Free-Living Ice-Nucleating Active Bacteria from High Mountain Lake Habitats

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We collected the culturable heterotrophic bacteria from oligotrophic high mountain lake habitats and tested their ability to induce ice formation. Direct plating was carried out using low-nutrient medium at a temperature of between 3 and 4°C. As many as 84 isolates were recovered from glacier ice and natural biofilm growing on granite rocks surface. Six out of 84 isolates were capable of expressing the ice-nucleation phenotype. After autoclaving the cell suspension at 121°C for 15 min, isolate J78 was still able to retain the ability for ice formation. Heat-stable ice nuclei produced by ice-nucleating active bacteria have potential applications in biotechnology. Characterization of INA bacteria was performed employing live-dead Gram staining and molecular methods. Universal primers for Bacteria (S-D-Bact-0008-b-S-20 and S-D-Bact-1524-a-A-18) were used for PCR to amplify almost the full length of the 16S rRNA genes of selected INA isolates. Restriction fragment length polymorphism analysis resulted in 2 unique patterns, as represented by J34 and J83, respectively. Based on DNA sequencing of 16S rRNA gene, isolate J43 (GeneBank accession no. AJ864852) was closely related to Pseudomonas mephitica (99.2% sequence similarity) and Janthinobacterium lividum (99% similarity), whereas isolate J83 (GeneBank accession no. AJ864859) showed 100% sequence identity to Pseudomonas fluorescens.

Key words: high mountain lake habitats, ice nucleation, 16S rRNA gene, free-living bacteria

Various Gram-negative bacteria are capable of catalyzing ice formation at temperatures of -2 to -12°C in nature (Lindow et al. 1982; Hirano et al. 1985). Most of ice-nucleating-active (INA) bacteria are associated with plants (Lindow et al. 1978; Lindermann et al. 1982; Loper and Lindow 1994; Waturangi et al. 2008) or animals (Lee et al. 1995; Worland and Block 1999). Other microorganisms, including several genera of fungi (Pouleur et al. 1992) and lichens (Kieft 1988) have been reported in their ability to induce ice formation. Ice-nucleating active (INA) bacteria have been recovered from various geographical areas, such as Antarctica, temperate, subtropical or tropical regions. INA bacteria, which induce frost damage in various plants, can contribute to a very devastating loss in agricultural crops production (Lindow et al. 1982).

INA bacteria produce outer surface membrane protein that can act as a catalyst for the unusual transition of water from liquid to its solid phase (Gurian-Sherman and Lindow 1993). Droplets of pure water (INA-free water) remain in its liquid state (supercool) up to a temperature of -40°C. The higher temperature of ice catalysis conferred by bacterial ice-nuclei makes them useful in ice-nucleation-limited processes such as artificial snow production, the freezing of some food products and possibly in future weather modification schemes (Gurian-Sherman and Lindow 1993).

Up to today, reports on free living INA bacteria recovered from high mountain lake habitats remain limited. Microorganisms that survive and actively grow in these habitats might be good sources of cold-active-proteins, such as ice nucleation active protein, which are catalytically efficient at low temperatures (Gerday et al. 1997). In this study, we report our study on free living INA bacteria recovered from these extreme habitats.

MATERIALS AND METHODS

Samples Collection and Treatments. Samples were collected from the Joeri lakes catchment, located in the southeastern Swiss Alps at an altitude of approximately 2 750 m a.s.l (Yuhana et al. 2006). Lake water and biofilm samples were aseptically collected during the snow-free season. Subsurface water samples were obtained in sterile glass bottles and patches of biofilm samples were collected from the surface of submerged rocks. Aliquots of 50 and 100 µl of water were spread-plated and subcultured onto minimal growth agar medium (pH 7.1), whereas biofilm samples were spread aseptically onto the medium after mechanical disruption of the biofilm with a sterile loop. Growth temperature for all cultures was 4±1°C. The minimal growth medium (designated as MM) contained the following ingredients: 0.5 g l⁻¹ Bacto tryptone, 1.0 g l⁻¹ yeast extract, 0.5 g l⁻¹ NaCl and 15 g l⁻¹ bacteriological agar. The concentrated trace elements stock solution (10 000 x) contained the following: 850 µM ZnSO₄·7H₂O, 7100 µM MnCl₂·4H₂O, 86 µM Co(NO₃)₂·6H₂O, 1600 µM Na₂MoO₄·2H₂O, 29750 µM Citrate-H₂O and 21 480 µM Ferric ammonium citrate.

When growth occurred (after 14 to 21 days of incubation in the cold), single colonies of visibly dominant and different colony morphotypes were subcultured onto new minimal medium. Isolates were maintained in 10 x diluted Luria Bertani (LB) agar medium (pH 7.2) containing the following ingredients: 0.5 g l⁻¹ Bacto tryptone, 1.0 g l⁻¹ yeast extract, 0.5 g l⁻¹ NaCl and 15 g l⁻¹ bacteriological agar.

Cell Morphology and Characterization by Gram Staining. Fluorescent staining was performed to determine the Gram type, characterize cell morphology and to
distinguish dead cells from living ones. Staining was carried out as described by the manufacturer (Molecular Probes, Inc.) using the Viability Gram Staining Kit (V-7023 Molecular Probes Inc.). The treated samples on slides were observed with a Zeiss Axiosplan microscope (Carl Zeiss, Oberkochen Germany) employing 3 different excitation filters (365-395, 450-490 and 546-580 nm) and photographed with an Optronik digital camera.

**Ice Nucleation Assay.** The ice nucleation capacity of all 84 isolates was tested qualitatively by the tube assay (modified from Hirano et al. 1985). Isolates were grown in 10 ml liquid MM and incubated at 4°C until the stationary phase was reached. Cells were then suspended in 10 ml autoclaved PBS solution (10 mM potassium phosphate-buffered saline, pH 7.0) and kept in a cooling-bath at -2 to -10°C for 5 to 10 min. A suspension of E. coli cells containing plasmid pJL1703 (Loper and Lindow 1994) was used as a positive control, whereas a cell-free PBS solution served as a negative control. The ice nucleation activity of each INA isolate was observed by droplet-freezing assay and the ice nucleation frequency was calculated by the following formula: \( N(t) = \frac{1}{V} \times (\text{No}) \) (Vali 1971) where \( N(t) \) is the frequency of ice nucleation at \( T \) temperature, \( f \) indicates the proportion of droplets unfrozen and \( V \) is the volume of individual droplets.

**Genomic DNA Extraction.** Total genomic DNA from pure isolates was extracted with cetyltrimethyl ammonium bromide (CTAB) (modified from Murray and Thompson 1980). This provided a simple, non toxic and inexpensive method and yielded enough DNA template for PCR amplification. After pelletting the cells, extraction buffer (2% w/v CTAB, 100 mM Tris-Cl pH 8.0, 1.4 M NaCl, 20 mM EDTA) was added and mixed. The solution was incubated at 60°C for 30 min and centrifuged at 12 000 xg (4°C) for 10 min. After transferring the supernatant into a fresh tube, an equal volume of chloroform:isoamyl alcohol (24:1) was added, mixed gently and the solution was centrifuged at 12 000 xg (4°C) for 10 min, these steps were repeated one more time. The resultant supernatant was transferred into a new tube and 1/10 volume of ammonium acetate was added. DNA was then collected by precipitation in ethanol. DNA extracts were then washed with 70% ethanol and the solution was centrifuged at 12 000 xg (4°C) for 10 min. After autoclaving the cell suspension at 121°C for 15 minutes, isolate J78 still showed its ability to initiate the ice formation even at lower temperatures (Fig 2).

**RESULTS**

Eighty-four isolates were recovered from lake water, glacier and biofilm growing on granite rocks surface. Generally they are cream-pigmented morphotypes and some of them were capable of producing water-soluble yellow-greenish fluorescent pigments. Test tube assay showed that 6 out of 84 isolates were capable of catalyzing the ice nucleation in the tubes at temperatures between -2 to -5°C. Fluorescent microscopy determination after live-dead Gram staining, showed that all of the 6 INA isolates were Gram negative and rod shaped (Fig 1).

After autoclaving the cell suspension at 121°C for 15 minutes, isolate J78 still showed its ability to initiate the ice formation at lower temperatures, ranging from -8 to -10°C. Other INA isolates (J43, J71, J77, J83 and J84) and autoclaved-positive control, were no longer capable of catalyzing ice formation even at lower temperatures (Fig 2).
isolates J43, J71, J77, J78 and J84 (Fig 3). 16S rRNA genes of isolates J83 and J43 (as a representative of other 5 INA isolates) were selected for sequencing. The DNA sequence of isolate J43 (1484 bp) showed 99.2% similarity to \textit{Pseudomonas mephitica} or \textit{Janthinobacterium lividum}. The nucleotides have been submitted to the GenBank with accession no. AJ864852. DNA sequence of isolate J83 (1491 bp) showed 100% similarity to \textit{P. fluorescens} CCM 2115 and has been submitted with accession no. AJ864859.

**DISCUSSION**

All INA isolates from high mountain lake habitats showed ice nucleation activity at temperatures warmer than -5°C. Similar results were shown in a previous study (Kieft 1988) of INA associated with lichens isolated from high mountain habitats. His study showed that several epilithic lichen samples of the genera \textit{Rhizoplaca}, \textit{Xanthoparmelia} and \textit{Xanthoria} expressed ice nucleation activity at temperature as warm as -2.3°C. According to the ice nucleation proteins classification described by Turner et al. (1990), all of our INA isolates are characterized as class A, which showed ice nucleation activity between temperature range of -2 to -5°C. Waturangi et al. (2008) reported that

| Table 1 Ice nucleation activity of free living INA bacteria from high mountain lake habitats |
|-----------------------------------------------|-----------------------------------------------|
| Isolate | No of frozen droplets/ total droplets | N(t) (at temperature) |
| J43    | 17/20                                      | 38 nuclei/ml (-2°C)   |
| J71    | 16/20                                      | 32 nuclei/ml (-2°C)   |
| J77    | 17/20                                      | 38 nuclei/ml (-2°C)   |
| J78    | 18/20                                      | 46 nuclei/ml (-2°C)   |
| J83    | 17/20                                      | 38 nuclei/ml (-2°C)   |
| J84    | 19/20                                      | 60 nuclei/ml (-2°C)   |
| J78 (after autoclaving) | 18/20                                      | 47 nuclei/ml (-8°C)   |

N (t), ice nucleation activity; where N, frequency of ice nucleation at temperature t.
tropical INA bacterial isolates showed ice nucleation activity at cooler temperature of -8°C and categorized as class B. At this temperature, the ice nucleation activity values of tropical INA bacterial isolates ranges from 9 to 50 minutes to 5°C.

Isolate J78 showed its ice nucleation ability at warmer temperatures than that of INA expressed by R. chryssoleuca, i.e. at temperatures ranging from -8 to -10°C. This might indicate a more stable INA protein structure present in this isolate, however, further studies are required to determine the uniqueness of the J78 INA protein.

Based on their DNA sequences, our INA isolates phylogenetically fall into the â- and â-subgroups of Proteobacteria (Fig 4). Isolate J83, which is closest related to P. fluorescens strain IAM12022, belonging to the gamma-subdivision. P. fluorescens has been reported to be tolerant to the bacteria which are able to catalyze ice formation (Lee et al. 1995) while isolate J43 falls into the â- subdivision. This isolate is closely related to P. mephitica ATCC 33665T or J. lividum DSM 1522T. To our knowledge, there are no other reports on the ability of the bacteria belonging to P. mephitica or J. lividum in catalyzing the ice formation.

Bacteria which are able to catalyze water crystallization may have an advantage over those which cannot. Those which can will be able to survive in frozen environments through slow cellular dehydration (Baertlein et al. 1992). This is a very important property for microorganisms to survive in this cold and extreme habitat. Ice formation on the outside of the cell allows water molecules to move from the cytoplasm across the cell membrane to join crystals of pure water nucleated extracellularly. This increases the osmotic potential inside the cells, thereby preventing freezing and cell damage by internal ice crystals. The ice nucleation process allows for ordered propagation of ice throughout the cell rather than a rapid freezing, which can result in membrane rupture and cell death (Baertlein et al. 1992). Since this ability was only found in 6 out of the 84 isolates, we must assume that other bacteria use different strategies to survive periods of freezing, for instance by producing anti-freeze proteins (Feller et al. 1996) or other osmolytes.

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REFERENCES