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Ice Nucleation Active bacteria in Mount Lawu forest, Indonesia: 2. Identification and characterization of *ina* gene bacteria isolated from lichens

TEGUH NUR ARIFIN, ARI SUSILOWATI♥, SUTARNO
Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57126, Central Java, Indonesia. Tel./fax.: +62-271-663375, ♥email: arisusilowati@staff.uns.ac.id

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Abstract. *Arifin TN, Susilowati A, Sutarno. 2018. Ice Nucleation Active bacteria in Mount Lawu forest, Indonesia: 2. Identification and characterization of* ina *gene bacteria isolated from lichens. Asian J For 2: 39-46.* Ice nucleation active (INA) bacteria can catalyze ice formation. Moreover, these bacteria cause frost injury in plants. This study aimed to determine the INA bacteria species of Mount Lawu forest, Java, Indonesia, based on the 16S rRNA gene and the characters of ice nucleation active gene coding of INA bacteria on lichens used *ina* gene primer. First, the isolates of INA bacteria were grown in NAG, and the DNA could be isolated. After that, genes coding for 16S rRNA and *ina* gene were amplified, and the amplification products were sequenced. Furthermore, the sequences were analyzed with BLAST program to know the similarity of bacteria species and *ina* gene characters. The result showed that the isolated INA bacteria had similarities with *Pantoea*, *Pseudomonas*, and *Rahnella*. The *ina* gene from N.2.1.B-13 isolate consists of 189 amino acids, dominated by a common amino acid found in other *ina* genes, like Alanine, Glycine, Tyrosine, Serine, Leucine, and Threonine. N terminal and C terminal are not found because *ina* gene from N.2.1.B-13 isolate is similar to the ice nucleation protein gene *Pseudomonas borealis* from bases 2947 bp to 3491 bp or 871 to 1060 amino acids.

Keywords: Amplification, INA bacteria, *ina* genes, sequencing

INTRODUCTION

The microbial community on leaf surfaces is diverse and includes many different species, such as bacteria, filamentous fungi, yeasts, algae, and a few protozoa and nematodes (Andrews and Harris 2000). Bacteria with the largest population are phyllosphere bacteria which differ greatly in size and number in plant parts of the same species (Lindow et al. 1978). One of the components of the leaf bacterial community that can be found is ice-nucleation-active (INA) bacteria (Lindow and Brandl 2003).

INA bacteria are a group capable of catalyzing ice formation at temperatures above -10°C. These bacteria can express ice nucleation proteins on the cell surface, lowering the water temperature and freezing it. If there is no ice nucleation, pure cold water (H_2O) can only be supercooled and will not spontaneously freeze until the temperature reaches -40°C. The INA bacteria can function in helping to accelerate the ice freezing process (Stephanie and Waturangi 2011).

INA bacteria have been shown to cause frost damage in some plants (Hirano and Upper 2000). Bacteria commonly found in plants consist of several species (such as *Pseudomonas syringae* and *Erwinia herbicola*), which can produce a protein that can induce ice core formation at temperatures above -2°C. The population on the leaf surface of this species of bacteria is limited and can form supercooling in some parts of the plant where INA bacteria are present by forming ice that can damage at temperatures of -2 to -4°C. Since plants lack the intrinsic elements of ice core formation at these temperatures, these bacteria play an important role in initiating frost damage formation (Lindow et al. 1978).

Various Gram-negative bacteria can trigger ice formation at temperatures of -2 to -12°C in nature (Lindow et al. 1982a,b; Hirano et al. 1985). Most 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). However, other organisms, including several species of fungi (Pouleur et al. 1992) and lichens (Kieft 1988), have been reported for their ability to induce ice formation.

INA bacteria that have been found include *P. syringae*, *Pseudomonas viridiflava*, *Pseudomonas fluorescens, E. herbicola* (synonymous with *Pantoea herbicola*), and *Xanthomonas campestris* (Edwards et al. 1994). These five bacterial species can catalyze ice formation at a temperature of -1.5°C to -10°C. Even at temperatures above -5°C, these species can cause frost injury on the leaf surface. It is due to changes in the water between and inside the leaf cells to the ice at a temperature of -5°C (Gurian-Sherman and Lindow 1993).

Some INA bacteria are also considered important in condensation and ice core formation in clouds. Ice formation in tropospheric clouds is required to form snow and most precipitation. Studies on the biological formation of ice cores in snowfall have been carried out. Biological ice core-forming at warm temperatures is abundant in fresh

snow samples (Christner et al. 2008). According to Morris et al. (2004), INA bacteria also play a role in atmospheric processes that cause rain, given that these bacteria are easily spread in the atmosphere and have been found in clouds at an altitude of several kilometers. Furthermore, INA bacteria participate in the biological cycle of precipitation; they are transported to the cloud from the plant canopy.

Besides having an important role in bioprecipitation, INA bacteria can make artificial rain and snow by seeding clouds with INA bacteria as a substitute for salt sowing, which is now widely used. In addition, INA bacteria can be used in the food preservation industry by freezing, which is initiated by INA bacteria (Wahyudi 1995). It can be seen because INA bacteria can cause freezing at a temperature of -6°C in samples containing 10% sucrose. These results may indicate that with the application of INA bacteria, several types of food can be frozen through this process by modifying the slightly high temperature a few degrees below 0°C for periodic freezing, saving energy, and improving the quality efficiency of food products (Li et al. 1997).

Lichens are a symbiosis between fungi and algae that form a unified whole morphologically and physiologically. Lichenes can stick to rocks or other plants (Setyawan 2000). Samsali's research (2008) showed that along the hiking trail of Cemoro Sewu, Mount Lawu, Java, Indonesia, there were 12 epiphytic species, namely 4 from the Lichens division, 1 from the Bryophyta division, 5 from the Pteridophyta division, and 2 from the Spermatophyta division. A study by Kieft (1988) showed that some lichens in the southwest region of the United States could induce ice core formation. It indicates that the lichens may contain microorganisms capable of assisting the formation of ice cores, namely INA bacteria.

Most research on INA bacteria is carried out in subtropical areas, so research on INA bacteria from the tropics, especially in Indonesia, is very necessary (Stephanie and Waturangi 2011); considering the role of INA bacteria is quite a lot, one of which is to help the bioprecipitation process. The INA bacteria have been isolated from lichens on the climbing route of Cemoro Sewu, Mount Lawu by Fu'adah (2017), and isolates have been found in the Biology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Central Jawa, Indonesia. However, the isolates of INA bacteria have not been identified and characterized, so the information on these INA bacteria is still very little. Therefore, it is necessary to research the identification and characterization of INA bacteria from these lichens. This research data can be used to understand species diversity and the role of INA bacteria in nature.

The aims of this study were: (i) to determine the INA bacterial species found in lichens on the hiking trail of Cemoro Sewu, Mount Lawu based on the identification with the 16S rRNA gene; (ii) to know the character of the gene encoding the ice core-forming protein of INA bacteria in lichens on the hiking trail of Cemoro Sewu, Mount Lawu by using the *ina* gene primer.

MATERIALS AND METHODS

Materials

The materials used in this study include: bacterial isolates from research conducted by Fu'adah (2014) with isolate codes K.2.1.B-5, N.2.1.B-13, N.2.1.B-15, K .2.2.B-2, K.2.2.B-4, N.2.2.B-6, N.2.2.B-7, media

Procedure

Equipment and materials sterilization

Equipment and materials used for research must be sterilized first to prevent contamination. The sterilized types of equipment include test tubes, Erlenmeyer, measuring cups, microcentrifuge tubes, PCR tubes, and tips. In addition, sterilized materials were distilled water, Nutrient Agar Glycerol (NAG) media, and ddH_2O. Sterilization was carried out using an autoclave at a temperature of 121°C and a pressure of 1 atm for 15 minutes.

Preparation of media

NA medium was prepared by mixing 6 g of NA powder with 5 mL of glycerol in a 250 mL Erlenmeyer tube and adding distilled water to a limit of 200 mL. Erlenmeyer is placed on a hot plate until the solution boils and is clear. Erlenmeyer tube holes are covered with cotton and aluminum foil. Then, this tube was sterilized using an autoclave at 121°C for 15 minutes.

To make the slant media, the NA medium was left until the solution temperature was reduced. After being warm enough, 4 mL of media was poured into each test tube and autoclaved at 121°C for 15 minutes at a pressure of 1 atm. After sterilization, the tube is slanted at 45° until the media hardens.

Isolate rejuvenation

Seven isolates, namely K.2.1.B-5, N.2.1.B-13, N.2.1.B-15, K.2.2.B-2, K.2.2.B-4, N.2.2.B-6, and N.2.2.B-7 were rejuvenated to prolong the life of bacteria. The rejuvenation process was carried out by taking one ose of each isolate and scratching it on seven slant NAs. After that, the slant agar was incubated at 27°C for 3 days and then stored in a refrigerator for 4°C.

Bacterial DNA isolation

Before the DNA of the bacterial isolates was isolated, the bacteria were first cultured on NB media for 24 hours at a temperature of 27°C. After 24 hours, the bacterial culture is ready to proceed to the DNA isolation process.

The bacterial DNA isolation process is divided into several procedures based on the kit used, namely:

Sample preparation. A total of 1 mL of bacterial culture was put into a 1.5 mL microcentrifuge tube. The sample was centrifuged for 1 minute at 10,000 rpm, and the formed supernatant was discarded. Then, 200 µL of GT Buffer was added and homogenized with the pellet by shaking or using a micropipette. Then it was incubated at room temperature for 5 minutes and continued with the lysis process.

Cell lysis. 200 µL of GB Buffer was added to the sample and vortexed for 5 seconds. Then the samples were incubated at 60°C for 10 minutes. During incubation, shake the tube every 3 minutes. At the same time, the Elution Buffer (200 µL per sample) was heated to 60°C (for DNA elution).

DNA binding. 200 µL of absolute ethanol was added to the sample and vortexed. The GD Column is placed in a 2 ml Collection Tube. The mixed solution from the cell lysis process was then transferred to the GD Column and centrifuged at 10,000 rpm for 2 minutes. The 2 mL Collection Tube containing the liquid deposit was then placed back into the new 2 mL Collection Tube.

DNA cleansing. 400 µL of W1 Buffer was inserted into the GD Column. Then it was centrifuged at 10,000 rpm for 30 seconds, and the liquid residue formed was discarded. Next, 600 µL of Wash Buffer (which had been added by absolute ethanol) was added to the GD Column and centrifuged again at 10,000 rpm for 30 seconds, and the formed liquid deposit was discarded. Then the GD Column was placed back into the 2 mL collection tube and centrifuged at 10,000 rpm for 3 minutes under dry conditions.

Elution. The GD Column was transferred to a 1.5 mL microcentrifuge tube, then 100 L of Elution Buffer (which had been heated) was added to the center of the matrix in the GD Column and allowed to stand for 3 minutes. Then the GD Column was centrifuged at 10,000 rpm for 30 seconds to obtain pure DNA.

16S rRNA gene amplification

The 16S rRNA gene amplification was performed by mixing 9 µL of ddH_2O, 12.5 µL of 2X KAPA2G Fast ReadyMix (containing DNA Polymerase 0.5 U per reaction, PCR Buffer dNTP 0.2mM, and $MgCl_2$ 1.5 mM), 1.25 µL of forward primer 63f, 1.25 µL of reverse primer 1387r, and 1 µL of DNA template. The primers used in this amplification were forward primer 63f (5- CAG GCC TAA CAC ATG CAA GTC -3) and reverse primer 1387r (5- GGG CGG WGT GTA CAA GGC -3)

Amplification was carried out under PCR conditions, namely, initial denaturation at 95°C for 3 minutes, denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, extension at 72°C for 5 seconds, and final extension at 72°C for 5 minutes. Denaturation, annealing, and extension were carried out for 30 cycles.

ina gene amplification

Primers used to amplify this gene include *ina*Z F (5'GCA GAC TGC GGG TTA TGA GAG C 3'); *ina*Z R (5'CGC CGG TCA GTT TGC TTC TAT C 3'); *ina*A F (5'AGG CTT TGA GAA CGG ACT AAC G 3'); *ina*A R (5' TTT CTG TCG GCT GCG TAC TG 3'); *ina*W F (5'GCA GTA CGC AGA CGG CAC AG 3'); *ina*W R (5' TTT CGT AGC CAG CAG TTG ATG TG3'); *ina*X F (5'GCA AGG GCA GCG ATG TCA C 3'); and *ina*X R (5' TCT GCG TGC TGC CGT AAC C 3').

The *ina* gene amplification was carried out by mixing 9 µL of ddH_2O, 12.5 µL of 2X KAPA2G Fast ReadyMix (containing 0.5 U DNA Polymerase per reaction, 0.2 mM dNTP Buffer PCR, and 1.5 mM $MgCl_2$), 1.25 µL of forward primer, 1.25 µL of reverse primer, and 1 µL of DNA template.

PCR carried out amplification with the provisions of initial denaturation at 95°C for 3 minutes, denaturation at 95°C for 15 seconds, annealing for *ina*X at 55.2°C for 15 seconds, annealing for inaZ at 55°C for 15 seconds, annealing for inaW at 55°C for 15 seconds, annealing for inaA at 55°C for 15 seconds and extension at 72°C for 6 seconds, and final extension at 72°C for 5 minutes. Denaturation, annealing, and extension were carried out for 40 cycles.

Sequencing of 16S rRNA and ina *coding genes*

The PCR products of 16S rRNA and *ina* genes that were successfully amplified were sent to P.T. Genetika Science Indonesia, which will then be sequenced by 1st Base Singapore using the ABIprism™ 310 Automated DNA Sequencer (PE Applied Biosystem).

Data analysis

The results of the 16S rRNA and *ina* gene sequences were then compared with the database using the BLAST program on the National Center for Biotechnology Information website (http://www.ncbi.nlm.gov/BLAST/). The sequence similarity was then analyzed descriptively based on the existing database. Finally, the phylogenetic tree was created using MEGA 5.1 software.

RESULTS AND DISCUSSION

Isolate rejuvenation

This study used seven positive isolates of INA bacteria found in lichens on the climbing route of Cemoro Sewu, Mount Lawu, by Fu'adah (2014). The list of bacterial isolates used in this study and the classification of *ina* protein based on freezing temperature can be seen in Table 1.

Table 1 shows that all bacterial isolates came from lichens of the *Parmelia* sp. encountered at sampling station two. *Parmelia* sp. It lives as an epiphyte in pine trees (*Pinus* sp.). This station is located at an altitude of 2,532 m above sea level. Shrubs and large trees dominate it with a dense enough canopy, so sunlight is sufficiently blocked to reach plant vegetation. The temperature is around 20°C, and the light intensity is around 3676 Lux (Fu'adah 2014).

Table 1. List of bacterial isolates and their *ina* protein classification

Isolate code	Freezing temperature	INA protein class
K.2.1.B-5	-10°C	C
N.2.1.B-13	-5°C	B
N.2.1.B-15	-8°C	C
K.2.2.B-2	-5°C	B
K.2.2.B-4	-9°C	C
N.2.2.B-6	-10°C	C
N.2.2.B-7	-10°C	C

Note: K.2.1.B-5. K: Growth medium (K: King's B; N:NAG), 2: Sampling station number, 1: Repetition number, B: Lichen species name (A: Usnea; B: Parmelia), 5: Isolate number

The existing INA bacteria must be rejuvenated to be used in this study. Rejuvenation was carried out by regrowing INA bacteria on slant NAG media. NAG media is NA media added with 2.5% glycerol. Glycerol is a carbon source for bacterial growth and is commonly used to isolate ice-core bacteria (Lindow 1990). The rejuvenation result is a new bacterial colony with the same morphological characteristics as the previous bacterial colony.

Bacterial DNA isolation

DNA isolation is the first stage of various DNA analysis technologies. To extract DNA, laboratory steps are needed to break the cell wall, cell membrane and nuclear membrane, followed by the separation of DNA from other cell components (Fatchiyah et al. 2011).

In this study, DNA isolation was carried out on INA bacteria which were incubated for 3 days using Presto™ Mini gDNA Bacteria Kit (Geneaid Biotech Ltd.) and following the procedure recommended by the manufacturer. Presto™ Mini gDNA Bacteria Kit (Geneaid Biotech Ltd.) can optimally isolate the genomic DNA of Gram-negative and gram-positive bacteria.

The results of DNA isolation in Table 2 showed that the DNA of the seven INA bacteria was successfully isolated because the A260/280 ratio of isolated DNA ranges from 1.90 to 1.99. According to Sambrook et al. (1989), DNA isolates can be pure and meet the requirements to proceed to molecular analysis if the value of the A260/280 ratio ranges from 1.8 to 2.0.

16S rRNA gene amplification

Each INA bacterial DNA was amplified using PCR to obtain the 16S rRNA gene. The 16S rRNA gene is one of three types of ribosomal RNA present in prokaryotes. This gene is most often used for species determination. In addition, 16S rRNA can be used as a molecular marker because this molecule is ubiquitous (Pangastuti 2006). In this study, primers 63f (5- CAG GCC TAA CAC ATG CAA GTC -3) and 1387r (5- GGG CGG WGT GTA CAA GGC -3) were used to amplify the 16S rRNA gene. According to Marchesi et al. (1998), this primer was used because it can amplify the 16S rRNA gene better than other primers and consistently amplifies the 16S rRNA gene from various organisms.

The 16S rRNA gene amplification results by PCR were analyzed by agarose gel electrophoresis 0.8% (w/v) for 45 minutes at a voltage of 90 volts and a current of 400 mA. The results of the electrophoresis of the 16S rRNA gene amplification by PCR can be seen in Figure 1.

Table 2. Results of INA bacterial DNA isolation

Bacteria isolate	DNA concentration (µg/mL)	A260/A280 ratio
N.2.2.B-6	19.5	1.99
K.2.2.B-4	28.9	1.95
N.2.1.B-13	59.6	1.98
K.2.1.B-5	52.2	1.90
N.2.2.B-7	103.6	1.97
N.2.1.B-15	41.3	1.91
K.2.2.B-2	402.6	1.98

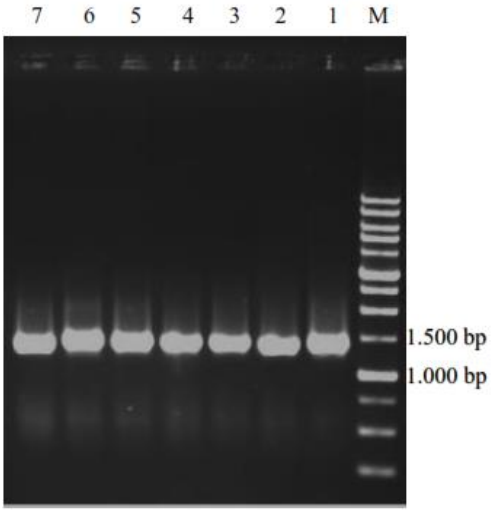

Figure 1. Electrophorogram of 16S rRNA gene using primers 63f and 1387r: M. *Marker* DNA 1 Kb, 1. N.2.2.B-6, 2. N.2.1.B-13, 3. K.2.2.B-2, 4. N.2.2.B-7, 5. K.2.1.B-5, 6. K.2.2.B-4, 7. N.2.1.B-15.

Figure 1 shows that bacterial DNA was successfully amplified. It is indicated by the presence of a bright and thick band. This success indicates that the primer attaches to a specific site on the DNA template with the optimum temperature used for primer annealing. The optimum temperature for the primer to anneal the DNA template can be known by looking at the information listed on the primer package.

The size of the PCR product can be determined by comparing the migration length of the DNA band with DNA markers of known size and concentration. For example, according to Marchesi et al. (1998), amplifying the 16S rRNA gene with primers 63f and 1387r was about 1,300 bp. Therefore, this study used a 1 Kb DNA marker. From Figure 1, it can be seen that in this study, all the obtained PCR products were about 1,300 bp in size with a concentration of about 92 ng/5 µL. Therefore, it can be known by comparing the DNA band with the DNA marker used.

***ina* gene amplification**

The *ina* gene possessed by INA bacteria has been identified according to its type. The known genes for each include inaA in Pantoea ananas (Abe et al. 1989), *ina*W in *P. fluorescens* (Warren et al. 1986), *ina*Z in *P.* syringae (Green and Warren 1985), and *ina*X on *X. campestris* (Zhao and Orser 1990). Therefore, different primers are needed for each isolate to detect the presence of this *ina* gene in it.

This study used four pairs of primers, namely inaZ F (5'GCA GAC TGC GGG TTA TGA GAG C 3'); inaZ R (5'CGC CGG TCA GTT TGC TTC TAT C 3'); inaA F (5'AGG CTT TGA GAA CGG ACT AAC G 3'); inaA R (5' TTT CTG TCG GCT GCG TAC TG 3'); inaW F (5'GCA GTA CGC AGA CGG CAC AG 3'); inaW R (5' TTT CGT AGC CAG CAG TTG ATG TG 3'); and *ina*X F (5'GCA AGG GCA GCG ATG TCA C 3'); and *ina*X R (5' TCT GCG TGC TGC CGT AAC C 3'). According to Nejad et al. (2006), these four pairs of primers can amplify four types of *ina* genes that are commonly found.

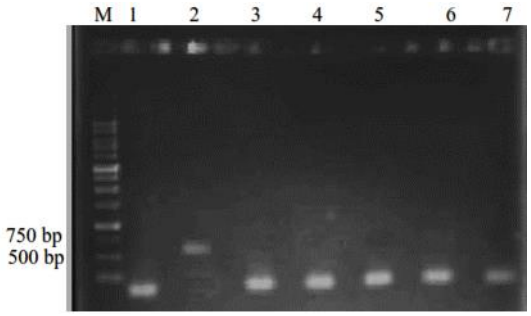

Figure 2. Electropherogram of the *ina*Z gene using primers *ina*Z f and *ina*Z r: M. DNA marker 1 Kb, 1. N.2.2.B-6, 2. N.2.1.B-13, 3.K.2.2.B-2, 4. N.2.2.B-7, 5. K.2.1.B-5, 6. K.2.2.B-4, 7. N.2.1.B-15

The amplification result of the *ina* gene showed that 1 of 7 bacteria whose genes were detected using four pairs of specific primers contained one of the *ina* genes. The bacteria had an isolate code of N.2.1.B-13. Figure 2 shows the results of agarose gel electrophoresis.

Based on the research conducted by Nejad et al. (2006), the four primary pairs of *ina* genes, namely *ina*Z, *ina*W, *ina*A, and *ina*X, can amplify the *ina* gene present in the sample. In this study, the researchers used PCR with program settings including pre-denaturation at 94°C for 5 minutes, post-extention at 72°C for 5 minutes, denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 1 minute. The PCR cycle lasted for 35 cycles. However, with this procedure, the amplification of the desired genes was not obtained in this study. Therefore, the researchers performed several PCR optimizations to produce the desired product.

The optimization of PCR was carried out according to the procedure given by Kapabiosystems as the master mix producer for PCR amplification. After PCR optimization, one pair of primers of the *ina* gene was obtained and amplified in the bacteria with the code N.2.1.B13. The amplified primer was the *ina*Z primer commonly used to amplify the *ina*Z gene (Nejad et al. 2006). The PCR conditions were carried out so that the *ina* gene was amplified, namely pre-denaturation at 95°C for 1 minute, post-extension at 72°C for 10 minutes, denaturation at 95°C for 15 seconds, annealing at 55°C for 15 seconds, and extension at 72°C for 6 seconds with a PCR cycle of 40 cycles. The comparison results of temperature used in PCR optimization to determine the best annealing temperature for amplifying the *ina* gene with *ina*Z f and *ina*Z r primers can be seen in Figure 3.

It can be seen from Figure 3 that the best temperature for annealing in the amplification of the *ina*Z gene is 55°C to 56°C. It is very different from the PCR conditions used in the study of Nejad et al. (2006) in amplifying the *ina* gene. Optimizing the use of temperature for annealing was also carried out on other *ina* gene primers but still did not produce the desired amplification product. The factor that allows this to happen is a primer that is not suitable. This mismatch can occur because no specific site on the DNA template matches the bases in the primer, so the annealing process during amplification does not occur, and PCR products are not formed (Zein et al. 2013).

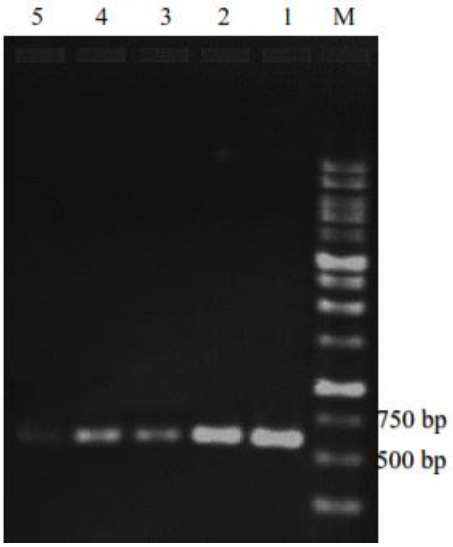

Figure 3. Electropherogram of *ina* gene in N21B-13 isolates using *ina*Z f and *ina*Z r primers with annealing temperature variation: M. *Marker* DNA 1 Kb, 1. 55°C, 2. 56°C, 3. 57°C, 4. 58°C, 5. 59°C.

Sequences analysis of 16S rRNA coding gene and *ina* gene

DNA sequencing is the process or technique of determining the sequence of nucleotide bases in a DNA molecule. These sequences are known as DNA sequences, which are the most basic information of a gene or genome because they contain the instructions needed for forming living organisms (Madigan et al. 1997).

In this study, the DNA sequencing process obtained from amplifying the 16S rRNA gene and the *ina* gene was carried out by PT Genetics Science Jakarta-1st Base Singapore. The sequencing cycle was performed on the ABIprism™ 310 Automated DNA Sequencer (PE Applied Biosystem).

The nucleotide base sequences of the 16S rRNA and *ina* genes were analyzed and compared with the database on GenBank using the BLAST program to determine the identity of the species being studied. According to Stephen et al. (1990), BLAST is a software algorithm for comparing primary information on biological sequences, such as amino acid sequences from different proteins or DNA sequences based on nucleotide bases. Following are the results of the analysis using BLAST. The results of the BLAST analysis can be seen in Table 3.

Table 3. Results of BLAST analysis based on 16S rRNA gene encoding of INA bacteria

Isolate code	Close relatives	Accession number	% Sim.
N.2.2.B-6	*Pantoea* sp. 57916	AF227860	99%
K.2.2.B-4	*Pantoea* sp. 57917	DQ094146	98%
N.2.1.B-13	*Pseudomonas* sp. BF81Fb	KC311267	96%
K.2.1.B-5	*Pseudomonas* sp. R1SpM3P2C2	KF147061	98%
N.2.2.B-7	*Pseudomonas* sp. R2SsM3P1C2	KF147058	95%
N.2.1.B-15	*Rahnella* sp. DmB 16	KF720908	95%
K.2.2.B-2	*Pseudomonas* sp. R2SsM3P1C13	KF147057	96%

Based on the database in GeneBank, four of the seven isolates had similarities with INA bacteria from several subtropical countries (Table 3). The four isolates were N21B-13 which was identified to have 96% similarity with *Pseudomonas* sp. BF81Fb, K21B-5 which was identified to have 98% similarity with *Pseudomonas* sp. R1SpM3P2C2, N22B-7 which was identified to have 95% similarity with *Pseudomonas* sp. R2SsM3P1C2 and K22B-2 which was identified to have 96% similarity with *Pseudomonas* sp. R2SsM3P1C13. *Pseudomonas* sp. BF81Fb was an *ina* bacterium isolated from the air at the moment of rain in the pine forest of Manitou, Colorado, USA, while *Pseudomonas* sp. R1SpM3P2C2, *Pseudomonas* sp. R2SsM3P1C2 and *Pseudomonas* sp. R2SsM3P1C13 were INA bacteria originating from the air of Lyon, France. The other three isolates have similarities with *Rahnella* sp. DmB 16, *Pantoea* sp. 57916 and *Pantoea* sp. 57917 were not INA bacteria. Although some *Pantoea* genera have *ina* capability, the emerging database showed they were not INA bacteria.

All the results of the BLAST analysis showed similarities to many INA bacteria originating from the subtropics (Table 4). It is because research on INA bacteria is mostly carried out in subtropical areas while the research in the tropics is still very little (Stephanie and Waturangi 2011), so the data in the NCBI database all come from research conducted in subtropical areas.

In general, the seven isolates belonged to 3 kinds of genera, namely *Pantoea*, *Pseudomonas*, and *Rahnella*. *Pantoea* is a genus of Gram-negative bacteria in the family Enterobacteriaceae. These bacteria are facultative anaerobes, rod-shaped, catalase-positive, and oxidase-negative. These bacteria can be found in soil, water, plants, and some animals (Paradis et al., 2005). In this genus, one species, Pantoea ananatis, is reported to cause browning of pineapple roots and is also classified as an *ina* bacterium with the *ina*A gene. In addition, *Pantoea* is the name of a new genus of *Erwinia* that is currently used so that bacteria that were previously in the genus *Erwinia*, such as *Erwinia ananas*, *E. herbicola*, and *E. uredovora*, are now in the genus *Pantoea* (Watanabe and Sato 1998).

Pseudomonas is one of the most well-known genera of INA bacteria. *Pseudomonas* is a Gram-negative bacterium, rod-shaped and oxidase negative. Soil, water, and air can be a habitat for these bacteria. Several species of this genus are plant pathogenic bacteria commonly found on leaf surfaces. In addition, these bacteria are thought to be able to be in the air around plants and contribute as a source of forming ice cores (Waturangi and Tjhen 2009). Several species of bacteria from this genus that have been reported to form ice cores include *P. syringae*, *P. fluorescens* (Maki et al. 1974), and *P. viridiflava* (Obata et al. 1989).

Rahnella is a genus of Gram-negative bacteria. This bacterium is rod-shaped, tolerant of low temperatures, and grows optimally at 30°C. This bacterium has been reported to cause decay in several plants (Oladoye et al. 2013), but no studies have reported that this bacterium can form ice nuclei.

Table 4. BLAST analysis results based on the ice nucleation gene of INA bacteria

Isolate code	Identified	Accession number	% Similarity
N.2.1.B-13	Complete sequence of ice core protein-coding gene in *Pseudomonas borealis*	EU573998.1	86%

DNA sequences using *ina*Z primers obtained from isolate N.2.1.B-13 had a similarity of 86% with the gene encoding the ice nucleation protein in *Pseudomonas borealis*. The *P. borealis*, used as a comparison in this study, is a bacterial isolate isolated from the Danu Daring Tundra Ecosystem Research Station, North West Territories, Canada. The gene encoding the ice nucleation protein in *P. borealis* isolates is similar to the *ina*Z gene in *P. syringae* (Wu et al. 2009).

The *ina* gene sequence using the *ina*Z primer showed the suitability of the base sequence with the *P. borealis ina* gene sequence in the database at GeneBank (access no. EU573998). The match starts from the base sequence 2947 to 3491 bp and is located in the middle region of the ice nucleation protein gene complete sequence of *P. borealis*.

The character of the *ina* gene obtained in this study has fewer amino acids compared to the *ina* gene in *P. borealis* in GeneBank (access no. EU573998). The *ina* gene fragment belonging to *P. borealis* encodes 1244 amino acids, each of which composes the N end of 163 amino acids, 41 amino acids make up the C end, and 1040 amino acids make up the core of the *ina* gene (Wu et al. 2009), while the *ina* gene present in isolate N.2.1.B-13 only amounted to 189 amino acids consisting of 16 amino acids. The *ina* gene from the N.2.1.B-13 isolate was not found to have an N tip and a C tip. It was because the amino acid sequencing of the *ina* gene from the N.2.1.B-13 isolate with the *P. borealis* gene showed compatibility at bases 2947 to 3491. bp or from the 871st amino acid to the 1060th amino acid. The translation product of the obtained *ina* gene structure is also dominated by amino acids commonly found in other *ina* genes, namely alanine, glycine, tyrosine, serine, threonine, and leucine.

The *ina*Z gene is one of the *ina* genes commonly found in INA bacteria. This gene is commonly found in the bacterium *P. syringae*. According to Green and Warren (1985), the product of translation of this gene structure is dominated by the octapeptide Ala-Gly-Tyr-Gly-Ser-Thr-Leu-Thr. The 24 bases that make up the octapeptide are GCCGGTTATGGCAGCACGCTGACC. The repeated presence of this octapeptide in the gene is thought to affect the catalytic function and the ability to form ice core formations in supercooled water. It has implications for the ability of INA bacteria to cause frostbite in plants.

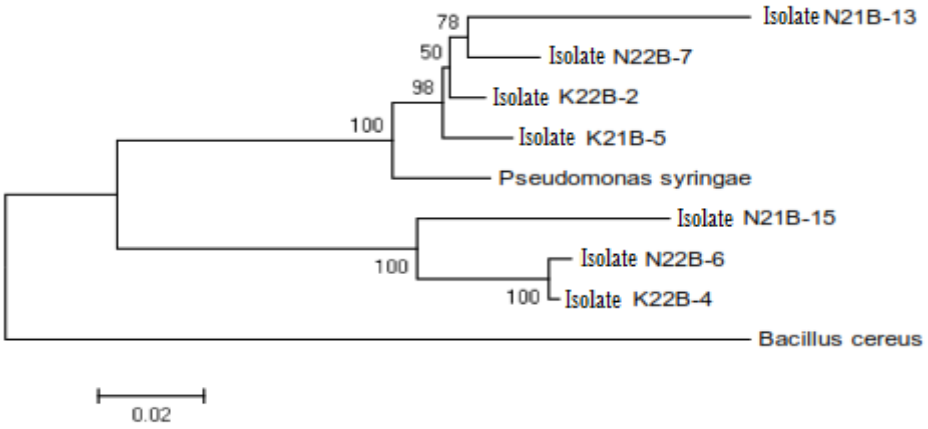

Figure 4. Phylogenetic tree of INA bacteria isolated from lichens on the climbing route of Cemoro Sewu, Mount Lawu, Jawa, Indonesia, with an outgroup of *Bacillus cereus* (Accession number AJ277908)

A phylogenetic tree was created based on the genetic distance between INA bacterial isolates using the MEGA 5.1 program. The purpose of making a phylogenetic tree is to determine the relationship of INA bacteria between existing isolates. The phylogenetic tree based on the 16S rRNA gene can be seen in Figure 4.

Based on the phylogenetic tree, *P. syringae*, known to form ice cores, are in the same group as bacterial isolates K21B-5, K22B-2, N22B-7, and N21B-13 which can form ice cores, while isolates N21B-15, N22B-6 and K22B-4 are in different group with *P. syringae*. Therefore, it indicates that K21B-5, K22B-2, N22B-7, and N21B-13 have a close relationship with INA bacteria of the *P. syringae* species.

The numbers in the branching of the phylogenetic tree in Figure 4 were bootstrap values. Bootstrap value is used to test how well the model data set is used in research. For example, the bootstrap value on branches of a phylogenetic tree is significant if the resampled data set repeatedly predicts the same branch for over 70% (Felsenstein 1988).

In conclusion, the INA bacterial species found in lichens on the climbing route of Cemoro Sewu, Mount Lawu, based on the results of identification with the 16S rRNA gene, respectively, were N.2.2.B-6 which had 99% similarity with *Pantoea* sp. 57916, N.2.1.B-13 which had 96% similarity with *Pseudomonas* sp. BF81Fb, K.2.2.B-2 which had 96% similarity with *Pseudomonas* sp. R2SsM3P1C13, N.2.2.B-7 which had 95% similarity with *Pseudomonas* sp. R2SsM3P1C2, K.2.1.B-5 which had 98% similarity with *Pseudomonas* sp. R1SpM3P2C2, K.2.2.B-4 which had 98% similarity with *Pantoea* sp. 57197, and N.2.1.B-15 which had 95% similarity with *Rahnella* sp. DmB 16. *Pseudomonas* sp. BF81Fb, *Pseudomonas* sp. R2SsM3P1C13, *Pseudomonas* sp. R2SsM3P1C2 and *Pseudomonas* sp. R1SpM3P2C2 were INA bacteria. The character of the protein gene coding for the ice core of INA bacteria in lichens on the climbing route of Cemoro Sewu, Mount Lawu, successfully amplified using *ina*Z primer from isolate N.2.1.B-13, was similar to the ice nucleation protein gene complete sequence from *P. borealis*. The *ina* gene from isolate N.2.1.B-13 had 189 amino acids consisting of 16 amino acids that were repeated, the N tip and the C tip were not found because the amino acid sequence of the *ina* gene isolate N.2.1.B-13 with the *ina* gene *P. borealis* showed compatibility at bases of 2947 to 3491 bp or from amino acids of 871 to amino acids to 1060, and was composed of amino acids commonly found in other *ina* genes, namely Alanine, Glycine, Tyrosine, Serine, Threonine, and Leucine.

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