

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

MUNTI YUHANA^{1*} AND KURT HANSELMANN²

¹Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Institut Pertanian Bogor, Darmaga Campus, Bogor 16680, Indonesia; ²Microbial Ecology Group, Institute of Plant Biology, University of Zurich, Switzerland

We collected the culturable heterotrophic bacteria from oligotrophic high mountain lake habitats and tested their capability 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 glacial 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 J43 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 south-eastern Swiss Alps at an altitude of approximately of 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: 6 mg l⁻¹ yeast extract, 10 µM CaCl₂, 20 µM Na₂CO₃, 14 µM NaNO₃, 10 µM NH₄Cl, 1.75 µM K₂HPO₄, 2.7 µM EDTA Na salt, 10 mg l⁻¹ yeast extract, 10 mg l⁻¹ peptone, 15 g l⁻¹ washed-bacteriological agar and 1 x trace elements, (added to the autoclaved basal medium). The concentrated trace elements stock solution (10 000 x) contained the following: 850 µM ZnSO₄·7H₂O, 7 100 µM MnCl₂·4H₂O, 86 µM Co(NO₃)₂·6H₂O, 1 600 µM Na₂MoO₄·2H₂O, 29 750 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

*Corresponding author, Phone: +62-251-8628755, Fax: +62-251-8622941, E-mail: myhn@gmx.ch

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 Axioplan microscope (Carl Zeiss, Oberkochen Germany) employing 3 different excitation filters (365-395, 450-490 and 546-580 nm) and photographed with an Optronic 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: $N(t) = [-\ln(f)] / V$, (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 pelleting 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 equivalent of 7.5 M ammonium acetate was added. DNA was then collected by precipitation in ethanol. DNA extracts were checked using the following electrophoresis conditions: agarose gel (1% w/v), [Tris-Acetate-EDTA] (TAE) buffer 0.5 x (20 mM Trizma base, 10 mM glacial acetic acid, 0.5 mM EDTA), running time 30 min at 5 V/cm. Successful extraction of DNA was verified by staining the gels in a 1 μg ml⁻¹ ethidium bromide solution.

16S rRNA Gene Amplification by PCR. Nearly full-length 16S rRNA genes were amplified by PCR using the bacterial primers: S-D-Bact-0008-b-S-20 (5'-AGA GTT TGA TCC TGG CTC AG-3') and S-D-Bact-1524-a-A-18 (5'-AAG GAG GTG ATC CAR CCG-3'). PCR amplification was performed in a 25 μ l reaction volume with a Techne Thermocycler (Techne LTD, Oxford, UK). Each reaction mixture contained (final concentration) *Taq* buffer (1x) (Sigma), 1.5-2.0 mM MgCl₂, 0.1 mg ml⁻¹ DNase-free Bovine Serum Albumin (Amersham, Pharmacia Biotech Inc.), 0.2 mM dNTPs, 200 nM of forward and reverse primer, respectively, 40 U *Taq* Polymerase (Sigma), ddH₂O and approximately of 20-100 ng of template DNA. The following touch-down PCR program was used: initial denaturation at 94°C for 2 min; 20 cycles of 94°C for

20 sec, 63°C for 30 sec with lowering temperature by 0.5°C in every cycle, 72°C for 80 sec with increasing duration by 1 sec in every cycle; another 20 cycles of 94°C for 20 sec, 53°C for 30 sec, 72°C for 100 sec with increasing period by 1 sec every cycle; followed by a final extension step at 72°C for 10 min. PCR products were analyzed by electrophoresis in 1% w/v agarose gels in 0.5x TAE running buffer, stained with ethidium bromide (1 μg ml⁻¹) and photographed under UV. For RFLP analysis, 8 μ l PCR products were double-digested with 1 U restriction enzyme *Hinf*I (5'-G/ANTC) and *Hae*III (5'-GG/CC) in a total reaction volume of 10 μ l.

Sequencing. The PCR products of the 16S rRNA genes from different RLFP patterns were purified through microcon centrifugal filter devices (Microcon YM 100, Millipore, Bedford, Mass., USA). Nearly full-length 16S rRNA genes were bidirectionally sequenced with a DNA sequencer (ABI Prism 377), using ABI Prism® Big Dye™ v2.0 (Applied Biosystems) as described by the manufacturer with the following 6 primers: S-D-Bact-0008-b-S-20 (5'-AGA GTT TGA TCC TGG CTC AG-3'), S-*.-Univ-0519-a-A-18 (5'-GWA TTA CCG CGG CKG CTG-3'), S-*.-Univ-0519-a-S-18 (5'-CAG CMG CCG CGG TAATWC-3'), S-D-Bact-1099-b-S-16 (5'-GYAACG AGC GCAACC C-3'), S-D-Bact-1099-b-A-16 (5'-GGG TTG CGT CCG TTR C-3') and S-D-Bact-1524-a-A-18 (5'-AAG GAG GTG ATC CAR CCG-3'). For a 10 μ l-single PCR reaction, 5 to 20 ng DNA template, 3 μ l Big Dye (Applied Biosystems) and 3 μ l of 1.5 μ M primer were used. After the sequencing PCR, the products were purified with Sephadex G-50 (Amersham, Pharmacia Biotech AB) and loaded onto a sequencing machine (ABI Prism 377 DNA Sequencer). The BLAST search tool available from NCBI (<http://www.ncbi.nlm.nih.gov/blast>) was used to list the closest neighbors of the sequences.

Phylogenetic Tree Construction. The phylogenetic tree was analysed online using the Phylip Interface, available at the ribosomal database project (<http://rdp8.cme.msu.edu/html/analyses.html>). Sequence data was imported and aligned to their closest neighbors by an automated alignment. The distance matrix was calculated by maximum likelihood method and a phylogenetic tree was constructed based on neighbor-joining analysis.

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).

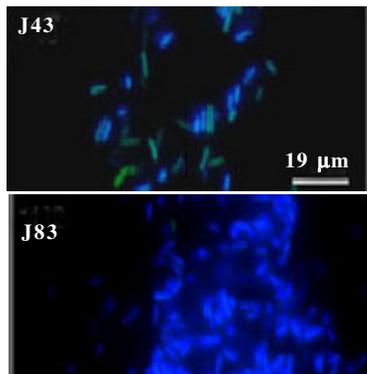


Fig 1 Fluorescent micrographs of isolate J43 and J83. Live cells fluoresce blue, whereas dead cells fluoresce green. Both isolates were Gram negative.

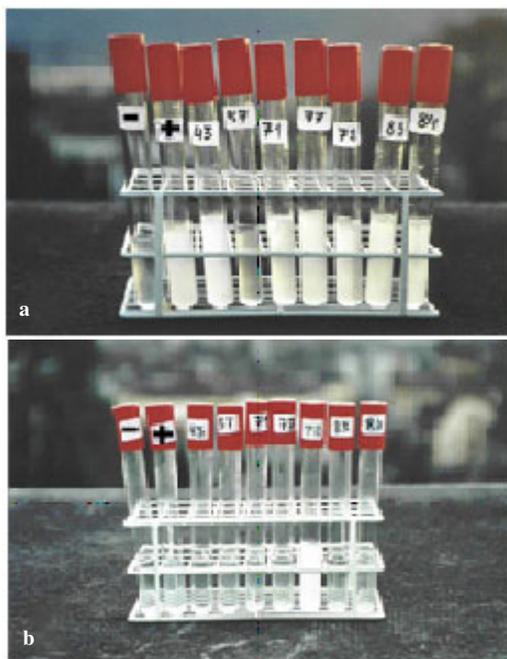


Fig 2 Ice nucleating assay tubes. (a) Assay carried out before autoclaving the cell suspension at the temperature ranging from from -2 to -5°C. Assay showed ice nucleation capability of isolate J43, J71, J77, J78, J83, and J84. J57 represents a non ice-nucleating-active isolate, remaining unfrozen after 30 minutes incubation at temperatures ranging from -8 to -10°C. Cell-free PBS solution (-) serves as a negative control, while cell suspension of *E. coli* containing plasmid pJL1703 was used as a positive control; (b) Assay carried out after autoclaving the cell suspension. Suspension of the isolate J78 remaining capable of inducing the ice nucleation at lower temperatures ranging from -8 to -10°C.

The highest ice nucleation activity among INA isolates was shown by J84 with frequency of 60 nuclei ml⁻¹ and the lowest was indicated by isolate J71 (32 nuclei ml⁻¹). Isolates J43, J77 and J83 had the same ice nucleation frequency of 38 nuclei ml⁻¹ and isolate J78 was 46 nuclei ml⁻¹ (Table 1). The temperature of the assay was warm (-2°C). Ice nucleation activity of isolate J78 after autoclaving was 46 nuclei ml⁻¹ at -8°C.

PCR amplifications of the 16S rRNA gene of selected INA isolates yielded products of approximately 1500 bp. By using double restriction with *Hin*fl and *Hae*III, two different RFLP patterns were obtained. J83 has a unique pattern, whereas J43 shows corresponding band patterns with

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.

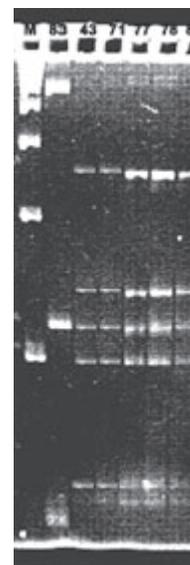


Fig 3 RFLP patterns of 16S-rRNA genes from the INA isolates. Lane 1, molecular marker 100 bp ladder (Pharmacia, Biotech); Lane 2-7, J83, J43, J71, J77, J78, and J84. Running conditions on Spreadex EL-800 gel (Elchrom Sci.): 70 Vcm⁻² for 90 min, running temperature 55°C in 30 mM TAE buffer.

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 *Pseudomonas mephitica* or *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 *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 *Rhizoplaca*, *Xanthoparmelia* and *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

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

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a lichen, *Rizoplaca chrysoleuca* (Kieft 1988) and from *Fusarium* (Pouleur *et al.* 1992). Kieft (1988) reported that control cell suspension (no heat treatment) and 10 min treatment at temperatures of 40 to 70°C , showed the ice nucleation activity at temperatures between -2.3 to -3.3°C , whereas the ice nucleation activity at a lower temperature of -14.6°C was detected after cell suspension exposed by heat treatment at temperature range of 80 to 95°C . Our isolate J78 showed its ice nucleation ability at warmer temperatures than that of INA expressed by *R. chrysoleuca*, 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 \hat{a} - and \tilde{a} -subgroups of *Proteobacteria* (Fig 4). Isolate J83, which is closest related to *P. fluorescens* strain IAM12022, belonging to the *gamma*-subdivison. *P. fluorescens* has been reported to belong to the bacteria which are able to catalyze ice formation (Lee *et al.* 1995) while isolate J43 falls into the \hat{a} - subdivison. 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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