expected to increase at a fast pace. There is no doubt too that progress in applied microbiology research has improved the quality of human life; with the development of vaccines and antibiotics, most dangerous bacterial diseases, such as cholera, tetanus, and tuberculosis, are now under control. Genetically-engineered bacteria, such as *E. coli*, have been broadly used to

produce recombinant therapeutic biologic drugs, from recombinant tumor necrosis factor to interferon and other cytokines. Metabolic products of microbes have also been applied in food and other industries, including two examples used in frequently referred to in this thesis, that of xanthan gum, which has been used as a food additive or in drug delivery, and Snowmax, which is used to save energy in low temperature applications including in ski areas.

Despite substantial advances, some old questions in the field of microbiology have still not been satisfactorily answered. One such question concerns Koch's rule, one of the biggest paradoxes in microbiology research. In Koch's rule or postulate, pure cultures of potential pathogenic bacteria have to be retrieved *in vivo* and disease has to be reproduced *in vitro* to be verified. Although many important pathogenic bacteria have been described using this principle, other pathogens cannot be cultured in artificial medium. Until recently, this was the case for *Mycobacterium leprae* and *Helicobacter pylori* (Marshall and Warren, 1983). Confirmation of the bacterial infection proved to be a big challenge. Today non-culturable microbes remain a large majority of the unknown microbial world.

Another great challenge in microbiology is imposed by bacterial interactions and mutualistic activities (Stuart *et al.*, 2007). The principles upon which natural selection and Darwin's theory of evolution are based deal, for the most part, with individual species. The interactions among different microbes, including horizontal gene transfer, or the interactions between microbes and other living organisms as well as abiotic factors (Walker *et al.*, 2006; Sullivan and Palmisano, 1984) are other challenges for microbiologists. In this thesis, I am concerned with how different species of bacteria survive and interact with each other and in their communities, while coping with low temperatures.

The discovery of biofilms and the development of biofilm theory in the last 30 years have enabled microbiologists to at least partially understand how microbes survive and interact in stressful environments (Caldwell and Costerton, 1996; Costerton *et al.*, 1978). Evidences for biofilms have been found in dental infections, transplant-related infections, wastewater treatment plants and chronic diseases. Biofilms are often associated with a stress response and result in resistance to antibiotics, phagocytes and radiation, amongst other trauma (Gilbert *et al.*, 1997; Elasri and Miller, 1999). ‘Synergistic biofilms’ appear to be formed by a combination of different biofilm-producing bacteria. These can show an increase in biomass to 1.16 times along with increased resistance to hydrogen peroxide and tetracycline treatment from 2 to 4 fold (Burmølle *et al.* 2006). Based on image analysis, a bacterial biofilm model was developed and shows at least 5 distinct steps (Stoodley *et al.*, 2002; Monds and O’Toole, 2009; Appendix 9). First, free living microbial cells swim to a surface, and secondly, they reversely attach to the surface. Subsequently, microcolonies begin to form on the surface followed by the fourth step, the potential formation of macrocolonies with the cells swarming to a new surface or dispersing to a free-swimming environment. In the fifth step, a complex heteroarchitecture forms, but the structure of biofilm may dissolve and cells are prone to swim to new habitats. To date, most studies on biofilms concentrate on bacteria with relative high adhesion to certain surfaces and their swarming motility. This explains why swimming, swarming and adhesion assays are part of the necessary description of any newly described biofilm.

In this thesis, Chapter 2 describes the initial characterization of biofilms that may be linked to the relative richness of the microbial community on frost-exposed leaves. Different culturable bacteria species (15) were identified, ranging from those found in the

classes Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, to Flavobacteria. Interestingly, the composition of bacterial genera found in this study was similar to some of those microbes isolated from the Arctic and other cold regions (Wynn-william, 1983; Bottos *et al.*, 2008; Marx *et al.*, 2009; Steven *et al.*, 2007; 2008; Walker *et al.*, 2006). Among the bacteria listed in Chapter 2, *P. syringae* J6 had INA activity and this may explain the INA activity observed when the whole culturable microbial community was assayed together. In fact, *P. syringae* is the best known and abundant INA⁺ bacterium in North American temperate regions (Zagory *et al.*, 1983; Lindow *et al.*, 1978). It has been isolated from leaves of almond, pear, oats, snap bean, corn and other plants (Arny *et al.*, 1976; Lindow *et al.*, 1978; Lindow *et al.*, 1982). After estimating the abundance of *P. syringae* from different water resources such as rain, snow, alpine streams, lakes and plants, Morris *et al.*, (2008) suggested that the life cycle for *P. syringae* was driven by environmental water cycles (Fig 5.1).

Among the described isolates derived from overwintering leaves studied here, *P. syringae* J6 had a relatively lower tolerance to freeze-thaw cycles than those bacteria with IR activity (*e.g.* *Erwinia billingiae*). It also may be relevant that there was no evidence of biofilm formation in the *P. syringae* isolate. However, after 24 freeze-thaw cycles, viability in the INA⁺ isolate was still two orders of magnitude higher than with *E. coli*. *E. billingiae* and *Flavobacterium sp.* lost only approximately three orders of magnitude in cell viability after 48 cycles, compared to no viable cells found in the *E. coli* control. The highest freeze-thaw resistance was observed with an isolate of *S. kitahiroshimense* Y2, which lost little viability over the entire course of the experiment (Chapter 2), and which showed evidence of biofilm (Appendix 4). Similar to the microbes studied in this thesis, Walker *et al.*, (2006) showed high levels of freeze-thaw resistance in microbes from

temperate soils. No attempt, however, was made to characterize biofilm formation in any of the recovered soil isolates in that study.

Biofilm formation has been suggested to be important for survival in diverse environments (Stoodley *et al.*, 2004; Hong and Marshall, 2001). Biofilm EPS provides an extra coat to protect cells from low temperature-related injury or damage and also keeps formed ice crystals small. It is known that flagella-driven swarming motility and swimming activity are related to biofilm expansion (Harshey 2003). The appearance of *E. billingiae* colonies in swarming agar assays suggested that these bacterial cells were motile and further indicated possible flagella involvement (Köhler *et al.*, 2000). Indeed, electron micrographs showed a single monotrichous polar flagellum on *E. billingiae* cell, while 1-3 polarized flagella were associated with *P. borealis*, a bacterium isolated by ice affinity and derived from low Arctic soil (Chapter 3; Appendix 5 and 6). Scanning electron micrographs showed a “slimy” EPS-like matrix associated with *P. borealis* and *S. kitahiroshimense*, but a thick matrix structure was linked to observations of *E. billingiae*. These studies provided direct evidence of biofilms in these three ice-associating microbes. Although the relationship between flagella and biofilms has not been extensively investigated, several biofilm-producing bacteria do have flagella that are localized to the poles and include *P. aeruginosa*, *H. pylori* and *E. carotovora*. As mentioned, there appears to be some correlation between flagella-driven motility, which may have an important role in cell migration and colony expansion. Swarming cells may be hyperflagellated, with a result that their continuous movement provides a quicker access to fresh nutrients (Allison and Hughes, 1991; Fraser and Hughes, 1999; Harshey, 2003), as flagella-associated swarming results in a faster movement than pili-driven twitching and gliding (Harshey, 2003; Köhler *et al.*, 2000; Mijan and Shinji, 2006).

Biofilms are composed of bacterial cells and EPS structures, and thus these bacteria might become cold resistant due to the presence of these extracellular macromolecules. The production of the extracellular matrix or EPS, the main component of biofilm, has reportedly increased when producing cultures were subjected to cold acclimation, desiccation and nutrient limitation (Wallner *et al.*, 1986; Roberson and Firestone, 1992). As well, EPS can enhance some microbial metabolism and survival at low temperatures (Wolfaardt *et al.*, 1999; Junge *et al.*, 2006; Kim and Yim, 2007). The experiments in this thesis showed that the EPS of *E. billingiae* was able to confer increased freeze-thaw resistance to *E. coli* and *P. syringae* J6 (Chapter 2). EPS has previously been shown to be associated with microbes in water and sea ice (Heukelekian and Heller, 1940; Jones *et al.*, 1969; Marx *et al.*, 2009), and as well, EPS appears to improve viability after several freezing events (Tamaru *et al.*, 2005; Hong and Marshall 2001; Kim and Yim, 2007). Although a similar mutual beneficial effect on freeze-thaw survival was produced by *Chryseobacterium* sp C14 on *Enterococcus* sp. C8 as well as *E. coli* (Walker *et al.*, 2006), in this case, the presence of biofilm was not investigated. Preliminary experiments (not shown), however, indicate that *Chryseobacterium* sp C14 does not form a biofilm under the conditions tested.

The presence of biofilm may also help explain the overall survival of microbes on the leaves from winter plants as well as the INA in leaf homogenates. It is known that biofilms are important to certain bacterial pathogenesis, but less attention has been given to biofilms on plants and in bacterial interactions in cold environments (Crossman and Dow, 2004; Marques *et al.*, 2002; 2003; Mijan and Shinji, 2006; Bodman *et al.*, 2003; Morris *et al.*, 1997; 1998; Morris and Monier, 2003). Only high concentrations of bacteria (over 10^4 CFU/mL) can nucleate ice effectively at temperatures close to 0°C

(Army *et al.*, 1976; Lindow *et al.*, 1978), yet because *P. syringae* is not very freeze-thaw resistant in isolated culture (viable cell number dropped to about 100 CFU/mL after 24 cycles of freeze-thaw cycles; Chapter 2), there may be insufficient cells to promote ice nucleation at temperatures close to 0°C. When *E. billingiae* EPS was added to *P. syringae*, the viable cell concentration was improved by 4 orders of magnitude to about 10⁶ CFU/mL at 24 freeze-thaw cycles (Chapter 2), which is a sufficiently high cell number to induce ice nucleation at a relatively high subzero temperature (Army *et al.*, 1976; Lindow *et al.*, 1978).

Biofilms appear to play an important role in quorum sensing (QS), which is critical in pathogenesis and cell communication, swarming motility, horizontal DNA transfer, and bacterial survival (Bodman *et al.*, 2003). QS may also be used by INA⁺ epiphytes as part of their survival strategy. Shepherd and Lindow (2009) identified two N-acyl-homoserine lactone acylases (HacA and HacB) from *P. syringae* B728a that hydrolyzed a QS signal, acyl homoserine lactone (AHL). Degradation of AHLs may be important for the pathogenesis of *P. syringae* as HacA may work as a quorum-quenching agent to repress the population of QS-dependent bacteria, and thus help *P. syringae* become dominant at low temperatures (Shepherd and Lindow, 2009). Similarly, the AHL-synthase gene was found in *Erwinia ananatis*, which may be involved in EPS production and biofilm formation (Morohoshi *et al.*, 2007; Coutinho and Venter, 2009). A new QS signal pathway was found in *Xanthomonas*, with its *rpf* gene cluster involved in the regulation of the production of virulence factors by the small diffusible factor (DSF) (Dow *et al.*, 2003). The *rpf*/DSF system controls its biofilm formation and dispersion (Dow *et al.*, 2003; He and Zhang, 2008; Crossman and Dow, 2004).

Similar to epiphytic INA⁺ bacteria, the soil bacterium *P. borealis*, promoted ice

nucleation at close to 0°C and showed evidence of a biofilm (Chapter 3; Appendix 5). INA assays revealed that *P. borealis* has type I INA activity, which is similar to *P. syringae* and other Type 1 INA bacteria (Chapter 3; Maki *et al.*, 1974; Arny *et al.*, 1976; Lindow *et al.*, 1978; Lindow 1987; Zhao and Orser, 1990). In this thesis, INA production by *P. borealis* was associated with continuous cell growth at low temperature, or after growing at room temperature to stationary phase with subsequent induction at 4°C (Chapter 3). In contrast, INA from *P. syringae* and *Pantoea ananatis* was reported to be associated with phosphate limitation and continuous growth at low temperatures (Fall and Fall, 1998; Nemecek-Marshall *et al.*, 1993).

In *P. borealis*, the gene that encodes its ice nucleation protein (*inaPb*) was cloned with PCR and chromosome walking, as described (Chapter 3). BLAST analysis of the conceptually-translated protein showed more protein sequence divergence than INPs from epiphytic bacteria, even though its repeat motif, AGYGSTxTAGxxSxLi/t (with highly conserved residues shown as capital letters, less well conserved residues in lower case letters and variable amino acids shown as the letter x), like other known INPs, occupy much of the protein (Wu *et al.*, 2009). Sequence analysis suggested that this repetitive region might fold to expose two relatively flat hydrophobic faces and we speculated that one face (Li/tA) could facilitate protein-protein interactions with the other face (TxT) interacting with ice (Chapter 3). Previously, the conserved 16 amino acid (aa) repetitive peptide found in *P. syringae* INP was modeled as a β -folded structure *in silico* (Graether and Jia, 2001). Likewise, the *inaPb*-encoded 16-aa repeat has been modeled as a left hand β -helix, similar to the structure of spruce budworm AFP (Chris Garnham, unpublished). The non-repetitive 163 residue amino-terminal of the protein may serve as a membrane anchor, so that INP can be attached to the outer membrane of the cells. The hydrophilic

carboxyl-terminal sequence is the most variable portion of the *P. borealis* protein compared to others in the databases, and this is true of most INPs. Interestingly, deletion of this sequence from the *P. syringae* protein caused the loss of INA (Edward *et al.*, 1994), but it is not known what role it plays in ice-association. Just as with other INPs, the recombinant expression of *inaPb* displayed some INA in recombinant *E. coli* but at a lower level than in the natural host, *P. borealis* (see Chapter 3 and 4). One possible reason may be that the accumulation of INP aggregates does not reach a sufficiently large size to act as active nucleation sites, and so do not promote ice nucleation at high, sub-zero temperatures.

Expression of the *P. borealis* INP in recombinant *E. coli* could be visualized under UV light because it was tagged with jelly fish green fluorescent protein (GFP) (Chapter 4). GFP is commonly used as a reporter for gene expression as well as a protein marker for subcellular localization experiments. GFP is composed of 238 amino acids, with an excitation peak at 394 nm, and an emission peak at 508 nm. It is generally considered relatively stable and a useful, non-invasive method for monitoring protein expression and cellular localization. However, GFP has some limitations too. For example, GFP has to be used in aerobic applications, it is not as sensitive as using an enzyme to monitor expression, it may aggregate, and it also can form dimers at high temperatures (Kaether and Gerdes, 1995). Expression of the *P. borealis* *INP-GFP* gene fusion was monitored using a fluorescent confocal microscope, and the green signal showed “polar” expression after treatment of the cells at low temperatures for many hours, but the reason for this localization remains unclear (Chapter 4). It is possible that the apparent polar expression is an artifact caused by aggregation and misfolding of the fusion protein, but previous studies have reported that the GFP folds well below room

temperature (Kaether and Gerdes, 1995; Tsien, 1998). Alternatively, the bipolar appearance of the INP-GFP fusion could be due to the aggregation of the INP sequences at low temperatures since the assembly of large-sized templates is necessary to initiate ice growth at higher subzero temperatures (Southworth *et al.*, 1988; Ruggles *et al.*, 1993). In this thesis, INA was routinely detected only after shifting cultures to low temperatures (Chapter 3). Although INPs are known to anchor in the membrane in *P. syringae*, the distribution of the proteins on the surface of this natural host is unknown. It is possible that the “polarized” localization of INP-GFP observed here could be related to the functional architecture of the poles in *E. coli*, where larger molecules, such as autotransporter-secreted proteins, receptor proteins, and polysaccharides, are exported out of the cellular membrane (Jain *et al.*, 2006; Scott *et al.*, 2001; Judd *et al.*, 2005; Ping *et al.*, 2008; McNulty *et al.*, 2006). Thus the bipolar expression of INP-GFP at low-temperatures may be the result of the presence of a membrane transport system coupled with the temperature-induced aggregation of the recombinant protein.

Similarities among different bacterial INPs sequences suggest horizontal gene transfer between different species (Edward *et al.*, 1994) and possible conjugation or transduction mechanisms on plant leaves provided a basis for gene transfer (Kidambi *et al.*, 1994). However, such a model also suggests that new hosts would derive some benefit from the transfer, which has been assumed to be enhanced pathogenicity. *P. borealis* is not known to be a plant pathogen and the presence of INA in this species suggests that host organisms may benefit in other ways. It is possible that the INP-mediated ice nucleation at high subzero temperatures may confer some benefit for overwintering. The INA⁺ *P. syringae* and *P. borealis* isolates did not show high levels of freeze-thaw resistance, yet they showed a higher rate of survival than other bacteria,

including *E. coli* (Chapter 2 and not shown). It is possible that INA, which results in the formation of large ice crystals at high subzero temperatures could benefit bacteria by the release of the heat of crystallization, or it could promote vitrification during freeze dry events. Using radioisotope labeling and molecular techniques, it has been shown that some microbes in ice found in glaciers, the Arctic, the Antarctic, sea ice and plants are not always quiescent; transient bacterial movements have been noticed at -12°C (Junge *et al.*, 2003). As well, the synthesis of DNA and protein has been found at temperature as low as -15°C to -34°C (Christner, 2002; Junge *et al.*, 2006; Marx *et al.*, 2009).

Extracellular polymeric substance, as a cryopreservative, may also affect freeze-thaw survival. There are two main different types of cryopreservatives: penetrating cryopreservative and non-penetrating cryopreservative additives (Hublek, 2002). Penetrating cryopreservative additives such as glycerol, dimethyl sulfoxide (DMSO) and propylene glycol can move across cell membranes and achieve osmotic balance. They are among the most effective antifreezes. In contrast, non-penetrating cryopreservative agents, including skimmed milk, serum, sucrose, trehalose and other peptides and polymers, do not move across cell membranes. It is not known how these agents interact with INPs. I suggest that a thermal dynamic analysis of bacterial INP and biofilm preparations may provide further information on subtle temperature changes (or heat change) during freeze-thaw processes.

Some investigations on these questions were initiated in this thesis, but not completed due to time constraints. However, preliminary experiments showed, as expected, that the penetrating cryopreservative, glycerol, decreased both the INP-mediated ice nucleation temperature and ice melt temperature (Appendix 1). A similar effect on the freeze-thaw curve in the presence of INP was observed with the addition of

bacterial EPS and AFP (Appendix 1). The nearly identical thermodynamic curves generated using either EPS-INP and AFP-INP argue that both agents are non-penetrating cryoprotectants. “Superheating” of AFPs and other polymers such as polyvinyl alcohol and polyethylene glycol (PEG) have been observed by different groups (Knight and Devries, 1989; Zobrist *et al.*, 2003; Wang *et al.*, 2009). Thus these polymers, including EPS or AFPs may protect bacteria in freezing-thaw events, whether or not INPs are present, by decreasing the ice nucleation temperature (either by supercooling or slowing down freezing speed), and by superheating the ice to lower the equilibrium melting point, thus creating an ordered ice propagating and melting process. Clearly, more experiments need to be done on these mixed systems.

Open questions and future perspectives

In future studies, more characterization in addition to the introduction of more reliable methods should be applied to EPS analysis. Osmotic conditions should be assessed by including a comparison with standard curves, such as could be generated using PEG. Analysis of the composition of the *E. billingiae* EPS, including the sugar identification remains an interesting goal in order to provide more information on the properties and future applications of this biofilm product. In the classic phenol-sulfuric acid assay (Marx *et al.*, 2009), the total concentration of EPS is determined using a glucose standard curve, but compared to PEG controls, the concentration can be seriously underestimated, to about 1/300 of the real concentration of the solution (w/w). Considering the complexity of polysaccharides and EPS, it is not surprising that EPS concentrations may not be correctly estimated using traditional methods. In some other studies, xanthan gum has been used as a standard, but the purity of this reagent can also influence effectiveness of the concentration determination (Krembs *et al.*, 2002).

The MS analysis of the main protein products in *E. billingiae* EPS is an interesting challenge as well, since only the major protein band was analyzed in this thesis. BLAST analysis of the *E. billingiae* EPS protein after MS sequencing suggested a protein homogenous to bacterial outer membrane protein A (OmpA), yet its carboxyl terminal peptides were more closely related to OmpF. It is possible that these results derived from evolutionary divergence, but it is also possible that these results reflect a mixture of two proteins with similar size that were not sufficiently separated by electrophoresis. Cloning of the corresponding genes and recombinant expression in *E. coli* would be likely an effective method to address this question. The importance of this protein by itself in conferring freeze-thaw resistance was also not tested in this thesis. *E. billingiae* with an *OmpA* knockout could also be created in order to do a functional study of the protein, although this is likely to present some technical problems. Nevertheless, it would be of interest to study its potential freeze-thaw protective activity or its role in biofilm formation in a future investigation.

A most important goal would be to develop a simple plant biofilm model. This is needed in order to investigate the function of biofilm under low temperature stress. Bacteria may have different phenotypes on plants than in artificial culture media and successful laboratory models are not necessarily associated with field experiments. If such a model were achieved, it would help in our ability to combine traditional microbial theory with relatively new biofilm theory. It is also hoped that such a future model may assist in the study of ice associating microbes, and microbe-microbe interactions in their natural environments.

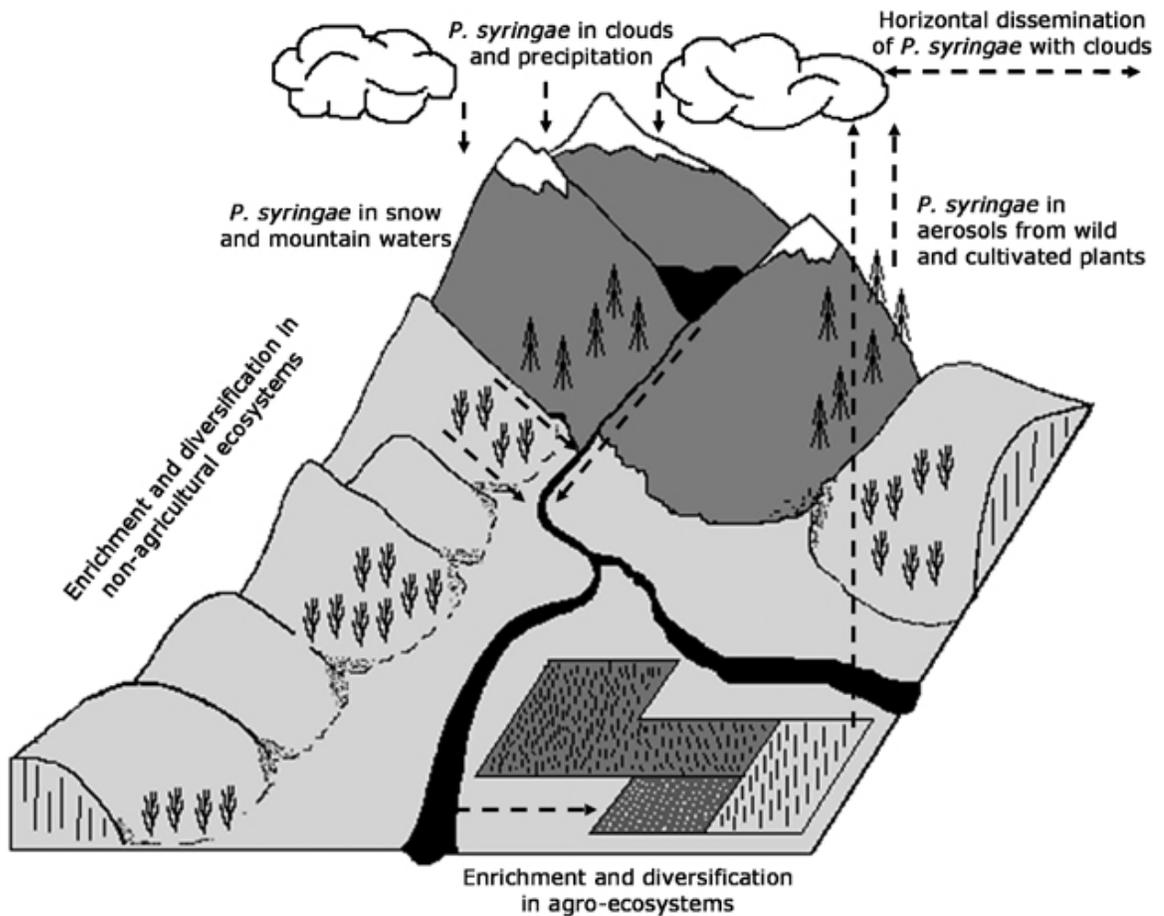


Fig. 5.1. *P. syringae*'s life history and its association with the water cycle (reproduced with permission from Morries *et al.* (2008) The life history of the plant pathogen *Pseudomonas syringae* is linked to the water cycle. ISME J 2:321-34. Copyright Macmillan Publishers Ltd). *P. syringae* is driven by air from wild and cultivated plants to clouds, horizontally disseminated, and precipitated as snow and water in diversity of non-agricultural ecosystems and agro-ecosystems.

5.2 References

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Appendix 1

The effects of cryoprotectants on *P. syringae* INP: a thermal dynamic analysis

*(Note: although this Appendix is written as a short manuscript, it is not in the main body of my thesis since the INP under study was from a commercial source, and does not represent the continuing analysis of the INP cloned from *P. borealis* as reported in Chapters 3 and 4)*

A1.1 Abstract

Ice nucleation activity (INA) and ice recrystallization inhibition (IRI) are two different ice associated activities. INA is promoted by bacterial ice nucleation proteins (INPs) at temperatures close to 0°C, whereas IRI is mediated by some cryopreservative macromolecules, such as antifreeze proteins (AFPs) or polysaccharides. The thermal dynamic interactions of a cryoprotective extracellular polymeric substance (EPS) with INPs have been studied by purifying EPS from *Erwinia billingiae* and mixing this preparation with bacterial INP. This mixture was compared to the results obtained after adding AFPs, polyethylene glycol or glycerol to a commercial source of bacterial INP. Differential scanning calorimeter and a thermal analyzer were used to independently examine the freeze and thaw process by following exothermic and endothermic curves, respectively. It was found that the addition of EPS decreased the ice nucleation temperature of INP and promoted an earlier melting curve than did the addition of AFPs. It is speculated that by slowing the freezing speed, increasing the premelting temperature and decreasing the freeze-thaw time, EPS may protect bacteria from freeze-thaw injuries.

A1.2 Introduction

Microorganisms with ice nucleation activity (INA) prevent supercooling of the surrounding liquid and thus prevent freezing until the temperature drops below -10°C.

The explosive growth of ice crystals at low temperatures can cause lethality or serious damage since the growing ice crystal can pierce cell membranes and result in ionic leaking or other metabolite imbalance. When water crystallizes, the solute concentrations rise and result in osmotic stress, viscosity changes, metabolism and pH alterations (Russell 1990; Amato and Christner 2009; D'Amico *et al.*, 2006). Presumably, partially due to the stress of freeze related damage, two different types of proteins evolved in microorganisms: Ice nucleation proteins (INPs) promote ice nucleation at temperature close to 0°C and antifreeze proteins (AFPs), polyols or extracellular gums or biofilms may modify ice crystal sizes, shapes and inhibit ice recrystallization (Kawahara *et al.*, 2007; Kim and Yim, 2007; Muryoi *et al.*, 2004; Regand and Goff, 2002). INPs are extracellularly displayed in some epiphytial bacteria that colonize the surface of leaves and stems of plants, and they may have a common evolutionary origin (Maki *et al.*, 1974; Army *et al.*, 1976; Lindow *et al.*, 1978; Edward *et al.*, 1994). The repetitive scaffold in INP structures may allow ice crystal to form at temperatures as high as -2°C. It is speculated that unscheduled ice formation allows plant pathogens such as *Pseudomonas syringae* ready access to nutrients from damaged plants. Whether or not INPs contribute to bacterial overwintering survival, besides this nutritional benefit, is another question.

In stressful environments certain microbes form a biofilm, which can increase the probability of survival. For example, some INP-bearing bacteria were found to inhabit freeze-tolerant organisms, and they then can prevent supercooling in their host and possibly confer a benefit by freezing at higher subzero temperatures (Lee *et al.*, 1985). Other microbes with INA were found to have AFPs or extracellular polymeric substance (EPS) which can inhibit ice recrystallization (IR) (Feil *et al.*, 2005; Zhao and Orser, 1990; Muryoi *et al.*, 2004). In this thesis a microbial community from winter plants was studied

and individual isolates with INA and IR inhibition were partially characterized (Chapter 2). EPS extracts from one isolate, *Erwinia billingiae*, appeared to confer freeze-thaw protection to *E. coli* and *P. syringae*. Since bacterial INA can be reduced by agents that kill bacteria, as well as cryoprotective polymers, this motivated a re-evaluation of such interactions.

A1.3 Materials and methods

Snowmax (INP from *P. syringae*) was purchased from Wards Natural Science Establishment (NY, USA), myoglobin, glycerol and PEG 6000 were bought from Sigma (MO, USA), recombinant LpAFP-GFP and type III AFP-GFP were kindly provided by Raymond Gordienko in our lab, and *E. billingiae* EPS was partially purified as described previously (Chapter2).

A1.3.1 Ice nucleation activity assay, sample preparation and data analysis

A thermal analyzer was used to determine the ice nucleation temperature, equilibrium melting temperature and melting finishing point using exothermic and endothermic monitoring of the phase changes (Borchardt, 1957; Wu *et al.*, 2009). Samples were kept at 1 mL volume and were constituted as follows: INP (SM) included 1 mg/mL of Snowmax INP; Snowmax INP and LpAFP-GFP sample (SA) contained 1 mg/mL of INP and 1 mg/mL of LpAFP-GFP; Snowmax INP and polyethylene glycol sample (SP) contained 10% polyethylene glycol (PEG 6000) and 1 mg/mL of INP; Snowmax INP and glycerol (SG) were composed of 1 mg/mL INP and 10% glycerol; the combination of Snowmax INP, PEG and glycol (SPG) was made up using 1 mg/mL INP in 10% PEG and 10% glycerol; Snowmax INP, PEG, glycerol and AFP sample (SPGA) were composed of 1 mg/mL INP, 10% PEG, 10% glycerol, and 1 mg/mL LpAFP-GFP; and ethylene glycol alone was used as a reference sample. It is clear from these

descriptions that no attempt was made to keep a consistent solute concentration and thus similar theoretical colligative nonequilibrium freezing or melting point depression values. Thus, this may represent a flaw in these experiments, and those described below. The temperature of the sample chamber was decreased from 5°C to -20°C at a rate of -0.2°C/min using a programmable circulation system and was automatically recorded in spread sheet for subsequent plotting analysis. Microsoft Excel was used to analyze the ice nucleation temperature, equilibrium melting point and melting finishing points.

A1.3.2 Differential scanning calorimeter (DSC) assay

DSC assay (Q100 V9.9, Dept of Chemical Engineering, Queen's University) was performed in a liquid nitrogen-cooled calorimeter. EPS, Type III AFP-GFP and myoglobin were adjusted to about the same concentrations (0.7 mg/mL) and added to Snowmax INP (0.7 mg/mL). Each sample (10 µL) consisting of INP, EPS, EPS/INP or Type III AFP-GFP/INP was placed in a sealed aluminum pan, and run with a “cool-warm-cool-warm” cycle. Samples were first cooled down to -40°C at a rate of 5°C/min and after a 2 min annealing period, samples were warmed back to 15°C at the same rate. After a second 2 min annealing period, the samples were cooled down to -40°C again. The collected data were plotted in one graph to facilitate the comparison of samples.

A1.3.3 Ice recrystallization inhibition

IR inhibition was performed using published methods (Knight *et al.*, 1998). Briefly 10 µl of each sample (INP, LpAFP-GFP/INP, LpAFP-GFP, Type III AFP, and Type III AFP-GFP/INP) were loaded into microcapillaries (10 µL), sealed with silica gel, and placed in a microcapillary holder. Samples were frozen quickly to -50°C, and then transferred in a -6°C chamber for 16 h. Each sample was replicated in 5 microcapillaries and photographed through crossed polarized film at the beginning and the end of the

assay using a micro-photography system composed of a Cannon digital camera and a Zeiss dissecting microscope.

A1.4 Results

A1.4.1 Calorimeter results

As seen in Fig. A1.1, freezing and melting processes appeared to be two asymmetric processes which differed in temperature and time. Generally, the addition of glycerol, LpAFP-GFP or PEG reduced the ice nucleation temperature of the Snowmax INP. Without additives, the INP induced nucleation at -1.51°C . However, the temperature was lowered in the presence of AFP (SA) by 0.18°C , with PEG (SP) by 0.22°C , with glycerol (SG) by 2.56°C , with the glycerol and PEG mixture (SGP) by 4.40°C , or in SPGA by 4.46°C (Fig. A1.2). The melting temperatures of INP samples were also affected, and are represented as the equilibrium melt temperatures and the melting finishing points (Fig. A1.1; Table A1.1): the melting finishing points were inhibited from 6.12°C with INP alone to 5.49°C , 5.20°C , 2.4°C , -2.09°C , and -2.18°C in the SA, SP, SG, SGP and SPGA samples, respectively. The major heat of fusion (exothermic reaction) tracings for the PEG, and LpAFP samples were detected between 1,000 s to 2,000 s, and 2,000-4,500 s; and the endothermic profiles (melting) were seen between 14,000-16,000 s and between 11,000-15,000 s. It was observed that the times for melting periods were about 2 times longer than freezing times in each case. The combination of PEG, glycerol, with or without LpAFP-GFP had similar freezing and melting times (2,500-4,500 s; 12,000-14,000 s; Fig. A1.1). The temperature differences (between ice melting finishing points and ice nucleation temperature) of each sample were plotted against the time differences (between ice melting finishing points and ice nucleation starting points) in Fig. A1.3. It can be seen that the combination of PEG, glycerol and LpAFP-GFP to the INP

solution had the least temperature and time differences between the freezing and melting processes.

Asymmetric freezing and melting curves were also observed in the DSC assay (Fig. A1.4). The EPS preparation and the myoglobin addition inhibited the Snowmax INP-mediated INA from -3.9 to -5.3°C, but Type III AFP-GFP increased INA to -3.3°C (Table A2; Fig A1.4). The different effects of EPS, Type III AFP-GFP and myoglobin additions were also observed on the equilibrium melting temperatures and melting finishing points (Table A1.2). The melting finishing point of INP-induced freezing ranged over 3°C, depending upon the additive (Fig. A1.5). Earlier melt events were shown in AFP and EPS samples with samples containing the EPS preparation showing melting at temperatures starting at -12.5°C (Fig. A1.4).

A1.4.2 The results of IR inhibition assay

Both Type III AFP-GFP/INP and LpAFP-GFP/INP combinations showed IR inhibition activity and featured the formation of initial smaller ice crystals than the INP sample alone. The INP samples showed larger ice crystals than any of the other samples. Mixing AFPs with Snowmax INP slightly decreased the initial ice crystal size, while the smallest ice crystal size was formed in with Type III AFP having a TH value 0.5. All of the samples tested seemed to show IR inhibition in that the sizes of the ice crystals did not change after annealing, but this is very hard to assess when the crystals are initially large.

A1.5 Discussion

Microbial low temperature adaptations can be complex and may involve the utilization of unsaturated fatty acids, changing the carbohydrate content, reducing water concentrations and the production of polyols (see Chapter 1 for a review of the literature). Some of these bio-products may act to modify ice formation by either promoting or

inhibiting the ice nucleation process. The phrase “adaptive ice nucleator” was used by Lundheim (2002) to explain some freeze-tolerance related ice nucleation and to differentiate this from incidental ice nucleators. High INA activity induced by microbial INPs can be used as in a freeze-tolerant strategy by certain insects, perennial plants, tree frogs and even some reptiles (Rajashekar *et al.*, 1983; Wolanczyk *et al.*, 1990; Storey, 2006). Extracellular ice nuclei can form at high subzero temperatures, and possibly to protect cells from damage (Lundheim, 2002). A seemingly opposite mechanism for microbial low temperature adaption is the production of cryoprotectants including AFPs, glycerol, and EPS amongst others (see Chapters 1 and 5 for a discussion of this literature). It appears from the current study, as well as some previous experiments, that certain of these cryoprotectants can decrease the temperature of ice nuclei formation as well as the initial ice crystal size. In this thesis, bacteria with INA and those with IR inhibition properties were found together in an epiphyte community isolated from *Chrysanthemum indicum* (see Chapter 2), thus suggesting experiments to study the possible interaction between these two different microbial freeze-survival mechanisms.

The effect of cryoprotectants on bacterial INA can be studied with traditional experiments such as comparing viability of the cells after freeze-thaw events, or examining cell membranes microscopically after freezing (Kim and Yim 2007; Hong and Marshall, 2001). Decreased ice crystal sizes were observed after freezing when AFP was added to INP samples, compared to INP samples alone (Fig A1.6). Similar results have been obtained with bacterial EPS (Regand and Goff in 2002; Regand and Goff, 2006; Kim and Yim 2007; Amornwittawat *et al.*, 2009). A functional cryoprotectant effect of EPS from *E. billingiae* was demonstrated by the increased viability of INA⁺ *P. syringae* (Chapter 2), which may imply that INP activity by itself is not an adaptation for low

temperature alone, and thus may be beneficial with respect to access to nutrients (see Chapter 3) .

A thermal dynamic analysis of the freeze-thaw process in the various samples showed asymmetrical freezing and melting processes. DSC has been previously used to study phase changes and to analyze the melting process of different AFPs (Hansen and Baust, 1988, Hansen and Carpenter, 1993; Amornwittawat *et al.*, 2008; 2009), and a calorimeter has also been used to study the freezing process (Parody-Morreale *et al.*, 1988b; Zobrist *et al.*, 2003; 2008; Wu *et al.*, 2009). The analysis of both these processes using combination of cryoprotectants with INP may reveal more information on temperature and time differences. The melting process may involve different heat absorption such as metastable eutectic, stable eutectic or the beginning ice melting point (Zobrist *et al.*, 2003). No apparent different curves were shown in the calorimeter results, so the equilibrium melting temperature (T_{m1}) and melting finishing temperature (T_{m2}) were used to describe melting process. Asymmetrical single ice crystals are formed during freezing and melting processes (Pertaya *et al.*, 2007). In the current study the INA and melting finishing points of bacterial INP samples were found to be inhibited by LpAFP-GFP, PEG and glycerol, which broadly agree with other reports (Wowk and Fahy, 2002; Duman, 2002; Zobrist *et al.*, 2003; Parody-Morreale *et al.*, 1988a). In the DSC assay, bacterial INA activity was inhibited by EPS from *E. billingiae*, but was only slightly increased by Type III AFP-GFP, perhaps due to its relatively high concentration and high TH activity (Fig. A2; Holt, 2003). Both cryoprotectants (EPS and AFPs) reduced the INP equilibrium melt temperature (Table A1.2), with the EPS equilibrium melting temperature at 1.6°C; lower than that seen for glycerol, a penetrating

cryoprotectant. This suggests that EPS may be a non-penetrating cryoprotectant, as previously suggested (see Chapter 2).

Cryoprotectants also were shown to reduce the temperature difference between ice nucleation temperature and the melting finishing points, as well as the time spent in the freezing state. Compared to INP alone, the mixture of glycerol, PEG and AFP together reduced the freezing time about 26%. With glycerol addition to the INP, the freezing time was reduced about 13%, and EPS addition to INP samples reduced the freezing time about 5%. A shorter time to freezing state may improve organism's viability (Storey, 2006).

In conclusion, the thermal dynamic interaction between AFP or EPS and INP suggests complicated adaptive strategies within the difference species making up a microbial consortium. The presence of EPS, AFP, and glycerol, bacteria, and frost-tolerant plants together may enhance the survival of all members of this environmental niche, both eukaryote and prokaryote, by inhibiting the ice nucleation temperature, equilibrium melting temperature, melting finishing points and reducing freezing time.

A1.6 Figure legends

Fig. A1.1. Typical thermal dynamic curves of the freezing (exothermic) and melting (endothermic) processes of INP and various cryoprotectants. The Y-axis represents the temperature difference between samples and reference (ethylene glycol), and the X-axis represents time (seconds). The slowest freezing times, and the fastest warming times, were seen when Snowmax INP was mixed with glycerol and PEG (purple), or mixed with a glycerol/PEG/LpAFP-GFP mixture (mahogany). Broad intermediate melting and freezing peaks are seen for INP mixed with glycerol (aqua blue), with a sharper intermediate melting peak for the INP and PEG mixture (yellow). The addition of LpAFP-GFP to INP (red) showed only slight differences to the freezing and melting curves shown by INP alone (dark blue).

Fig. A1.2. Typical curves used to assess the INA activity in the presence of various additives. The ice nucleation temperature of Snowmax INP (blue) was slightly lowered by LpAFP-GFP (red) and PEG, (yellow), lowered more by the addition of glycerol (aqua blue), and more so by glycerol/PEG (purple) and glycerol/PEG/LpAFP-GFP (mahogany). The Y-axis represents the temperature difference between samples and reference (ethylene glycol), and the X-axis represents time (seconds). The ice nucleation temperatures were -1.51°C, -1.69°C, -1.71°C, -4.07°C, -5.91°C, -5.97°C respectively.

Fig. A1. 3. Freezing time and temperature differentials between melting finishing points and ice nucleation points in various samples. The X-axis shows the time differences between samples and reference (ethylene glycol) and the Y-axis shows the temperature

difference between samples and reference (ethylene glycol). The Snowmax INP samples (dark blue) showed the biggest temperature difference and freezing time span, followed by INP + LpAFP-GFP (red), INP + PEG, (yellow), INP + glycerol (aqua blue), INP + glycerol/PEG (purple), and INP + glycerol, PEG and LpAFP-GFP mixture (mahogany).

Fig. A1.4. Thermal dynamic curves of solutions containing Snowmax INP solutions containing EPS from *E. billingiae* (red), Type III AFP (black) and myoglobin (blue). The Y-axis represents the temperature difference between samples and reference (ethylene glycol), and the X-axis represents the temperature (time) in the DSC assay. The EPS sample was the last to nucleate and the first to melt. The AFP was slower to freeze than myoglobin but melted at the same time, as would be expected due to thermal hysteresis.

Fig. A1.5. The endothermic curves of Snowmax INP solutions containing EPS from *E. billingiae* (red), Type III AFP-GFP (black) and myoglobin (blue). For each sample in the DSC assay, the Y-axis represents the temperature difference between the samples and reference (ethylene glycol), and the X-axis represents the temperature (time). EPS from *E. billingiae* melted earlier than either Type III AFP-GFP or myoglobin.

Fig. A1.6. Ice recrystallization inhibition of AFP and Snowmax INP samples. The top image shows the capillaries (size = 10 μ L) after freezing and the bottom image shows the capillaries after annealing at -6°C , overnight. From left to right are Type III AFP-GFP/INP, Type III AFP, LpAFP-GFP/INP, LpAFP-GFP and INP respectively.

Table A1.1 Comparison of the ice nucleation temperature, equilibrium melt temperature (Tm1), and melting finishing point (Tm2) in the presence the Snowmax INP and different additives (data obtained from the thermal analyzer; see Methods).

Temperature (°C)	INP	INP+AFP	INP+PEG	INP+Glycerol	INP+glycerol+PEG	INP+Glycerol+PEG+AFP
Nucleation temperature	-1.51	-1.69	-1.73	-4.07	-5.91	-5.97
Equilibrium melting (Tm1)	1.67	1.46	0.84	-2.2	-2.63	-2.68
Melting finishing point (Tm2)	6.12	5.49	5.20	2.40	-2.09	-2.18

Table A1.2 Comparison of the ice nucleation temperature, equilibrium melt point (Tm1) and melting finishing points (Tm2) of the Snowmax INP with the addition of EPS preparation, type III AFP-GFP or myoglobin (data from the differential scanning calorimeter; see Methods).

Temperature (°C)	EPS	EPS+INP	INP	Type III AFP+INP	Myoglobin+INP
Ice nucleation temperature	-12	-5.3	-3.9	-3.3	-5.3
Equilibrium melt point temperature (Tm1)	1.2	1.6	4	3.8	4.4
Melting finishing temperature (Tm2)	5.2	4	6.3	5.5	7.2

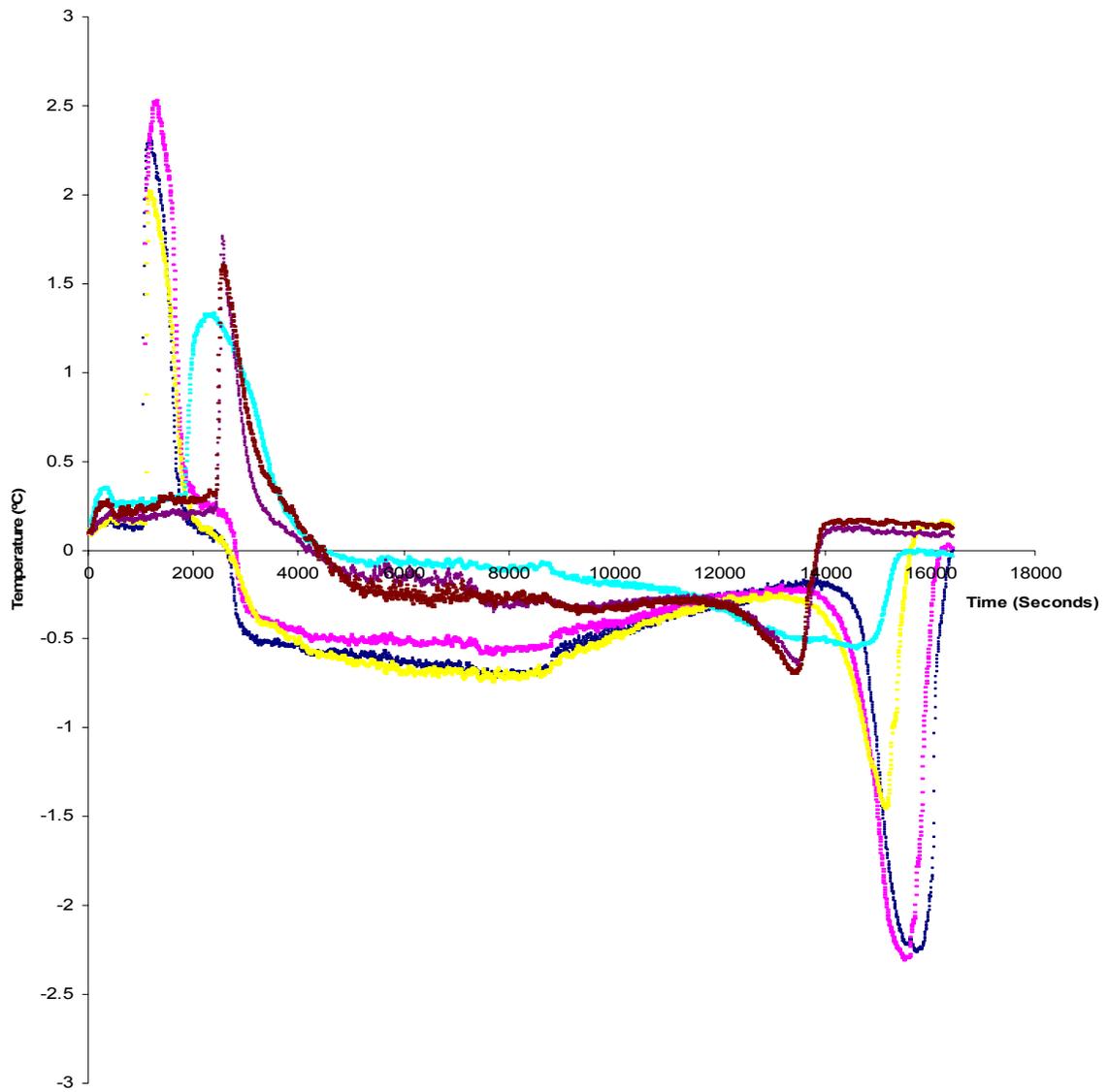


Fig. A1.1

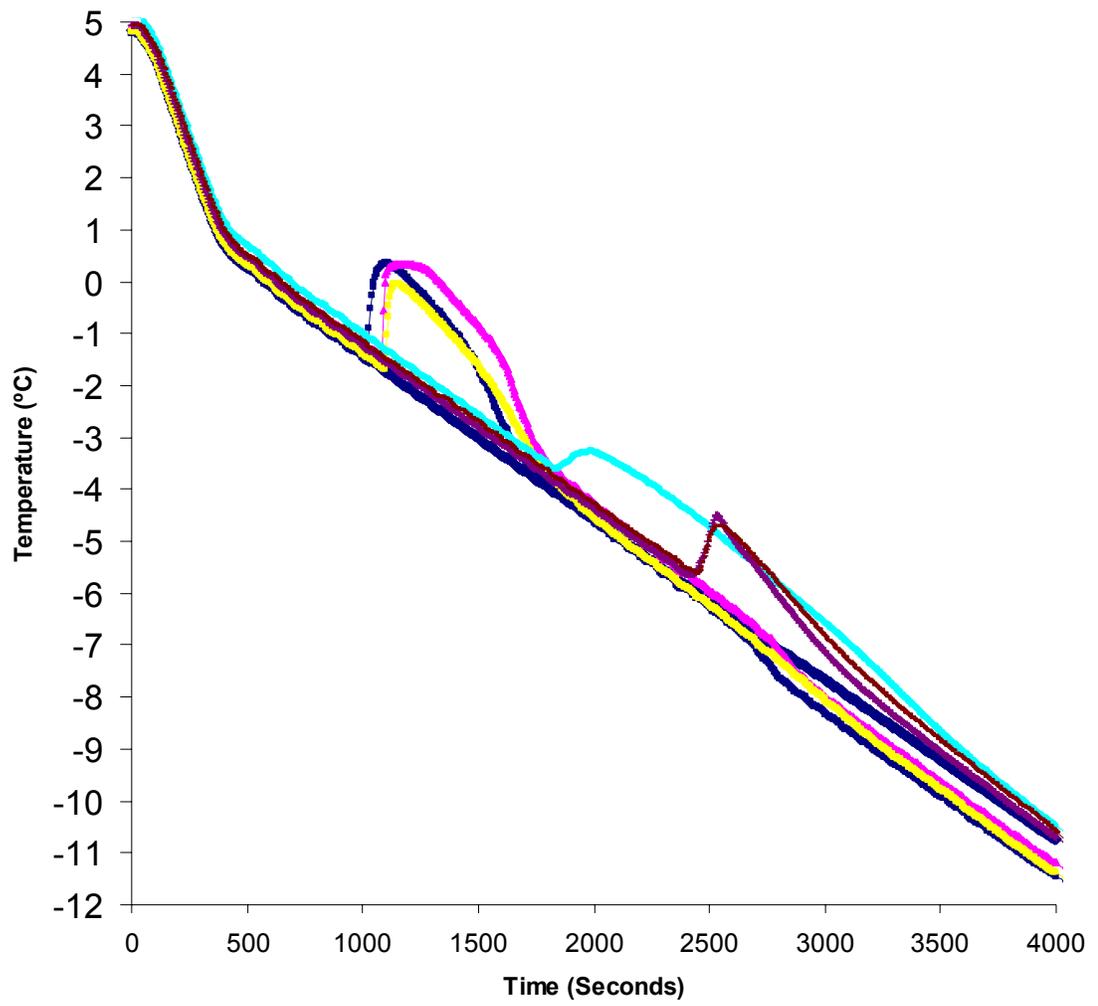


Fig. A1.2

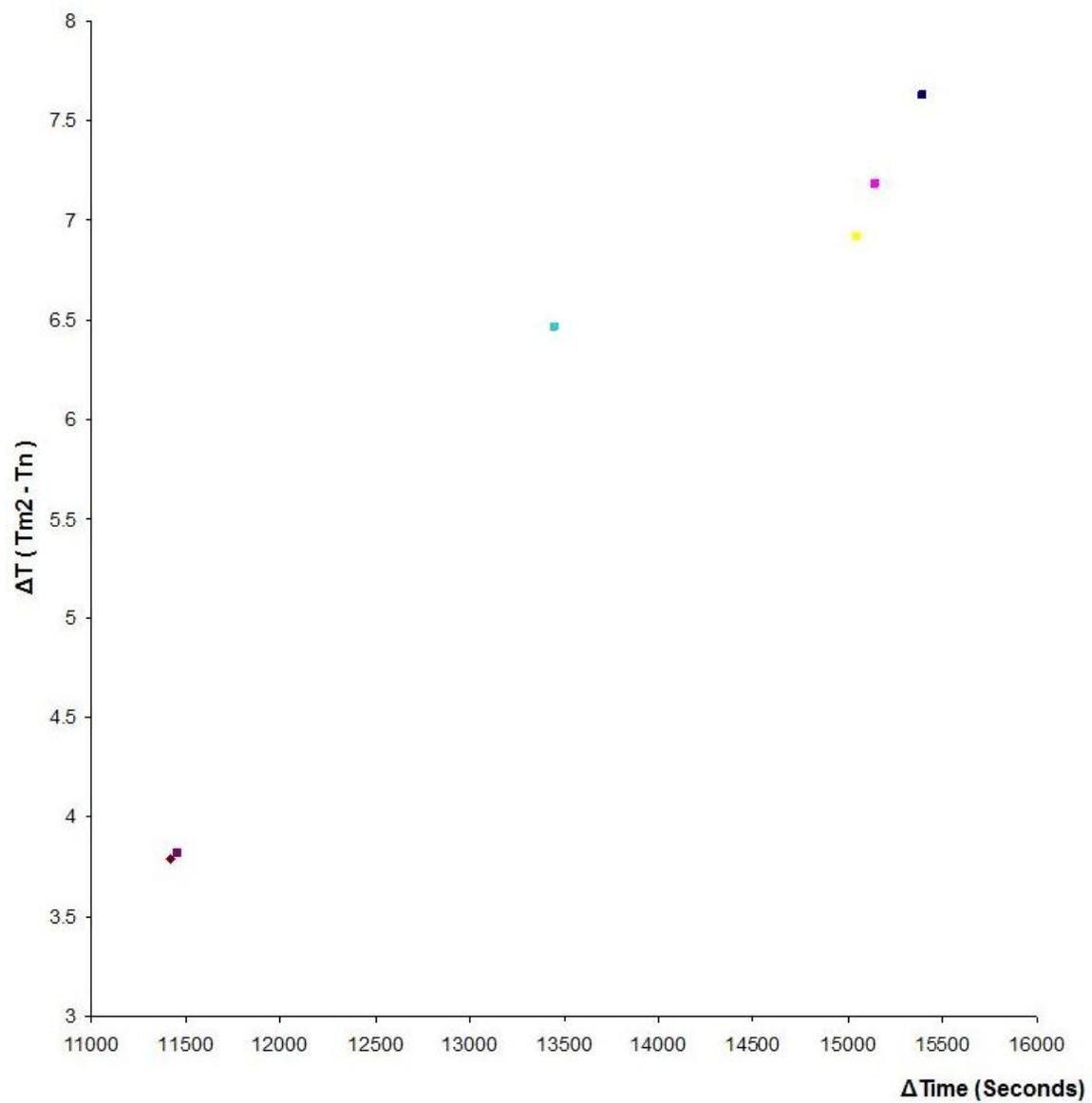


Fig. A1.3

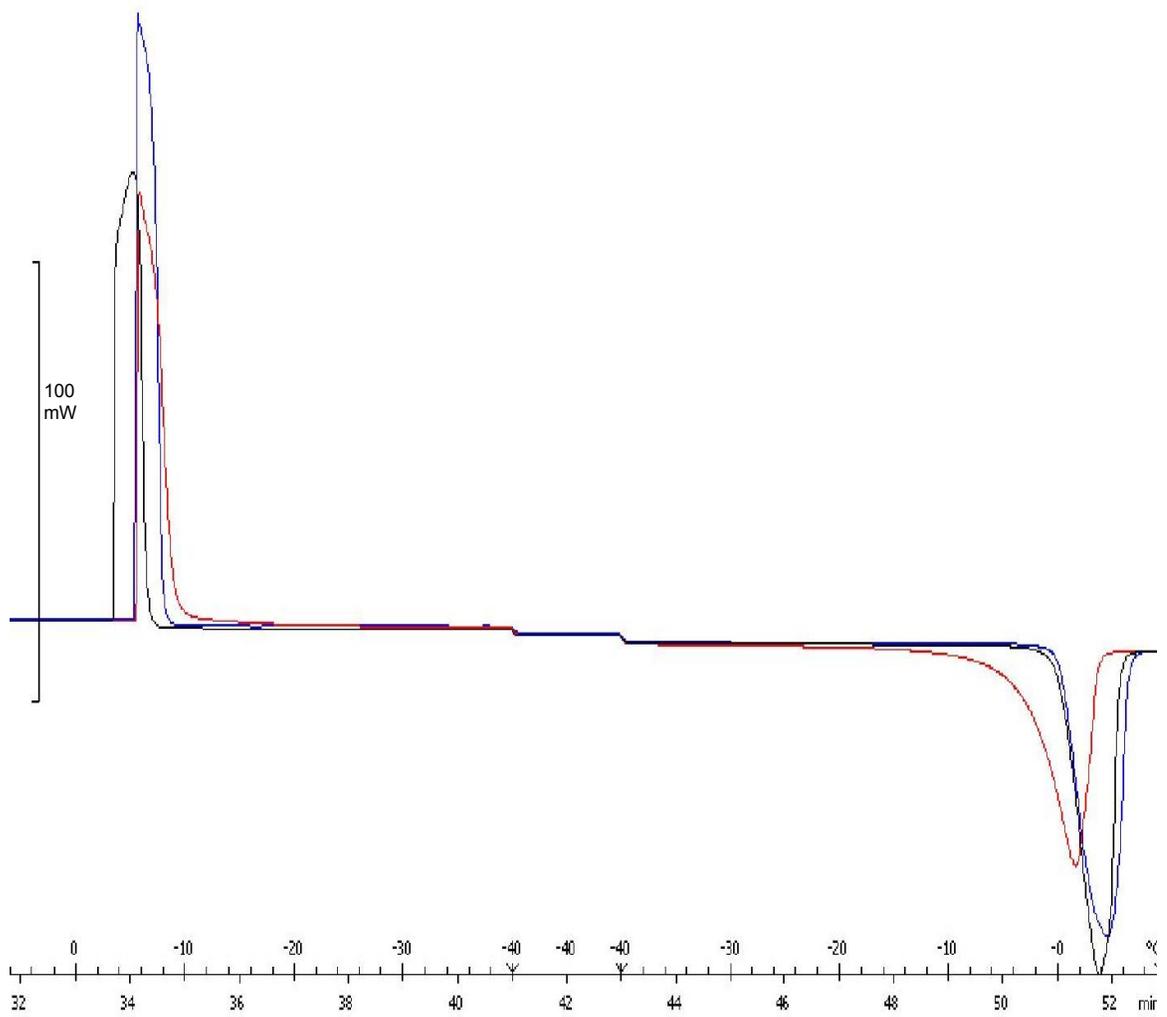


Fig. A1.4

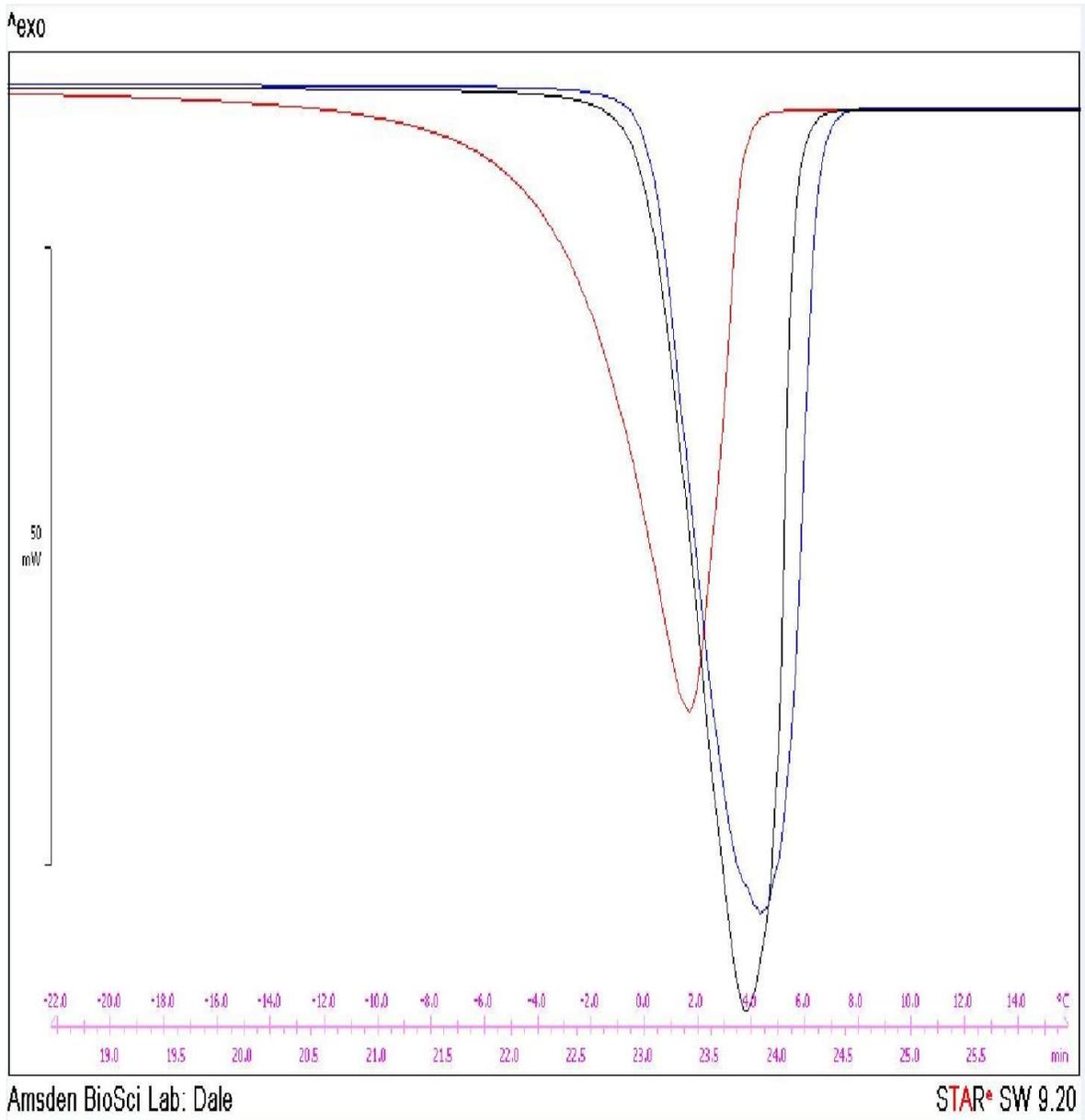


Fig. A1.5

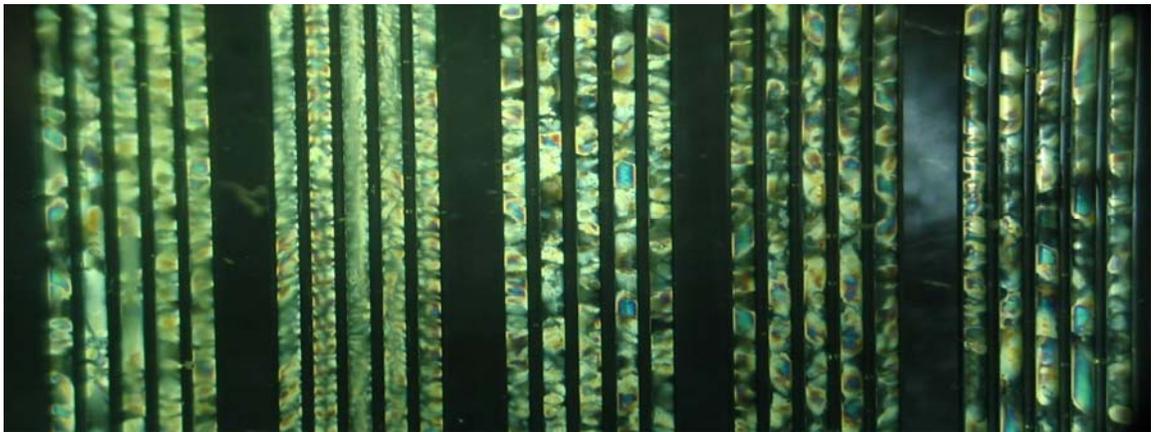
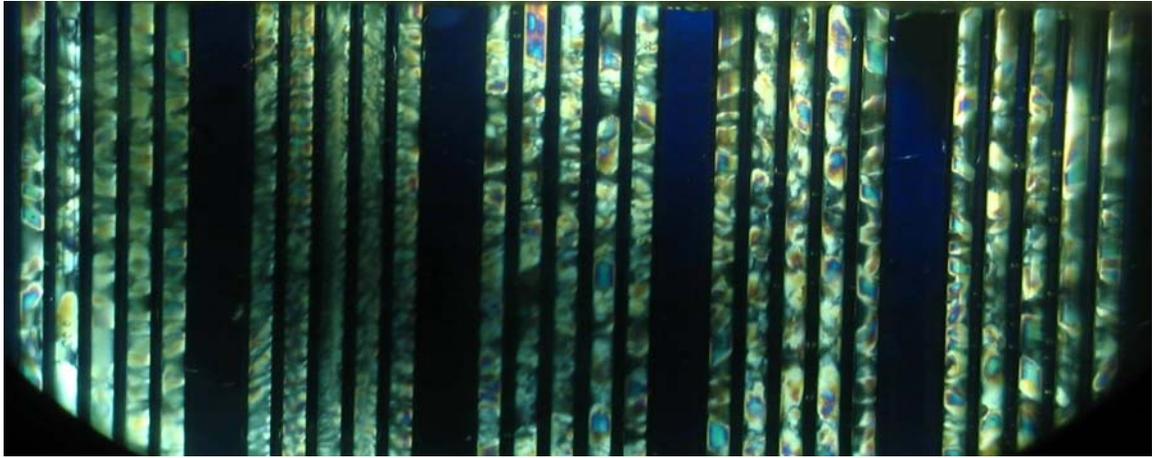


Fig. A1.6

A1.7 References

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Appendix 2. Comparisons of biofilm adhesion to PVC microtitration plates in some of the bacterial strains studied in this thesis.

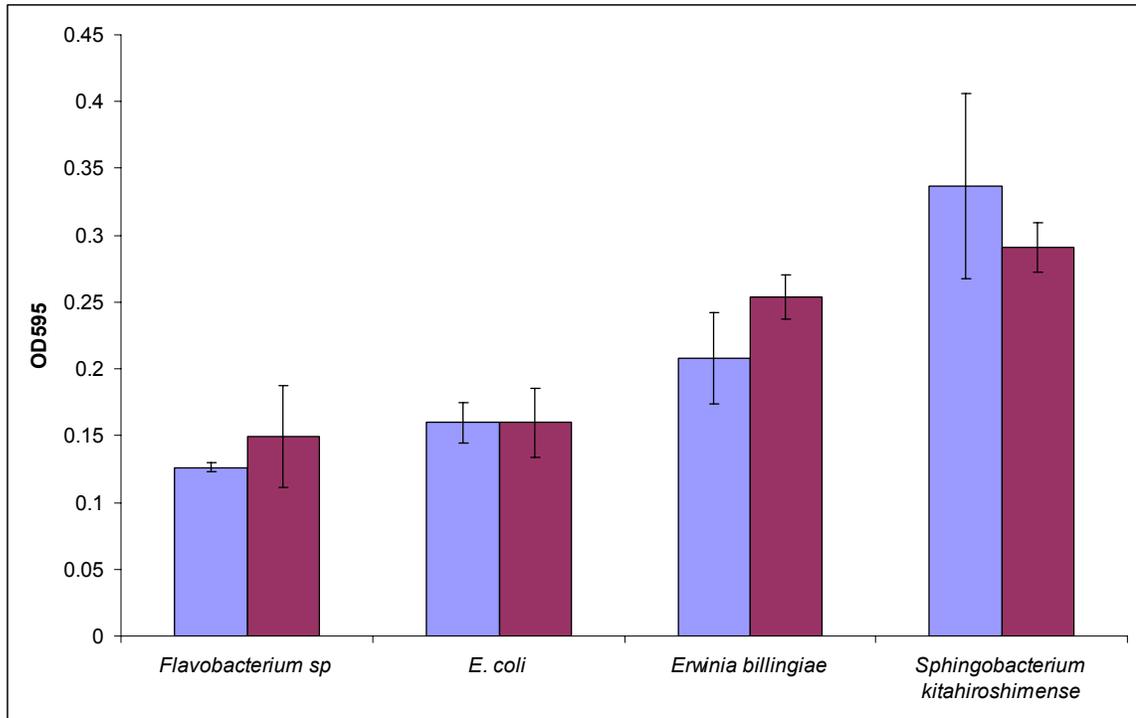


Fig. A.2. Comparison of bacterial adhesion to PVC microtiter plates among *Flavobacterium sp* YIN, *E. coli* TG2, *Erwinia billingiae* J10 and *Sphingobacterium kitahiroshimense* Y2 at 48 h (blue) and 72 h (red) of incubation (see Chapter 2, Materials and Methods for details of the experiment). Error bar represents standard deviation. *Erwinia billingiae* and *Sphingobacterium kitahiroshimense* showed significantly higher adhesion than *E. coli* and *Flavobacterium sp*.

Appendix 3 Swarming motility of *Erwinia billingiae*.

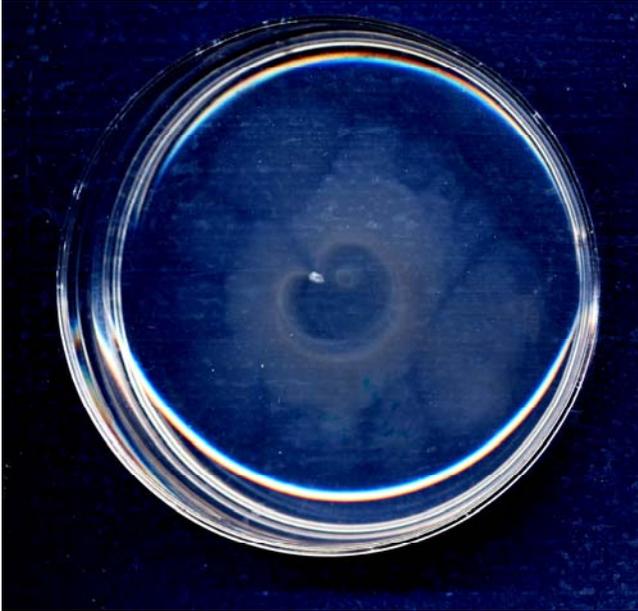


Fig. A.3. Swarming motility of *Erwinia billingiae* J10 on a 0.5% agar plate as described in Chapter 2, Materials and Methods. The light colour represents bacterial growth from an inoculation point (white) at the centre of the 9cm petri plate. This figure is of a representative plate.

Appendix 4. Electron micrographs of *Sphingobacterium kitahiroshimense* and *Flavobacterium sp.*

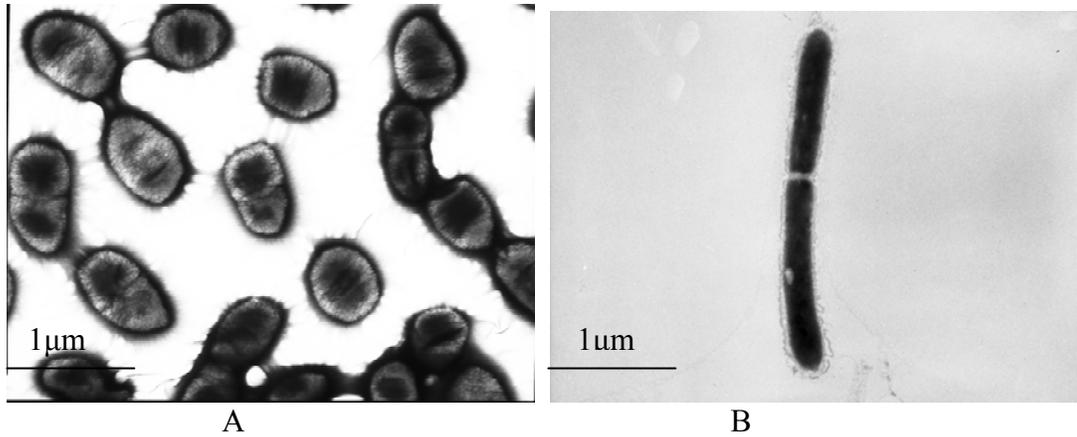


Fig. A.4. Electron micrographs of *Sphingobacterium kitahiroshimense* Y2 and *Flavobacterium sp.* Yin after negative staining with PTA. Details of the methods used can be found in Chapter 2. Fimbriae appear to be visualized around *S. kitahiroshimense* Y2 cell (A), while no flagella or fimbria can be seen in *Flavobacterium sp.* Yin (B). The bars — represent 1 μm.

Appendix 5. Electron micrograph of *Pseudomonas borealis*.

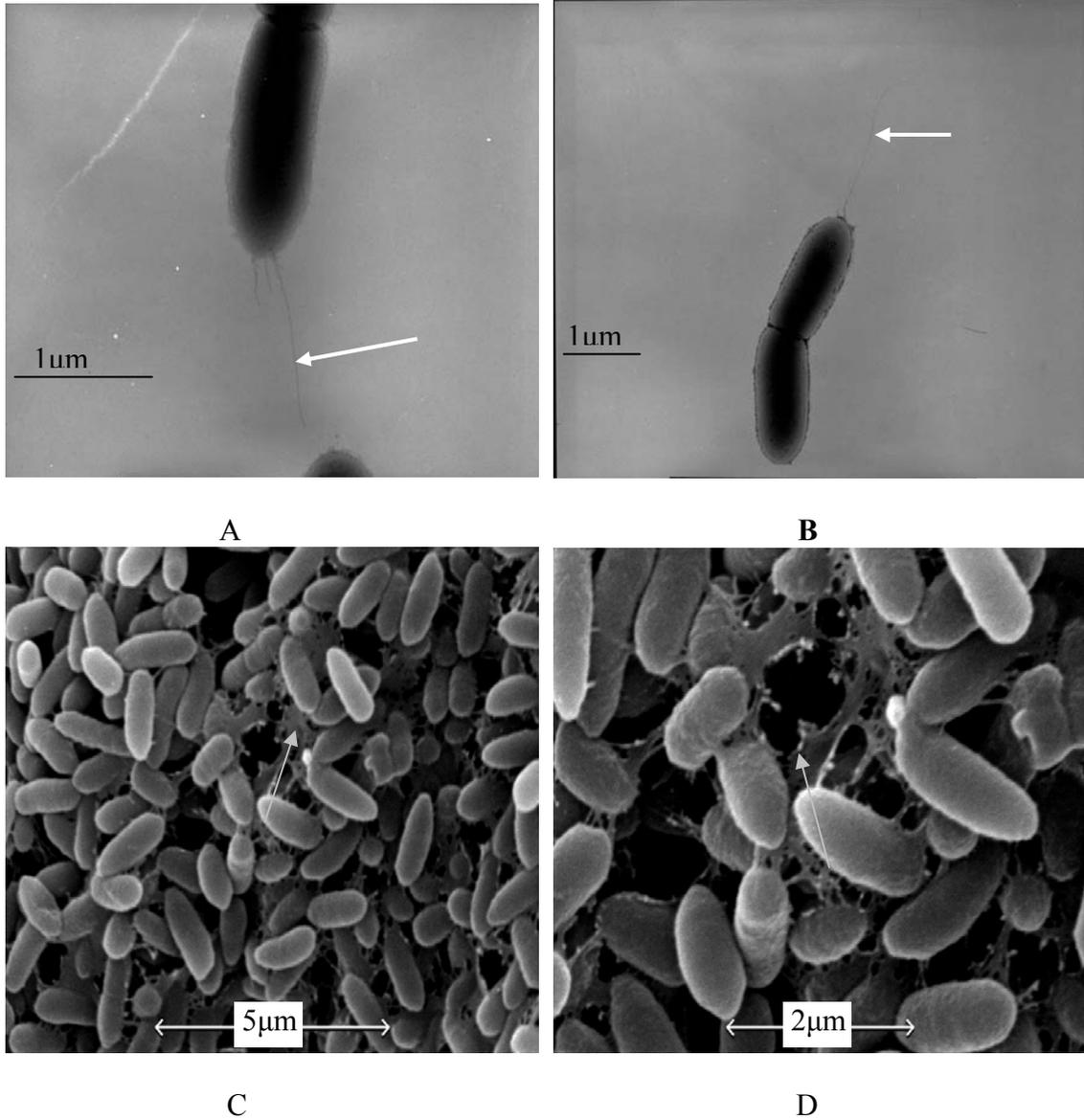


Fig. A.5. Electron micrographs of *Pseudomonas borealis* cells and their associated biofilm. The arrows show the presence of flagella (flagellum; A, B) that were negatively stained and examined under transmission electron microscopy (TEM). Single headed arrows show evidence of a slime matrix (C, D) that was seen using scanning electron microscopy (SEM). The magnifications are indicated as bars (A and B) or double-headed arrows (C and D). Details of the TEM and SEM methods used are described in Chapter 2.

Appendix 6. Viability of *P. borealis* after 12 freeze-thaw cycles alone or in the presence of *E. billingiae* or with *E. billingiae* and *S. kitahiroshimense*.

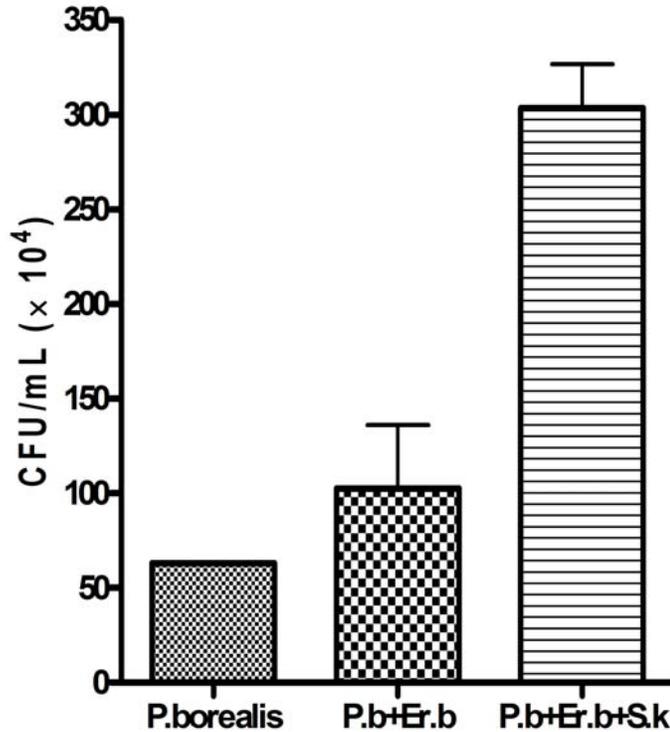
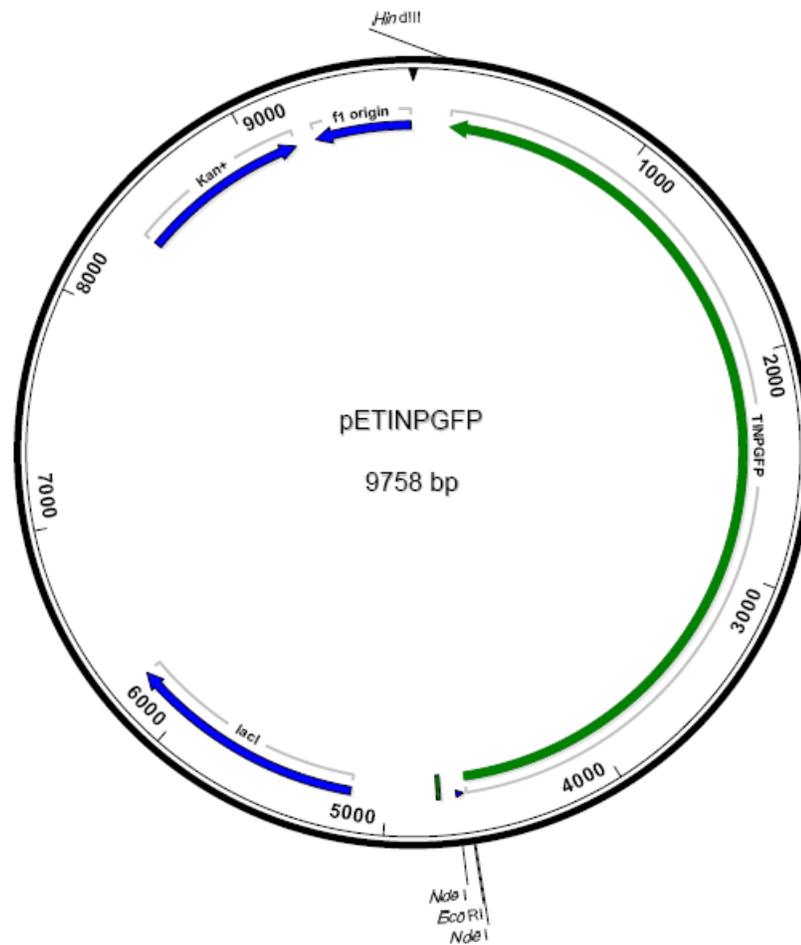


Fig. A.6. Freeze-thaw resistance of *P. borealis* after multiple freeze-thaw cycles in the cryocycler (see Chapter 2 for details of the methods). Viability was assessed after single cultures (initiated at 1×10^8 cfu/mL) were subjected to 12 freeze-thaw cycles either alone (*P. borealis*) or in the presence of *Erwinia billingiae* (P.b + Er.b) or in the presence of *Erwinia billingiae* and *Sphingobacterium kitahiroshimense* (P.b + Er.b + S.k).

Appendix 7. pEINPGFP map



NdeI NheI T7-Tag pET-24α BamHI EcoRI SacI SalI Hind III EagI NotI XhoI His-Tag
 TATACATATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGCACCACCACCACCACCCTGA
 MetAlaSerMetThrGlyGlyGlnGlnMetGlyArgGlySerGluPheGluLeuArgArgGlnAlaCysGlyArgThrArgAlaProProProProProLeu

Fig. A.7. Molecular map of pEINPGFP. The map shows the vector sequences of interest including kanomycin resistance, the f1 origin and lactose operon sequences (blue line) as well as the insert of the *P. borealis* INP coding region (green line and designated T1NPGFP). The multiple cloning sites and the sequence of the junction are shown below the circular map. Details of the cloning strategy can be found in Chapter 4. This figure was prepared by Denian Miao and used with permission.

Appendix 8. Phylogenic tree of the isolates from frost-exposed chrysanthemum leaves.

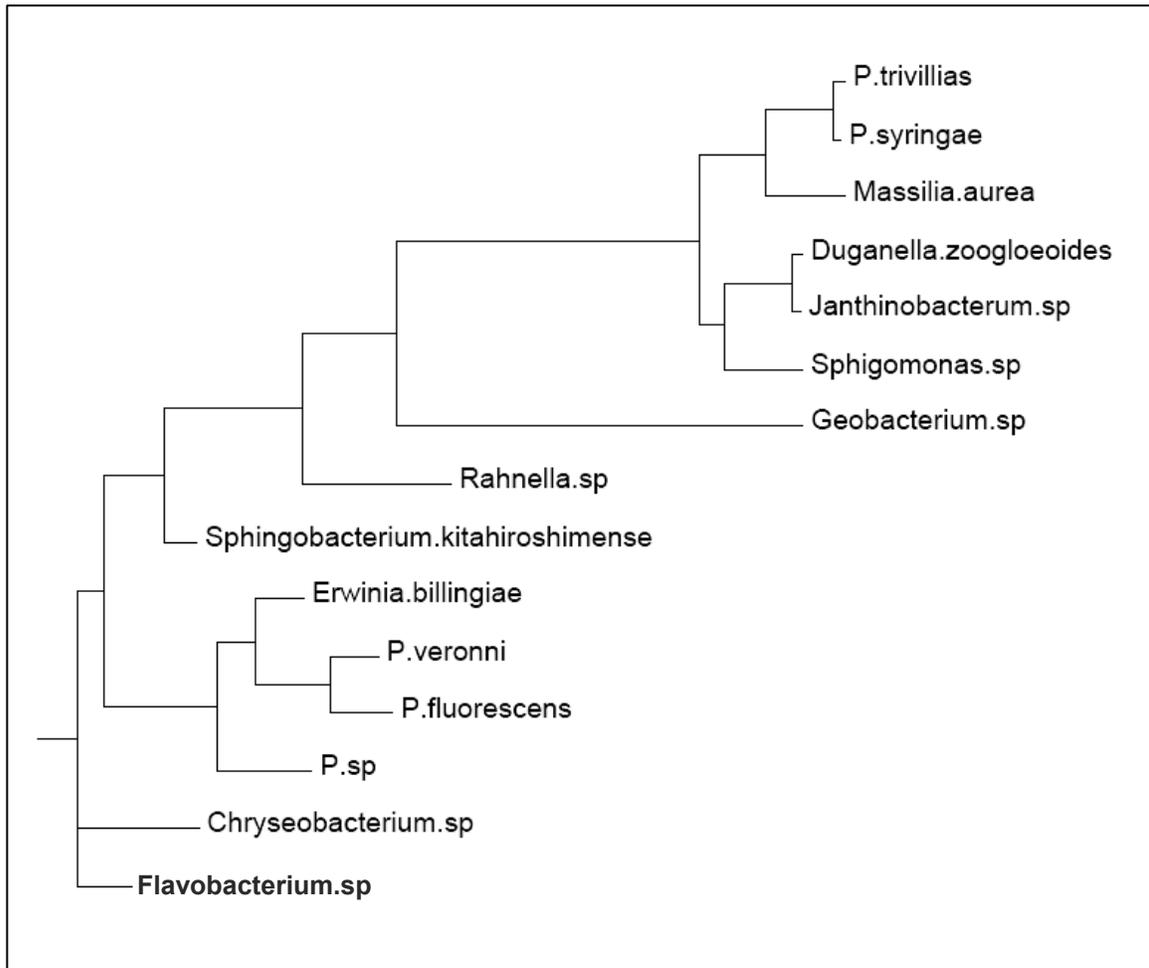


Fig. A.8. A phylogenetic tree of various bacteria isolated from the frost-exposed chrysanthemum leaves. Sequences were obtained from single cultured isolates originating from the winter-collected plant material as described in Chapter 2. The phylogenetic tree was constructed by using rRNA sequences, multi alignment and phylogenetic tree building software of Bioedit with some modifications. It shows that the epiphytes were from a variety of unrelated bacteria, many of which were not investigated in this thesis. Interestingly, the *Pseudomonas* species found were separated in this particular tree; the reason for this is unknown.

Appendix 9. A model of biofilm (picture was reproduced with the permission from Monds and O'Toole. (2009) The developmental model of microbial biofilms: ten years of a paradigm up for review. Trends Microbiol 17:73-87. Copyright Elsevier Limited)

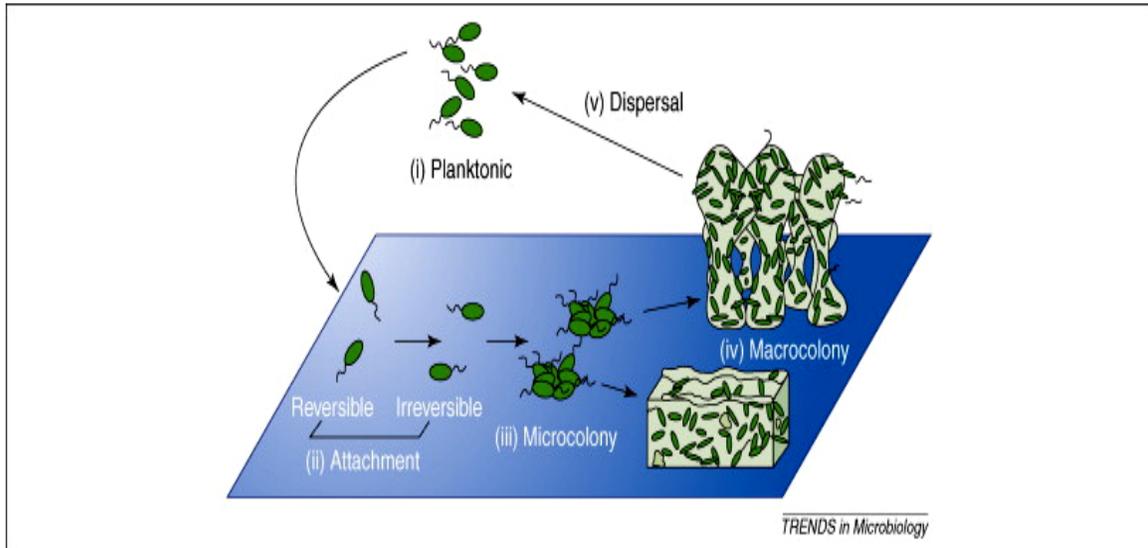


Fig. A.9. Biofilm formation begins with planktonic cells; they move to a certain surface, attach to it by forming microcolonies or macrocolonies, and form a complex biofilm directly.